The Regulation and Function of Phosphatidylinositol 3-kinase

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Abstract

Phosphatidylinositol 3-kinase (PI 3-kinase) is a lipid kinase that is likely to play an important role in lymphocyte biology and immune function. PI 3-kinase is activated by the T cell antigen receptor (TCR) and the interleukin-2 (IL-2) receptor which govern T cell activation and growth, respectively. Moreover, accessory receptors such as CD28 and CTLA-4 or other cytokine receptors regulate PI 3-kinase activity in T cells.

One aim of this thesis was to examine mechanism by which the TCR couples to PI 3-kinase. It was shown that the adaptor molecule Grb2 via its SH3 domains can bind to the p85 subunit of PI 3-kinase and provides a potential link between the TCR and PI 3-kinase. Importantly, these studies led to the first identification of a novel haematopoietic-lineage specific 75 kDa protein that associates with Grb2 SH3 domains. p75 is tyrosine phosphorylated in response to TCR engagement. In parallel studies by other investigators, a 76 kDa Grb2 associated protein was cloned, termed SLP-76. I show that p75 and SLP-76 are identical. p75/SLP-76 is involved in TCR signal transduction pathways leading to IL-2 gene transcription.

In order to analyse the cellular functions of PI 3-kinase I generated a constitutively active form of the enzyme. The strategy I employed was to membrane localise its p110 catalytic subunit. p110 was fused to a truncated rat CD2 cell surface receptor giving rise to rCD2p110. Expression of the rCD2p110 chimera elevates the cellular levels of D-3 phosphorylated inositol lipids. Using rCD2p110 it was demonstrated that PI 3-kinase signals are sufficient to stimulate p70S6k but not the MAP kinases Erk or Sapk/Jnk in fibroblasts or T cells. However, in T cells, PI 3-kinase signals can contribute to Erk activation. Importantly, it was established that the protooncogene PKB/Akt is activated by IL-2 in a PI 3-kinase-dependent fashion. Active PI 3-kinase can substitute for IL-2 in stimulating PKB and an active form of PKB can substitute for rCD2p110 or IL-2 in activating p70S6k.
Rac/Rho-dependent effector pathways play a role in cytoskeletal changes and regulation of gene transcription. PI 3-kinase is a putative upstream regulator of Rac and hence Rho. Here, I show that PI 3-kinase only regulates a subset of Rac/Rho-mediated cellular responses. PI 3-kinase signals are sufficient to induce Rac/Rho-controlled actin rearrangements but fail to trigger Rac/Rho-mediated effector pathways for activation of transcription factors. I propose that specific subcellular compartmentalisation mechanisms exist that localise different exchange factor/GTPase complexes to divergent downstream effector pathways.

Finally, I describe a previously unrecognised function for PI 3-kinase during T cell activation. PI 3-kinase can act as a selective negative regulator of TCR-mediated induction of the transcription factor NF-AT. This involves a novel uncharacterised effector of PI 3-kinase. The significance of these findings for immune homeostasis will be discussed.
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Abbreviations

aa  amino acid
Ab  antibody
APC  antigen-presenting cell
ATP  adenosine 5'-triphosphate
Bcr  breakpoint cluster region
bp  basepair
C  carboxy
C. elegans  Caenorhabditis elegans
CAT  chloramphenicol acetyltransferase
CD  cluster of differentiation
cDNA  complementary DNA
CMV  cytomegalovirus
cpm  counts per minute
CRIB  Cdc42/Rac interactive binding
D. melanogaster  Drosophila melanogaster
DAG  sn-1,2-diacylglycerol
DMEM  Dulbecco's modified Eagle's medium
DMEM  Dulbecco's modified Eagles medium
DMSO  dimethyl sulfoxide
DN  double-negative CD4+CD8- thymocytes
DNA  deoxyribonucleic acid
DP  double-positive CD4+CD8+ thymocytes
E. coli  Escherichia coli
ECL  enhanced chemiluminescence
EDTA  ethylenediamine tetraacetic acid
EGF  epidermal growth factor
EGTA  [ethylene-bis(oxyethylenenitrilo)] tetra-acetic acid
Erk  extracellular signal-regulated kinase
f  fento (10^{-15})
FACS  fluorescence-activated cell sorting, or: flow cytometric immunofluorescence analysis
Fak  focal adhesion kinase
FCS  fetal calf serum
Frap  FKBP12-rapamycin associated protein
G protein  guanine nucleotide-binding protein
g  gram
GAP GTPase activating protein
GDI guanine nucleotide dissociation inhibitor
GDP guanosine diphosphate
GEF guanine nucleotide exchange factor
Grb2 growth factor receptor-bound protein 2
GST glutathione-S-transferase
GTP guanosine triphosphate
H7 5-[(2-methyl-1-piperazinyl)-sulphonyl]isoquinoline dihydrochloride
HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPLC high-pressure liquid chromatography
I(1,4,5)P3 inositol 1,4,5-trisphosphate
IFN interferon
Ig immunoglobulin
IL-2 interleukin-2
IL-2R interleukin-2 receptor
Iono ionomycin
IPTG isopropyl-2-D-thiogalactopyranoside
ITAM immunoglobulin family tyrosine-based activation motif
Jak Janus kinase
Jnk Jun N-terminal kinase
k kilo
kDa kilodalton
l litre
LPA lysophosphatidic acid
LPS lipopolysaccharide
M molar
m metre
µ micro (10⁻⁶)
m milli (10⁻³)
mAb monoclonal antibody
MALDIMS matrix-assisted laser-desorption ionisation mass spectrometry
Map kinase mitogen-activated protein kinase
MBP myelin basic protein
Mek Erk kinase
MHC major histocompatibility complex
mlg mouse immunoglobulin
min minute
mol mole
MOWSE molecular weight search (peptide-mass database)
mRNA messenger RNA
mTor mammalian target of rapamycin
MW molecular mass
N amino
n nano (10^-9)
NF-AT nuclear factor of activated T cells
NGF nerve growth factor
NP40 Nonidet P-40, p-tert-octylphenyl 9.6 ethoxylate
p pico (10^{-12})
p-NPP p-nitrophenol phosphate
p70S6k p70 S6 kinase
PAGE polyacrylamide gel electrophoresis
Pak p21-associated kinase
PBL peripheral blood lymphocyte
PBSA phosphate-buffered saline A
PCR polymerase chain reaction
PdBu phorbol 12,13-dibutyrate
PDGF platelet-derived growth factor
PH domain pleckstrin homology domain
Pl 3-kinase phosphatidylinositol 3-kinase
Pi inorganic phosphate
Pl(3)P phosphatidylinositol 3-phosphate
Pl(3,4)P_2 phosphatidylinositol 3,4-bisphosphate
Pl(3,4,5)P_3 phosphatidylinositol 3,4,5-trisphosphate
Pl(4,5)P_2 phosphatidylinositol 4,5-bisphosphate
PKB protein kinase B, also termed Akt or Rac protein kinase
PKC protein kinase C
PLC phospholipase C
POR1 partner of Rac1
PTK protein tyrosine kinase
PTP protein tyrosine phosphatase
PVDF polyvinylidene difluoride
Raft1 rapamycin and FKBP12 target 1
Rapt1 rapamycin protein target 1
rCD2 rat CD2
rIL-2 recombinant IL-2
RNA ribonucleic acid
RPMI Roswell Park Memorial Institute
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>S. pombe</td>
<td>Schizosaccharomyces pombe</td>
</tr>
<tr>
<td>Sapk</td>
<td>stress-activated protein kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SH</td>
<td>src homology domain</td>
</tr>
<tr>
<td>Ship</td>
<td>SH2-containing inositol 5-phosphatase</td>
</tr>
<tr>
<td>SHP</td>
<td>SH2-containing protein tyrosine phosphatase</td>
</tr>
<tr>
<td>SHP-1</td>
<td>previously known as: SHPTP-1, SHP, HCP, PTP1C</td>
</tr>
<tr>
<td>SHP-2</td>
<td>previously known as: SHPTP-2, SHPTP-3, Syp, PTP2C, PTP1D</td>
</tr>
<tr>
<td>SLP-76</td>
<td>SH2 domain-containing leukocyte protein of 76 kDa</td>
</tr>
<tr>
<td>Sos</td>
<td>son of sevenless</td>
</tr>
<tr>
<td>SP</td>
<td>single-positive T cells, either CD4^+CD8^- or CD4^-CD8^+</td>
</tr>
<tr>
<td>SRE</td>
<td>serum response element</td>
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<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Tc</td>
<td>cytotoxic T cell</td>
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<tr>
<td>TCF</td>
<td>ternary complex factor</td>
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<td>TCR</td>
<td>T cell antigen receptor</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<td>Th</td>
<td>helper T cell</td>
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<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiscott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>zeta-associated protein of 70 kDa</td>
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Chapter 1

Background

1.1 Overview

The ability to mount a specific immune response is dependent on antigen-specific B and T lymphocytes. They migrate in large numbers (~2 x 10^{12} cells in man) through the blood, the lymph and into specialised lymphoid tissues such as the thymus, the lymph nodes and the spleen in order to encounter foreign molecules - antigens - at their sites of invasion. Many of them (about 1 x 10^9 lymphocytes per day) never meet their purpose in life and eventually become programmed to die, mediated by a process termed apoptosis which is vital for immune homeostasis (Nagata, 1997). However, the few that are exposed to their particular antigen become activated, clonally expand and mount a specific immune response that eventually will eliminate the foreign invader. B lymphocytes secrete specific antibodies which will mark the foreign particle for destruction by complement, or macrophages and other scavenger cells. In addition, certain subsets of T lymphocytes (cytotoxic T cells) exhibit direct defence mechanisms and can kill intracellular microbes such as viruses, Leishmania major and mycobacteria. The activation of T lymphocytes is crucial for the coordination of the immune reaction by permitting these cells to proliferate and exert their potent regulatory or effector activities. One of the major challenges in immunology is to unravel the molecular events that will lead to T lymphocyte activation, proliferation and differentiation as these processes are ultimately responsible for a balanced immune response and immune homeostasis.

The recognition of antigens is accomplished by the T cell receptor for antigen (TCR)/CD3 complex - but cognate T cell activation will only occur when multiple distinct accessory molecules present on the surface of a T cell interacting with an antigen-
presenting cell (APC) participate in the process of antigen recognition (such as CD2, CD4, CD8, CD28, CD45 or integrins) (Cantrell, 1996; Lenshow et al., 1996; Rudd, 1996; Trowbridge and Thomas, 1994; Wange and Samelson, 1996; Weiss and Littman, 1994). The interactive engagement of these receptors are responsible for initiating a sequence of intracellular biochemical events that lead to G0 to G1 transition and is referred to as 'T cell activation'. During this initial process of differentiation over 60 gene products become expressed in T cells (Kelly and Siebenlist, 1995; Ullman et al., 1990). Among these molecules are the lymphokine interleukin-2 (IL-2) and its receptor, the interleukin-2 receptor (IL-2R) (and other cytokines such as interferon (IFN) γ, transforming growth factor (TGF) β, tumour necrosis factor (TNF) β, IL-3 - IL-6, IL-9 - IL-11, IL-13 or IL-15) which are not expressed by naive non-activated T cells (Crabtree and Clifton, 1994). However, T cell activation delivered by the TCR/CD3 complex does not result in T cell proliferation/differentiation and an additional signal is absolutely required for the T lymphocyte to proceed through G1 of the cell cycle and enter S phase. This is provided by haematopoietic cytokines such as IL-2 (Cantrell and Smith, 1984; Smith, 1988). The binding of IL-2 to its receptor serves as an important checkpoint in the maintenance of the immune response: (1) it communicates that T cell activation is completed and initiates events that trigger the termination of this phase; (2) it induces further gene expression and ultimately primes the T lymphocyte to become committed to DNA replication and mitosis. A molecular understanding of the events that culminate in T cell activation and growth will be intimately linked to a characterisation of the signalling pathways engaged. One enzyme which is activated at multiple stages of the immune reaction in T cells including engagement of the TCR and the IL-2R is a lipid kinase, phosphatidylinositol 3-kinase (PI 3-kinase) (Cantrell et al., 1993). PI 3-kinase is thus likely to play an important role in the processes leading to T lymphocyte activation and growth. When I initiated these studies little was known about the function of this enzyme. My goal was therefore to further our understanding of the contributions of PI 3-kinase to lymphocyte biology and immune function.
1.2 Receptors that initiate T cell activation, growth and differentiation and their role in immune function

In the present section I would like to introduce the major receptors which control peripheral T cell biology. I will briefly mention their predominant structural features but mainly concentrate on how the structural determinants are relayed to their possible function during an immune reaction. Reference to the mice deficient in these receptor molecules will be made. Signalling pathways involved in the execution of their functions will be mentioned however the players will be presented only in the next section.

1.2.1 The TCR and the discrimination of 'self' from 'non-self'

The TCR is a multichain molecular complex with a binding specificity that can detect two structurally distinct complexes expressed on the surface of most nucleated cells or APCs, respectively: (1) nonameric peptides bound to major histocompatibility complex (MHC) class I molecules (Jardetzky et al., 1991) and (2) proteolitically derived antigenic peptides, 13 to 17 amino acids in length, presented by MHC class II molecules (Rudensky et al., 1991). The specificity of the TCR lies in the clonally variable regions of the disulphide-linked αβ chains, or in some rare cases (less than 3%) in the related TCR γδ glycoproteins, which form immunoglobulin-like structures (Bentley and Mariuzza, 1996; Padlan and Margulies, 1997). A clone of T cells, characterised by a unique αβ TCR, is generated by the somatic rearrangements of TCR α and β genes during thymocyte development, along with the timed expression of signal transducing receptor chains and importantly the coreceptor molecules CD4 or CD8, which define T cells that recognise class II or class I MHC molecules, respectively. It is here in the thymus where the foundation of immune recognition is laid and T cells acquire the structural determinants to discriminate 'self' from 'non-self' - a function encoded by the TCR: T cells bearing TCRs which exert too high avidity to self MHC molecules are eliminated in a process termed negative selection. T cells with TCRs which have at least a low
affinity for self MHC become positively selected. Thus, the T cell repertoire which reaches the periphery is shaped in a way that the TCR cannot react with 'self' but can recognise the structural features of MHC molecules.

The molecules which transduce the signals from these engagements of the TCR αβ chains and relay them into vital information are the CD3 complex (γ, δ and ε) and the non-covalently associated ζ chains. Assembly of all receptor chains is required for efficient surface expression of the TCR/CD3 complex. The intracellular tails of the CD3 and ζ molecules contain a common 17-amino acid sequence motif, D/EX7D/EX2YX2L/I/IX7YX2L/I (Reth, 1989), which is present in a single copy in each of the CD3 chains and triplicated in the ζ subunit (Fig. 1.1). The consensus sequence is also found in immunoglobulin- and Fc receptor-associated chains and therefore is thought to be an evolutionary conserved activation motif in component chains of haematopoietic cell antigen receptors named immunoglobulin receptor family tyrosine-based activation motif (ITAM). This activation motif becomes tyrosine phosphorylated after triggering of the TCR and allows coupling of intracellular protein tyrosine kinases (PTKs), in particular ZAP-70, to the TCR (Chan and Shaw, 1995).

Figure 1.1. The TCR/CD3 complex. The domains of the extracellular immunoglobulin structures and the intracellular ITAM motifs are schematically depicted.
More recently it has become evident that ITAMs also may serve as docking sites for adaptor or effector molecules which bind differentially to specific ITAMs, the degree of affinity being encoded in the 'X' residues of each motif (Wange and Samelson, 1996). ITAMs are hence important to link the ligand-binding subunits to intracellular signalling pathways. An additional function may derive from the multiplicity of these motifs, there are 10 ITAMs in the TCR, which may serve as a signal amplification mechanism by permitting the recruitment of multiple PTKs/effectors to one receptor complex. Indeed, in experiments using recombinant receptor chains containing ITAMs the number of ITAMs present reflected the intensity of the signalling response (Irving et al., 1993). (The study of the individual roles of the TCR/CD3 chains using mice deficient in TCR components has been hampered by the fact that hardly any T cells develop and reach the periphery in these mice (Pfeffer and Mak, 1994).)

1.2.2 Accessory molecules: integrins, CD2, CD4, CD8, and CD45

Although the TCR/CD3 complex mediates the initial encounter with the APC and is crucial for T cell activation, multiple accessory molecules participate in the interaction between the T cell and the APC. At the beginning these molecules were simply pictured as adhesives, solely ensuring efficient cell-cell contact and convey some information regarding the character of the apposing cell. Now a more complete picture has emerged from the findings that most of these molecules not only play an accessory role but also provide a costimulatory signal and transduce information obligatory for T cell activation (see below). Most of these auxiliary molecules belong to either the immunoglobulin (CD4, CD8, CD2, CD28) or the integrin (CD11a/CD18=LFA-1, VLA-3 to VLA-6) superfamilies; (LFA = lymphocyte function associated antigen; VLA = very late antigen).
Integrins and cell adhesion

Integrins such as LFA-1 enhance intercellular contacts between a T cell and an APC and increase both the surface contact area and the time of interaction between a T cell and the apposing cell, which in turn increases the chances of a productive TCR/Ag-MHC interaction (Sharpe, 1995; Springer, 1990). The ligands of LFA-1 are ICAM-1=CD54, ICAM-2, and ICAM-3. Moreover, integrins also participate in contacts with the extracellular matrix of the surrounding tissue.

CD2 is an adhesion molecule of 50 kDa which is heavily glycosylated in its extracellular domain. Its receptor is the widely distributed 'lymphocyte function associated antigen-3' (LFA-3). The CD2-LFA-3 pathway can contribute to antigen-independent T cell adhesion and also has been shown to enhance production of cytokines such as IFN and TNF (Bierer et al., 1989). CD2 is thought to function quantitatively by augmenting second messenger generation triggered by the TCR itself rather than by providing independent signals. During in vivo T cell responses CD2 function appears to be redundant: although CD2 is expressed early in thymic ontogeny, CD2 deficient mice do not appear to have any abnormality in thymic development; likewise, peripheral responses of Th or Tc cells appear to be normal (Killeen et al., 1992; Sharpe, 1995).

CD4 and CD8

CD4 and CD8 are mutually exclusive in their expression on peripheral T lymphocytes but both CD4 and CD8 cooperate with the TCR/CD3 complex in the recognition of Ag/MHC complexes. CD4 molecules recognises the monomorphic region of MHC class II molecules whereas CD8 engages MHC class I molecules. The nature of this distinction is of vital importance as recognition by CD8+ T cells leads to the destruction of the MHC class I bearing cell by the CD8+ T cell; in contrast, the association with a MHC class II molecule provides an activation signal for CD4+ T
lymphocytes and the delivery of help in the form of lymphokines, e.g. IL-4, IL-5, and IL-6, for the relevant antigen-specific B lymphocytes which eventually produce antibodies. It is for this reason that CD4+ cells are termed helper T (Th) cells and CD8+ cells are referred to as cytotoxic T (Tc) cells. Once contact has been made, the quaternary complex which forms between TCR, Ag, MHC and CD4/CD8 is thought to stabilise the interaction between the T cell and the APC (Garcia et al., 1996).

CD4 (55 kDa) is a single glycoprotein with four extracellular immunoglobulin-like domains. CD8 is a dimer of polypeptides - either α chain (34 kDa) homodimer or αβ heterodimer (with β: 30 kDa) - each having an amino-terminal immunoglobulin-like domain followed by an extended and heavily glycosylated region. Beyond their role in contact formation, CD4 and the CD8 α chain are important for initial activation events as both associate with the src tyrosine kinase p56lck (Veillette et al., 1988) via pairs of cysteine residues within their cytoplasmic domain (Shaw et al., 1990; Turner et al., 1990). Thus, during antigen activation the CD4/CD8 binding to MHCII/I leads to the intracellular coapproximation of Lck to the CD3 complex which delivers the first tyrosine phosphorylation signal leading to activation of effector cascades (Chan et al., 1994; Crabtree and Clipstone, 1994; Weiss, 1993). CD4, the CD8 α chain and the CD8 β chain are required in T cell development (Zamoyska, 1994). Disruption of the CD8α gene prevents expression of any CD8 protein - the β chain depends on α to be transported to the cell surface - and totally impedes generation of cells with MHC class I-restricted TCRs. Disruption of the CD4 gene also generates a severe impairment in T cell development, although a few double-negative (DN) Th cells do develop. Disruption of the CD8β gene has a profound effect also, as only 20%-30% of the normal number of CD8 cells develop.
**CD45**

CD45 (leukocyte common antigen) is a transmembrane tyrosine phosphatase which plays an important role in the activation of src tyrosine kinases in response to TCR engagement. Its regulation by ligands on the opposing cell is not clear, but the B cell adhesion molecule CD22 has been suggested as a possible partner (Stamenkovic et al., 1991). The cytoplasmic domain containing the phosphatase activity is highly conserved. In contrast, its extracellular moiety is generated by alternative splicing of three exons and posttranslational glycosylation which gives rise to a family of isoforms ranging from 180 kDa to 220 kDa molecular mass (Trowbridge and Thomas, 1994). Loss of CD45 expression abrogates TCR induced tyrosine phosphorylation, phosphatidylinositol (PI) turnover, and release of calcium from intracellular stores (Chan et al., 1994; Trowbridge and Thomas, 1994). The function of CD45 is to regulate the activity of src tyrosine kinases. Whether CD45 activity is regulated itself during T cell activation, is not known. During thymocyte development CD45 function is important for the transition of CD4−CD8− (double-negative (DN)) thymocytes into CD4+CD8+ (double-positive (DP)) thymocytes and for the maturation of the DP into mature, single-positive (SP), CD4+CD8− or CD4−CD8+, T cells (Byth et al., 1996; Kishihara et al., 1993).

1.2.3 **CD28, costimulation and helper T cell responses**

**CD28 and the concept of costimulation**

While TCR signal transduction is necessary for activation events, it is generally thought that under most circumstances additional TCR-independent events - referred to as costimulation - must occur to yield a sufficient immune response (Fig. 1.2). Triggering of the TCR/CD3 complex in the absence of a second signal is thought to render the clonal precursor T cell anergic or tolerant (Bretscher and Cohn, 1970). The major candidate to provide this independent or 'second' signal is CD28 (Bluestone, 1995; Jenkins, 1994; Lenshow et al., 1996; Thompson, 1995). CD28 is a homodimeric
glycoprotein (with the monomer having a predicted molecular mass of 44 kDa) that exhibits a immunoglobulin-like extracellular domain. CD28 is expressed constitutively in humans on all CD4\(^+\) T cells and on about 50\% of all CD8\(^+\) T cells. The interest in CD28 derives in particular from the discovery that triggering of CD28 activates a novel TCR/CD3 independent signal transduction pathway emphasised by its insensitivity to the immunosuppressant cyclosporin A (June et al., 1987). This is in contrast to stimulation via other accessory molecules which augment TCR/CD3-elicited responses (see above). Ligation of CD28 with anti-CD28 mAb or cells expressing the CD28 counterreceptors (B7-1 or B7-2, see below) in combination of limited concentrations of anti-CD3 or antigen promotes cell cycle progression and increases IL-2 production (~100 fold) by regulating IL-2 mRNA at the level of transcription, mRNA stability and translation (Fraser et al., 1991; Gimmi et al., 1991; Jenkins et al., 1991; June et al., 1994; Lindsten et al., 1989). This excess IL-2 has been suggested to prevent the T cell from clonal anergy (Janeway and Golstein, 1992) and administration of exogenous IL-2 during CD3 stimulation can act as 'second signal' (in particular for Th1 T cell clones, see below).
(Jenkins et al., 1991; Schwartz et al., 1989). More recent data suggests that CD28 - rather than having a proliferative role - may function as an anti-apoptotic signal by upregulating Bcl-xL expression which has been shown to prolong cell survival (Boise et al., 1995; Nagata, 1997; Sperling et al., 1996). Thus, the two-signal model can be viewed as the ability of CD28 to deliver biochemical signals that function in synergy with TCR-mediated signalling to initiate and maintain T cell responses. The nature of these biochemical events is not yet clear, but PI 3-kinase is one of the candidates, as upon ligation of CD28 the enzyme associates with a highly conserved phosphotyrosine motif in the cytoplasmic tail of the receptor. What are the consequences of these activation events for T cell biology? Is CD28 really required for primary T cell activation?

<table>
<thead>
<tr>
<th>Table 1.1 Th1 versus Th2 responses</th>
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<tbody>
<tr>
<td><strong>Regulatory cytokines produced</strong></td>
</tr>
<tr>
<td>Th1 responses</td>
</tr>
<tr>
<td>IFNγ, TNFβ, IL-2</td>
</tr>
<tr>
<td><strong>General effects</strong></td>
</tr>
<tr>
<td>Cell-mediated immunity</td>
</tr>
<tr>
<td><strong>Manifestations and executing cells</strong></td>
</tr>
<tr>
<td>'Help' for cytotoxic T cells</td>
</tr>
<tr>
<td>Macrophage activation</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Protection from autoimmune diseases</td>
</tr>
<tr>
<td>Intracellular pathogens eliminated, e.g. Leishmania major</td>
</tr>
</tbody>
</table>

**Activated Th cells can differentiate into distinct types of effector cells**

After the initial activation step by antigen and costimulatory signals CD4+ T cells (Th0 cell) can differentiate into either of two distinct types of effector cells: Th1 cells, which regulate cell-mediated immunity and Th2 cells, which regulate humoral immune responses (Paul and Seder, 1994). These subsets of Th cells can be
distinguished by their cytokine profile. Th1 cells produce IL-2, IFNγ and TNFβ, whereas Th2 cells produce IL-4, IL-5, and IL-10 (Table 1.1). Such a polarisation in cytokine production has major implications for the activation of other effector or regulatory cells and the course of an immune response (summarised in Table 1.1). Thus, in several models of autoimmune disease, a Th2 immune response is associated with disease resistance while induction of a Th1 response leads to progressive disease. In contrast, protective immunity from intracellular pathogens such as Leishmania major requires a Th1 response, while a Th2 response results in progressive disease.

The circumstances which determine the decision to follow one differentiation pathway or the other are outlined in Fig. 1.3 (in a simplified model). Activation of naive CD4⁺ T cells (precursor Th cells) results in the production of IL-2 with relatively little IFNγ or IL-4 being generated (Th0 cells). Subsequent events appear to bias the cell
toward differentiation into a Th1 or Th2 phenotype. Thus, the presence of IL-4 triggers a Th0 cell to become a Th2 cells whereas in the presence of IL-12 cells differentiate down the Th1 pathway. Subsequent challenge by antigen preferentially yields production of IL-4 by Th2 cells and Th1 cells generate IFNγ. The bystander cells which deliver these decision making cytokines are NK1.1 T cell subsets, FcεRI⁺ mast cells and basophils in the case of IL-4, while IL-12 is produced primarily by monocytes. However, even in the absence of exogenous lymphokines, T cells can be directed to either Th1 or Th2 differentiation (Kalinski et al., 1995). Finally, TCR transgenic mice have been used to show that high doses of peptide antigen can promote Th2-type cytokine production (IL-4), while low antigen doses preferentially can result in a Th1-type cytokine profile (IFNγ) (Hosken et al., 1995).

**CD28-deficient mice**

The first clues about the true role of CD28 in costimulation derived from studies of mice with a disrupted CD28 gene (Green et al., 1994; Shahinian et al., 1993). The first analysis of the immune responses in these mice indicated that CD28 costimulation is differentially required for cell-mediated and humoral immune responses in vivo (Shahinian et al., 1993). CD28-deficient mice show reduced Th2 cell-mediated responses which is manifested in poor B cell function (e.g. reduced Ig class switching). CD28 also provides a critical costimulatory signal for lectin-driven T cell responses (lectins aggregate cell membrane receptors), and this signal cannot be replaced completely in vitro by IL-2. In contrast, CD28⁻/⁻ mice have normal cytotoxic T lymphocyte activity which indicates that CD28 costimulation is not/less required for Th1-mediated responses. This suggests that distinct costimulatory signals - as yet unidentified (candidates are e.g. CD43, CD44 (Bluestone, 1995)) - may induce the production of cytokines. Alternatively, supraoptimal TCR-mediated signalling could in part substitute for CD28-delivered signals. For instance, purified CD28-deficient T cells can be stimulated with anti-CD3 but require about 10-fold more antibody to obtain an equivalent response (Green et al.,
1994). However, this latter study of the CD28-knockout mice also indicates that primary responses to anti-CD3 antibodies, natural APCs and alloantigens are significantly reduced (to one third of control levels). In addition, it has been suggested that CD28-mediated signalling may not affect initial T cell proliferation (24h-48h) but potentiates the late proliferative response (Lucas et al., 1995). Thus, a consistent theme emerging from the analysis of the CD28-deficient mice is that CD28 acts as the pivotal T cell costimulatory pathway but is not involved in all T lymphocyte responses and may be restricted to certain T cell populations at distinct stages of the differentiation process or immune reaction.

1.2.4 CTLA-4 and the downmodulation of an immune response

CTLA-4 (cytotoxic T cell-associated molecule-4) is a disulphide-linked homodimer (monomer ~35 kDa) with one immunoglobulin-like extracellular domain which shares sequence homology with CD28. CTLA-4 functions as a coreceptor but - in contrast to CD28 - CTLA-4 is expressed on T cells only after activation (peak at 48h). Occupancy of either CD28 or integrins may be involved in the upregulation of CTLA-4 (Lenshow et al., 1996). The amino-acid sequence of the cytoplasmic domain of CTLA-4 is completely conserved between man, mouse, rat and rabbit, strongly suggesting that the region has an important function. Interestingly, the cytoplasmic tail contains a PI 3-kinase binding site similar to the one in CD28. However, unlike CD28 CTLA-4 exhibits a sequence motif (TTGVVKMPPT) which retains the molecule primarily in Golgi or post-Golgi compartments resulting in higher intracellular than surface expression of CTLA-4 (Leung et al., 1995). Both CD28 and CTLA-4 are engaged by the same ligands, B7-1 and B7-2 but CTLA-4 binds to both with higher avidity than CD28 (~10-20 fold). In contrast to CD28, CTLA-4 is thought to have inhibitory or downregulatory function during an immune response (Lenshow et al., 1996; Linsley and Golstein, 1996). Thus, mice deficient for CTLA-4 die young (3-4 weeks of age) from massive infiltration of many organs by activated T cells, which highlights the essential inhibitory role the
coreceptor plays in the regulation of the immune response (Tivol et al., 1995; Waterhouse et al., 1995). The inhibitory role of CTLA-4 is supported by studies using purified cells which show that whole anti-CTLA-4 mAb (= stimulatory effect) prevent anti-CD3-mediated T cell activation in the presence of optimal CD28 costimulation (Krummel and Allison, 1995; Walunas et al., 1994).

1.2.5 Consequences of CD28 activation by its ligands B7-1 and B7-2: balancing Th1 versus Th2 responses during an immune reaction

The B cell activation antigen B7-1 (CD80) or B7-2 (CD86) function both as ligands for CD28 and CTLA-4 (Aruffo and Seed, 1987; Azuma et al., 1993; Freeman et al., 1993; Linsley et al., 1990). B7-1 and B7-2 belong to the immunoglobulin superfamily, are related to each other (26% amino acid identity) and are always coexpressed on 'professional' APCs: activated B cells, activated macrophages, and dendritic cells. This raised the question as to whether B7-1 and B7-2 could generate qualitatively different biochemical signals in the opposing T cell upon engagement of CD28. However, in comparative studies both ligands initiated identical biochemical events (Nunes et al., 1996; Nunes et al., 1996). Detailed studies of the expression levels of B7-1 and B7-2 in vivo revealed that B7-2 is constitutively expressed at high levels on dendritic cells while high level expression of B7-1 is only induced after activation. In general, B7-2 is more rapidly expressed than B7-1 upon activation of APCs. Thus, these data and other studies led to the conclusion that activation of CD28 versus CTLA-4 is achieved by timely restricting and regulating the cellular expression of B7-2 versus B7-1 and it is thought that B7-2 acts as the major ligand for CD28 in vivo due to their concurrent peaks of expression (for review see (Lenshow et al., 1996; Thompson, 1995)).

Finally, recent studies designed to address the role of B7-1 versus B7-2 in promoting costimulation in in vivo models indicate that these counterreceptors differ in
their ability to potentiate the development of T helper cells into either Th1 or Th2 cells. Although somewhat contradictory these studies promoted a model in which the balance of two major parameters influences the outcome of Th differentiation to either a Th1 or Th2 subset: the degree of TCR ligation (antigen dose) and the degree of costimulation. This model would predict that when TCR-ligand density/affinity is intermediate the presence of costimulatory signals promotes an Th2 response whereas absence of costimulation favours an Th1 response (Bluestone, 1995; Lenshow et al., 1996; Thompson, 1995).

1.2.6 The IL-2R and other cytokine receptors

The cytokine receptor superfamily

Many cytokine receptors consist of at least two polypeptide chains, a private ligand-specific receptor with a small and dispensable intracytoplasmic domain, and a public class-specific signal transducer with a large intracellular domain. Cytokines which share a common signal transducing subunit can be grouped into subfamilies. Three such subfamilies have been described (Fig. 1.4) (Kishimoto et al., 1994; Sato and Miyajima, 1994; Stahl and Yancopoulos, 1993; Sugamura et al., 1996).

Members of the first subfamily of which the receptors are ubiquitously expressed are IL-6, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OM) and IL-11. The common signal transmitting molecule of these receptor systems is called gp130. In the haematopoietic system a common signal transducer βc was discovered in the IL-3, IL-5, and granulocyte/macrophage colony-stimulating factor (GM-CSF) receptor systems. The lymphoid subfamily shares a common subunit, termed γc, which was first described as a component of the IL-2R complex (Sugamura et al., 1996; Takeshita et al., 1992). Other members of this subfamily are the IL-4, IL-7, IL-9, IL-13 and IL-15 receptor systems. Furthermore, the IL-
2R and the IL-15R share the IL-2R β subunit (Giri et al., 1994; Grabstein et al., 1994). A unique IL-15Rα chain has recently been cloned (Giri et al., 1995).

Figure 1.4. Cytokine receptors. Cytokine receptors share common signal-transducing receptor components.

The IL-2R

The IL-2R consist of three receptor chains (Fig. 1.5): the α chain, and the signal transducing common 64 kDa γ chain and the 75 kDa β chain (Taniguchi and Minami, 1993). The βγ heterodimer binds IL-2 with intermediate affinity and cells expressing the βγ heterodimer are responsive to IL-2. Expression of the α chain confers high affinity
binding of IL-2 to the $\alpha\beta\gamma$ complex. Characterised as a member of the cytokine receptor superfamily, IL-2Rβ has the common features of two pairs of the conserved cysteine residues near the N-terminus and a sequence of tryptophan-serine-'unconserved amino acid'-tryptophan-serine (WSXWS, WS motif) in the extracellular domain. Heterodimerisation of the βγ is required for the initiation of cytokine signalling processes including phosphorylation of the β and γ subunits (Nakamura et al., 1994). The cytoplasmic domain of IL-2Rβ contains unique regions such as the serine-rich, acidic, and proline-rich regions. The Box1 motif is also found in other cytokine receptor chains including gp130, IL-4R, IL-7R or IL-9R subunits. IL-2Rγ contains in its intracellular tail two Src homology (SH) 2-like subdomains. These are insufficient for phosphotyrosine binding and their role is unknown. Similar to the TCR or growth factor receptor tyrosine kinases, the tyrosine residues in the IL-2R β and γ chains - upon receptor activation and phosphorylation - provide potential binding sites for intracellular signalling molecules (Taniguchi, 1995).

**Figure 1.5.** Schematic of the IL-2R subunits. The numbers refer to amino acid positions from the N-terminus. The two pairs of cysteine residues and the tryptophan-serine motif common to the cytokine receptor superfamily are indicated. TM, transmembrane domain; SH2, SH2 subdomain; C30, C-terminal 30 amino acid residues.
IL-2Rγ is constitutively expressed on various populations of human and mouse haematopoietic cells, while expression of IL-2Rα and IL-2Rβ are restricted to lymphocytes and monocytes/macrophages. IL-2α and IL-2β expressions are different among cell populations; IL-2Rα is generally not expressed on quiescent lymphocytes but is induced (within one day) after TCR ligation or mitogen stimulation whereas low level expression of IL-2β can be detected which are further increased in response to T cell activation. Interestingly, IL-2 stimulation induces suppression of the IL-2Rγ chain on normal activated T cells which may result in IL-2-dependent growth cessation (Ohbo et al., 1995).

1.2.7. Common versus individual functions of cytokines; the unique functions of IL-2, IL-4 and IL-7

Cytokine receptors induce signals specific to each cytokine as well as signals common to various stimuli. This redundancy of effects has been a puzzle. Although it is not clear as yet what the underlying functions are, the structural determinant appears to lie in the common receptor chains which connect to similar signalling pathways. In this respect, particularly cytokine receptors which contain gp130 or βc in their receptor complex display similar functions. In contrast, however, the cytokines which share the common γ chain activate quite unique signalling pathways and display different functions even in the same cell: IL-2 stimulates B cell proliferation whereas IL-4 induces differentiation of B cells. IL-2 and IL-4 also are secreted by and act upon different subsets of T cells, Th1 and Th2 cells, respectively. Thus, IL-4 has an immunomodulatory role by regulating antibody production, haematopoiesis and inflammation, and the development of effector T cell responses. In particular, IL-4 is thought to direct the immunoglobulin class switch to IgG1 and IgE. IL-2 is most important for T cell proliferation and the regulation of cell-mediated immunity including activation of cytotoxic T cells, natural killer (NK) cells and macrophages (due to this capacity IL-2-treatment is applied in the immunotherapy of cancer, immunodeficiency, and infectious
IL-4 affects cells of most haematopoietic lineages whereas IL-2 stimulates mainly T lymphocytes and NK cells.

In contrast to IL-2 and IL-4 which are important for peripheral T cell function but do not play a critical role in T cell development, IL-7 is required for thymic ontogeny and lymphopoiesis (Peschon et al., 1994; Pfeffer and Mak, 1994; Von Freeden-Jeffry et al., 1995). IL-7 is secreted by stromal cells in the bone marrow and thymus and exerts its effects on immature B and T lymphocytes. However, IL-7 can also costimulate the proliferation of mature T cells (Grabstein et al., 1990; Morrissey et al., 1989).

Experiments using mice with a targeted disruption of the genes encoding IL-2, IL-4, IL-7, IL-2Ra, IL-2Rβ, IL-7Rα or the common γ chain have contributed to our understanding of the distinct roles of these cytokines:

**IL-2-deficient mice**

In mice lacking IL-2, lymphoid development is normal, and so is the composition of peripheral T cell populations during the first 3-4 weeks of age (Sadlack et al., 1993; Schorle et al., 1991). IL-2 deficiency, however, was manifested at the functional level: T cells isolated from these mice (~4 weeks of age) responded poorly to proliferative/polyclonal stimuli such as concavalin A and anti-CD3, an effect which could be overcome when exogenous IL-2 was added (Schorle et al., 1991). The differentiation of B cells was affected in as much as the isotype levels of serum immunoglobulins changed - drastic increase in IgG1 and IgE levels - suggesting an at least skewing of T cell help toward Th2-dependent isotypes. Older mice, however, become severely compromised, and about 50% of the animals die between 4 and 9 weeks after birth of an unknown cause (Sadlack et al., 1993). Of the remaining mice, 100% develop an inflammatory bowel disease which is similar to ulcerative colitis in humans. The alterations of the immune system are characterised by a high number of activated hyperproliferative T cells and B cells, elevated immunoglobulin secretion, anti-
colon antibodies, enormous T cell infiltrates in the colon, and aberrant expression of MHC class II molecules. Unexpectedly, this implicates IL-2 as a cytokine with a unique function in the negative regulation of the immune reaction while proliferative responses, at least in part, can be compensated by other mediators; as IL-15 can bind to the intermediate affinity IL-2R consisting of the IL-2Rβ and γc chain, it is possible that IL-15-binding can compensate for some IL-2-mediated responses.

**IL-4-deficient mice**

In IL-4-deficient mice, T cells and B cells develop normally (Kopf et al., 1993; Kuhn et al., 1991). Consistent with its predicted role in class switching of immunoglobulin production by B cells, IL-4−/− mice exhibit a strongly reduced serum level of IgG1 and produce no IgE (Kuhn et al., 1991). In addition, the levels of Th2-type cytokines such as IL-5 and IL-10 was decreased (Kopf et al., 1993).

**IL-7-deficient mice**

Mutant mice are highly lymphopenic in the peripheral blood and lymphoid organs (Von Freeden-Jeffry et al., 1995): thymic cellularity is reduced 20-fold and splenic T cell cellularity 10-fold. Interestingly, thymocytes retain a normal distribution of CD4 and CD8 cells which indicates that IL-7 is most important for the proliferative expansion (or protection from apoptosis) of thymocytes. Similarly, splenic B cells are decreased in numbers but also an abnormal population of immature B cells is present in adult IL-7−/− animals.

**IL-2Rα-deficient mice**

Although IL-2Rα expression occurs at specific stages of early T cell and B cell development, young mice that lack IL-2Rα show no phenotypic abnormalities in T cell and B cell development (Willerford et al., 1995). However, as adults, similar to adult mice with an ablated IL-2 gene, these mice have a polyclonally expanded T and B cell
population resulting in massive enlargement of peripheral lymphoid organs. T cells from these mice show impaired activation-induced cell death \textit{in vivo} (Willerford et al., 1995). Older IL-2R$\alpha$-deficient mice also develop autoimmune disorders including inflammatory bowel disease. Thus, IL-2R$\alpha$ appears to be essential for the regulation of clonal expansion and cell death following lymphocyte activation, and in particular is important for the termination phase of the immune reaction.

\textit{IL-2R$\beta$-deficient mice}

As predicted from the IL-2 and IL-2R$\alpha$ knockout mice, thymic development in mice lacking the IL-2R$\beta$ chain appears to be normal (Suzuki et al., 1995). Similar to these mutant mice the IL-2R$\beta$-deficient mice have spontaneously activated T cells, infiltrative granulocytopenia, autoantibody production, and high serum concentrations of IgG1 and IgE indicative of a defect in B cell class switching and a shift towards Th2-type subsets. The animals die at an age of about 12 weeks. In marked contrast, however, to the IL-2- or IL-2R$\alpha$-deficient mice, T cells isolated from the IL-2R$\beta$ knockout mice do not proliferate in response to polyclonal activators, nor could antigen-specific immune responses be elicited. This suggests that IL-2R signalling is essential for the proliferation and clonal expansion of T lymphocytes and that the low level proliferation observed in IL-2 or IL-2R$\alpha$ knockout mice is due to substituting effects of another cytokine, possibly IL-15, which signals \textit{via} the intermediate affinity IL-2R (B$\gamma$c).

\textit{IL-7R$\alpha$ deficient mice}

Similar to the IL-7-deficient mice, ablation of IL-7R$\alpha$ yields a phenotype where thymic cellularity ranges from 0.01 to 10% of that of the IL-7R$^{+/+}$ thymus (Peschon et al., 1994). B cell development is severely impaired at a pro-B cell stage in the bone marrow. In contrast to the IL-7 knockout, however, in which the thymus contains normal subsets of thymocytes, these mice show two distinct thymus phenotypes. One group (35% of all IL-7R$\alpha$-deficient mice) exhibits a similar phenotype as the IL-7-deficient mice
and displays normal CD4/CD8 subpopulations. The other group though (65% of all IL-7Rα-deficient mice) is composed exclusively of CD4+CD8+CD3+ thymocytes indicating an early block at the double-negative (DN) stage. To explain this discrepancy it has been postulated that IL-7Rα may function in an additional - as yet unknown cytokine receptor - and may be important to initiate TCR gene rearrangement (Crompton et al., 1997). IL-7Rα has also been reported to be required for the development of γδ+ T cells, while the receptor subunit is not essential for NK cell differentiation (He and Malek, 1996). Studies using T cells from IL-7Rα−/− mice which reach the periphery demonstrate that these T cells do not respond to proliferative stimuli including receptor-independent stimuli such as phorbol ester or ionomycin and the majority of these cells undergo programmed cell death (Maraskovsky et al., 1996). It has therefore been suggested that IL-7Rα-deficient T cells have an intrinsic proliferative defect and IL-7Rα is important for the functional maturation of T cells.

**γc chain-deficient mice (XSCID)**

As the γc receptor chain is a component of e.g. the IL-2R, the IL-4R and the IL-7R, gene inactivation of this subunit is likely to result in a cumulative defective phenotype. Indeed, mutations in the γc subunit in humans results in X-linked severe combined immunodeficiency (XSCID) (Noguchi et al., 1993). This disease is characterised by the absence or greatly reduced numbers of T cells and NK cells and the presence of non-functional B cells. Mice lacking the γc receptor chain exhibit defects in lymphoid development that appear more severe than human XSCID (Cao et al., 1995). Thus, these mice also have greatly diminished numbers of B cells which is in contrast to human XSCID. Other populations/tissues which are virtually absent in XSCID mice include NK cells, γδ+ T cells, dendritic epidermal T cells, peripheral lymph nodes, and gut-associated lymphoid tissue.
1.3 Protein tyrosine kinases utilised by the TCR, CD28 and the IL-2R to connect to downstream effector cascades

Protein tyrosine kinases (PTKs) play a crucial role in cellular proliferation and differentiation. Tyrosine phosphorylation by PTKs serves at least three main purposes:

1. Tyrosine phosphorylation of a signalling enzymes can increase/decrease its catalytic activity.
2. Tyrosine phosphorylated amino acid residues contribute binding sites for Src homology (SH) 2 and PTB '-phosphotyrosine binding'- domains (see below) that specifically recognise the phosphotyrosine moiety. Therefore, proteins containing SH2/PTB domains can be recruited to the site of phosphorylation and signalling complexes can be formed in a phosphotyrosine-dependent manner.
3. Moreover, (tyrosine) phosphorylation can contribute to the subcellular localisation of an effector molecule. The first intracellular biochemical events detected after ligation of the TCR, CD28 or the IL-2R, is the activation of cytoplasmic PTKs. Numerous PTKs have been implicated in mediating these initial tyrosine phosphorylation events which will ultimately orchestrate downstream effector pathways:

1.3.1 Src family tyrosine kinases: Lck and Fyn

T cells express at least three members of the src family of tyrosine kinases, p56lck, p59fyn(T), and p62yes, both p56lck and p59fyn(T) being primarily T cell-specific. Both Lck and Fyn contain a N-terminal myristoylation/palmitoylation site followed by an unique region, one SH3 domain and one SH2 domain (see Fig 1.6) (Peri and Veillette, 1994). During TCR signalling their catalytic activity is regulated by the balanced action of p50csk and CD45 which phosphorylate and dephosphorylate a tyrosine residue in the C-terminus of Lck/Fyn, respectively (Bergman et al., 1992; Mustelin and Altman, 1990; Mustelin et al., 1992). Phosphorylation of this C-terminal tyrosine residue is thought to attenuate Lck/Fyn activity by binding through an intramolecular interaction to their SH2 domain. Whilst Lck is associated with CD4/CD8
via its N-terminal unique region (Shaw et al., 1990; Turner et al., 1990), Fyn may bind to phosphorylated ITAMs via its SH2 domain although binding does not appear to depend on specific residues within the ITAM motif (unlike ZAP-70) (Gauen et al., 1994). However, Fyn has also been reported to interact with the unphosphorylated ζ or CD3 chains which requires at least 4 amino acid residues (Gly2, Cys3, Lys7, Lys9) in its N-terminus (Gauen et al., 1996). These residues are also essential for sufficient fatty acylation and plasma membrane targeting of Fyn. A primary function of the src family kinases is to phosphorylate key tyrosine residues within the ITAMs (Iwashima et al., 1994). Other functions of these kinases include the phosphorylation and concurrent activation of ZAP-70 (Wange and Samelson, 1996), autophosphorylation to increase their own catalytic activity (Chan and Shaw, 1995), and phosphorylation of downstream substrates. Here, an interesting example is the phosphorylation of the I(1,4,5)P₃ receptor by Fyn (the I(1,4,5)P₃ receptor regulates intracellular Ca²⁺ levels) (Jayaraman et al., 1996). Genetic experiments in mice, in which Lck- or Fyn-function is either ablated or enhanced, suggest unique roles for them during development - in particular Lck appears to be important for the normal maturation of thymocytes (Perlmutter et al., 1993; Weiss and Littman, 1994). Nevertheless, in Lck-deficient mice a few T cells reach the periphery which raised the question whether Lck and Fyn serve some redundant functions. Analyses of the Fyn/Lck double knockout suggest that there is indeed redundancy in pre-TCR signalling as these mice have completely abolished αβ T cell development (Groves et al., 1996; Van Oers et al., 1996).

Figure 1.6. A selection of signalling molecules and their modular structure. Reference to the structural features of these molecules will be made throughout the text. The predicted molecular mass is indicated to the right. SH, Src homology domain; PH, pleckstrin homology domain; PH-1/PH-2, split PH domain in PLCγ; P, tyrosine phosphorylation sites; Cys, cysteine rich motif providing diacylglycerol-binding site; proline, proline-rich region providing SH3 domain binding site; PTB, phosphotyrosine-binding motif (Asn-Pro-Xxx-Tyr); Gly/Pro, glycine/proline rich region with homology to collagen; Rho GAP, homologous region to rhoGAP domain in Bcr; GRP33, hetero nuclear RNA binding motif; Arg/Gly, motif containing demethylated arginine residues found in RNA binding proteins. α2-β2-chimaerin is an alternate splice variant of n(α1)-chimaerin and contains an SH2 domain not present in n-chimaerin.
Lck, Fyn 56,59 kDa
ZAP-70 70 kDa
Itk, Btk, Tec 62-77 kDa
PLC\(\gamma\)1 145 kDa
SHP-1, SHP-2 70 kDa
p120 GAP 120 kDa
Vav 95 kDa
C3G 121 kDa
Grb2 26 kDa
Crk II, CrkL 34 kDa
Nck 45 kDa
p85 of PI 3-kinase 85 kDa
p52 Shc 52 kDa
p66 Shc 66 kDa
p130CAS 130 kDa
Cbl 120 kDa
Stat5 90 kDa
\(\alpha\)2-, \(\beta\)2-chimaerin 45 kDa
p190 190 kDa
p62 dok 62 kDa
SAM68 68 kDa
Akt 59 kDa
An additional role for src family PTKs in T cells exists in mediating IL-2R signal transmission (Miyazaki et al., 1995). Lck is activated by IL-2 in peripheral blood lymphocytes (PBLs) (Hatakeyama et al., 1991), and other src family PTKs such as Fyn or p53/p56lyn are activated in an analogous manner in BAF-B03-derived cells (Kobayashi et al., 1993). The physical interaction between Lck and IL-2Rβ is mediated by the catalytic domain of Lck and the acidic region in the IL-2Rβ chain (see Fig. 1.5) (Hatakeyama et al., 1991). Furthermore, the activation of src family PTKs by IL-2 requires the serine-rich region of IL-2Rβ (Minami et al., 1993), and this activation correlates with p21ras activation (Satoh et al., 1992) followed by the induction of possibly c-fos and c-jun gene transcription, which suggests a role for src family PTKs in the induction of these proto-oncogenes (Minami et al., 1993; Shibuya et al., 1994; Shibuya et al., 1992). Lck phosphorylates the IL-2Rβ chain at either or both of two tyrosines (Tyr355 and Tyr358) within the acidic region (Hatakeyama et al., 1991). The mechanism of IL-2-induced Lck activation has not been resolved; however, by analogy with CD4 and CD8, it is possible that ligand binding causes IL-2R oligomerisation or clustering and that this in turn allows associated Lck molecules to transphosphorylate each other which stimulates their catalytic activity (Kolanus, 1993; Weiss and Littman, 1994). Whether Lck activity after IL-2R triggering is controlled by phosphatase activity - in analogy to the regulation by CD45 in TCR signalling - awaits analysis.

1.3.2 ZAP-70 and Syk

ZAP-70 is a PTK that is required for both T cell development and TCR function (Chan et al., 1994; Chan and Shaw, 1995; Howe and Weiss, 1995). ZAP-70 is expressed exclusively in T cells and NK cells and is structurally related to the Syk PTK. ZAP-70 and Syk contain two N-terminal SH2 domains but no myristoylation motif for membrane-recruitment (see Fig. 1.6). Activation of ZAP-70 and Syk is thought to be achieved by (1) binding of their two SH2 domains to the doubly phosphorylated ITAMs (especially, in the case of Syk) (Iwashima et al., 1994; Weiss, 1995) and (2) by specific
tyrosine phosphorylation - in particular, the tyrosine residue at position 493 in ZAP-70 (519 in Syk (Kurosaki et al., 1996)) is critical for its activation as mutation of this residue to phenylalanine abrogates the ability of ZAP-70 to become activated by src family PTKs and interrupts the activation cascade initiated by the TCR (Chan et al., 1995; Wange et al., 1995). Thus, beyond regulating the catalytic activity of ZAP-70, tyrosine phosphorylation of ZAP-70 may provide docking sites for downstream SH2 domain-containing effector molecules (Neumeister et al., 1995). The substrates of ZAP-70 are not well characterised but, T cells from ZAP-70-deficient mice do not respond to TCR ligation by an increase in intracellular calcium, which argues that ZAP-70 may mediate the tyrosine phosphorylation and activation of PLCγ1 (Negishi et al., 1995). Other possible effector candidates include Vav (Deckert et al., 1996; Katzav et al., 1994), rasGAP (Neumeister et al., 1995), Cbl (Fournel et al., 1996) or SHP-1 (Plas et al., 1996). Patients lacking ZAP-70 develop an autosomal-recessive form of severe combined immunodeficiency (SCID) (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994). These individuals lack peripheral CD8+ T cells, and the TCRs expressed on the peripheral CD4+ T cells are non-functional (in contrast, ZAP-70 deficient mice are arrested at the CD4+CD8+ stage). The lack of an intact cellular immune system in these patients increases their risk for a variety of opportunistic infections and contributes to the high mortality rate in their first year of life. In addition, the significance of ZAP-70 for functional TCR signalling has been shown in experiments analysing the molecular events that lead to induction of anergy (Madrenas et al., 1995; Sloan-Lancaster et al., 1994). Thus, anergic stimuli such as altered-peptide ligands lead to different phosphorylation patterns of the TCRζ chain which in turn prevents ZAP-70 phosphorylation and/or its recruitment to the TCR.

Syk is coexpressed with ZAP-70 in T cells, but plays a prominent role in B cells. Mice deficient in Syk arrest early in B cell development (pro- to pre-B cell stage) and die shortly after birth from severe haemorrhaging (Cheng et al., 1995; Turner et al.,
1995). The role of Syk in thymic development is not known, but interestingly Syk expression levels are downregulated as T cells leave the thymus (Chan et al., 1994). The affinity of ZAP-70 or Syk is similar for each of the ITAMs represented in the TCR. These data suggest that multiple ZAP-70 or Syk PTKs can be recruited to a single receptor complex. Moreover, Syk is likely to substitute for ZAP-70 in TCR-controlled signalling pathways, but whether these two tyrosine kinases serve redundant or unique functions in TCR signal transduction awaits analysis. Nevertheless, a unique function for Syk in T cells may exist in transmitting signals delivered by ligation of the IL-2R (Minami et al., 1995; Taniguchi, 1995). Thus, Syk has been shown to associate with the serine-rich region in the IL-2Rβ chain (see Fig. 1.5) which may contribute to the proliferative signals emanating from this region that lead to c-myc gene transcription and cell cycle progression.

1.3.3 The Tec family tyrosine kinase Itk

Recent studies have identified the Itk/Btk/Tec family of PTKs as possible players in T cell signalling (Desiderio and Siliciano, 1994). This family of PTKs is characterised by having an N-terminal pleckstrin homology domain (PH) followed by an SH2 and SH3 domain, the catalytic activity resides in the C-terminal region (see Fig. 1.6). Btk is expressed predominantly in B lymphoid and myelomonocytic lineages and is regulated by the B cell antigen receptor (BCR) (Aoki et al., 1994; DeWeers et al., 1994). Its importance for haematopoietic cell function is exemplified by the notion that mutations in the btk gene at the XLA locus cause X-linked agammaglobulinemia in humans (xid in mice). Itk (Emt) is predominantly expressed in T lymphocytes. Itk−/− mice produce fewer thymocytes, and mature T cells isolated from these mice proliferate poorly in response to TCR-triggering, but respond normally to phorbol ester plus ionomycin treatment (Liao and Littman, 1995). In Jurkat cells, TCR ligation leads to transient tyrosine phosphorylation of Itk which may increase its catalytic activity (Gibson et al., 1996). Similarly, Itk is also tyrosine phosphorylated after CD28
stimulation (August et al., 1994; Gibson et al., 1996). Tyrosine phosphorylation of Itk in both TCR or CD28 signalling requires functional Lck.

1.3.4 Jaks

In cytokine signalling much attention has been focused on a novel kinase family which share the unusual feature of having two kinase domains, and therefore were termed Jaks - Janus kinases (originally cloned as 'just another kinase'). At least four members have been identified: Jak1, Jak2, Jak3, Tyk2. Whilst Tyk2, Jak1, and Jak2 are ubiquitously expressed, Jak3 expression is restricted to the myeloid and lymphoid lineages. Jak1 (135 kDa) and in particular Jak3 (120 kDa) are important for IL-2 signal transduction (Asao et al., 1993; Beadling et al., 1994; Boussiotis et al., 1994; Johnston et al., 1994; Kirken et al., 1995; Miyazaki et al., 1994; Russell et al., 1994; Witthuhn et al., 1994). Jak1 associates with the IL-2Rβ chain and Jak3 binds the IL-2Rγc chain. These interactions require the serine-rich region of IL-2Rβ and the C-terminal 48 amino acid residues of IL-2Rγc, respectively, and both regions are critical for Jak activation and proliferative signal transmission. On Jak3 the N-terminal portion of the protein has been implicated in the interaction with the IL-2Rγc chain (Taniguchi, 1995). The importance of Jak3 for IL-2 / cytokine signalling is underlined by the finding that patients lacking a functional jak3 gene have an autosomal-recessive form of severe combined immunodeficiency (SCID), which is phenotypically similar to XSCID of patients deficient in the γc chain (see above) (Russell et al., 1995). Mice with ablated Jak3 function exhibit a similar phenotype, and the residual T cells and B cells are functionally defective (Nosaka et al., 1995). The precise activation mechanism of Jak1 and Jak3 has not been elucidated, but it is thought that ligand-induced receptor dimerisation or oligodimerisation brings about the local aggregation of these molecules, resulting in the stimulation of the catalytic activity by transphosphorylation (Ihle, 1995). Once activated, the Jaks phosphorylate certain tyrosine residues in the IL-2Rβ and IL-2Rγ chains which provide binding sites for SH2 domain containing downstream signalling molecules, that in turn
become tyrosine phosphorylated themselves. In particular, one group of substrates which is recruited into the activated IL-2R complex has been in the centre of the attraction due to their direct line to the nucleus: the STATs - signal transducers and activators of transcription (see section 1.4.4).

1.3.5 PTKs in immediate TCR signalling

At least three cytoplasmic PTKs are important for the initiation of TCR signal transduction, Lck, Fyn and ZAP-70. While Lck is associated with the cytoplasmic tail of the CD4/CD8 coreceptor in quiescent cells, Fyn and ZAP-70/Syk are in the proximity of the TCR/CD3 complex and become recruited to the tyrosine phosphorylated receptor chains upon engagement of the TCR (in the case of Fyn, it is possible that Fyn is already associated with the ITAMs). The proposed sequence of events in the TCR-PTK cascade is: (1) CD4-Lck (or CD8-Lck) are coapproximated into the TCR/CD3 complex and CD45 removes a negative regulatory phosphate at amino acid residue 505 and 528 in the C-terminus of Lck and Fyn, respectively. (2) Activated Lck and/or Fyn phosphorylate the ITAMs in the TCR/CD3 cytoplasmic tails. (3) ZAP-70 (and/or Syk) associates with the doubly-phosphorylated ITAMs - phosphorylation of both tyrosines present within the ITAM is required for sufficient binding of ZAP-70. Moreover, recruitment of ZAP-70 to the ITAMs is essential for its stimulation and for T cell activation, since agents that block recruitment prevent these events (Qian et al., 1996; Wange et al., 1995). (4) The phosphorylation of ZAP-70 on Y493 by a src family PTK results in the activation of ZAP-70 kinase activity (Chan et al., 1995; Wange et al., 1995). (5) Both ZAP-70 and src family PTKs are thought to phosphorylate downstream effector molecules and/or proteins associated with the cytoskeleton (Cantrell, 1996; Wange and Samelson, 1996) (see section 1.4.2). Although quite a few effector molecules are known which become tyrosine phosphorylated, further studies are necessary to unravel which PTK is responsible for a specific/single phosphorylation event.
1.3.6 PTKs in immediate CD28 signalling

The precise makeup of PTKs which are responsible for phosphorylating the tyrosine residues in the cytoplasmic tail of CD28 has not been established. One candidate is Lck (Gibson et al., 1996; Raab et al., 1995). Once CD28 is tyrosine phosphorylated, Itk (Emt) can bind via its SH2 domains to the receptor (August et al., 1994). In addition, it has been reported that serine phosphorylation of the CD28 intracellular region may be important for its signalling function (Hutchcroft et al., 1995; Hutchcroft et al., 1996) (Steve Ward, personal communication).

1.3.7 PTKs in immediate IL-2 signalling

At least two distinct tyrosine kinase families are essential for IL-2R signal transmission, the src family PTK(s) Lck (and/or Fyn) and the Jak family of PTKs, Jak1 and Jak3. Furthermore, the Syk PTK may also contribute to IL-2R signalling. However, the interplay of these kinases is not yet known. Thus, both Lck and Jaks have been reported to be essential to couple the IL-2R to downstream signalling events by mediating IL-2R chain phosphorylation which creates docking sites for effector molecules that contain SH2 and/or PTB domains. Proteins that associate with the phosphotyrosines in the IL-2R chains and the signalling pathways they initiate will be eluded to in section 1.4.3 and 1.4.4.

1.4 Connections to the cellular intranet

Extensive research has concerned the molecular links which connect the phalanx of PTKs at the plasma membrane to the intracellular signalling networks. As briefly mentioned in the previous section, the formation of molecular networks is highly dependent on the modular structure of the effector proteins involved and the establishment of specific protein-protein interactions - or as more recently demonstrated
the founding of lipid-protein associations. The nature of such complex formation will be discussed in the following section, reviewed in (Cohen et al., 1995; Gibson et al., 1994; Koch et al., 1991; Pawson, 1995; Pawson and Gish, 1992; Van der Geer and Pawson, 1995). Moreover, a brief discussion on the cellular substrates of TCR-controlled PTKs will follow and how they connect to effector cascades. Proteins with modular structure are also important for IL-2R coupling to downstream signalling pathways. Finally, the STAT pathway used by the IL-2R and other cytokine receptors to connect to gene transcription will be described.

1.4.1 Signalling modules: SH2 domains, SH3 domains, PTB domains, PH domains

The archetypal interaction modules are the SH2 and SH3 domains, found in a large array of signalling components (see Fig 1.6). The Src homology domains were identified in the Rous sarcoma virus oncogene, Src, which contains an SH2 and SH3 domain, and noted as regions of homology between Src, Crk and PLCγ. SH2 domains bind phosphorylated tyrosine residues in the context of the three amino acids C-terminal to the phosphotyrosine (Tyr\(^{P}\)-Xxx-Xxx-Xxx). SH3 domains bind proline-rich sequences. Another binding module (of about 40 amino acids) which interacts with motifs rich in proline is the WW domain (consensus Xxx-Pro-Pro-Xxx-Tyr) (Sudol, 1996). Recent additions to the modular kit for the creation of the perfect signalling molecule, are Pleckstrin-homology (PH) domains, which are predominantly present in a variety of membrane-associated proteins. PH domains are thought to play a role in both protein-protein or lipid-protein interactions. A novel phosphotyrosine-binding (PTB) domain has been identified in the adaptor protein Shc and in IRS-1 ('insulin receptor substrate-1') that appears to be responsible for protein-protein interactions. Whilst SH2 domain-binding is directed by the amino acids C-terminal to the phosphotyrosine, PTB domain recognition is mediated by the amino acid residues N-terminal to the
phosphotyrosine (consensus motif Asn-Pro-Xxx-Tyr\(^P\)). All of the above domains are real protein domains in that they have the capacity to maintain their structure in isolation.

**SH2 and PTB domains**

The SH2 domain is a motif of about 100 amino acid residues. All SH2 structures solved so far contain a large central antiparallel \(\beta\)-sheet, two flanking \(\alpha\) helices, and follow a general pattern of \(\beta-\alpha-\beta-\beta-\beta-\beta-\alpha-\beta\) protein structure. A conserved arginine surrounded by hydrophobic/basic residues is essential for phosphotyrosine binding (often a Phe-Leu-Val-Arg (FLVR) motif is present). SH2 domains bind to phosphopeptides of optimal sequence with relatively high affinity: \(K_d=10-100\) nM. Binding to phosphotyrosine can affect SH2 domain-containing proteins in many ways, including direct stimulation of the enzymatic activity, conformational changes, alteration of subcellular localisation, and enhanced tyrosine phosphorylation. The PTB domain is a motif of about 160 amino acid residues, a high-resolution of its structure has not been determined. It is not known whether the PTB domain has specific functions not encoded by SH2 domains.

**SH3 domains**

SH3 domains which consist of about 80 to 100 amino acids are found in many proteins involved in PTK signalling, but also in cytoskeletal components. Indeed, one of the first reports on the function of SH3 domains showed that SH3 domains localise proteins to components of the actin cytoskeleton (Bar-Sagi et al., 1993). The structure of SH3 domains shows a bigger variety than the one of SH2 domains, nevertheless, an overall topology is conserved which consists of five antiparallel \(\beta\) strands that pack to form two perpendicular \(\beta\) sheets. A hydrophobic patch that contains a cluster of aromatic residues and is surrounded by two charged and variable loops forms the ligand-binding pocket. SH3 binding sites consist of proline-rich peptides of approximately 10 amino acids which bind to isolated SH3 domains with dissociation
constants of $K_d$=5-100 μM. The core ligand appears to be a seven-residue peptide containing the consensus Xxx-Pro-pro-Xxx-Pro, where X tends to be an aliphatic (Phe, Leu, Ala, etc.) residue and the two conserved prolines are crucial for high affinity binding. The intervening scaffolding amino acid residue (pro) also tends to be a proline. SH3-binding peptides are pseudo-symmetrical and can potentially bind in either orientation. Specificity of SH3 binding is apparently conferred by the interaction between non-proline residues in the ligand and the two variable SH3 loops flanking the main hydrophobic binding surface. Many SH3 domain to proline-ligand interaction in the cell are constitutive and not regulated by receptor activation itself - rather additional binding modules in either partner may relocalise the protein complex to a different site in the cell. Nevertheless, the conformation of the SH3 domain containing protein or its binding partner may control accessibility of the SH3 domain or its binding site. Potential association partners may also be spatially separated, only interacting when juxtaposed by relocation. In addition, several SH3-binding sites have consensus sites for proline-directed kinases, such as Erks, which may affect the contacts made with the SH3 domain (Cherniack et al., 1995; Cherniack et al., 1994).

**PH domains**

The PH domain is a region of approximately 100 amino acids, first recognised in the pleckstrin protein, the major protein kinase C (PKC) substrate in platelets (Haslam et al., 1993; Mayer et al., 1993). The sequence homology of the PH domain among proteins is generally low, but despite this fact their overall structure is virtually the same. The basic PH fold contains two antiparallel β sheets and a (long) C-terminal amphipathic α helix. The PH structures differ most in the loop region between the β strands - both in length and sequence. Many of the conserved hydrophobic residues found in PH domains are located in the interior of the protein, while the highly conserved charged residues are solvent-exposed. PH domains have been reported to interact with two distinct ligands: phosphatidylinositol-polyphosphates and the $\beta\gamma$ subunits of
heterotrimeric G proteins. How can one domain accommodate two structurally so
different molecules? The answer to this question is not entirely clear yet, but these
ligands may interact with two different regions present in PH domains. Thus,
phosphatidylinositol-polyphosphates could bind to one side of the PH domain, where
excess to the hydrophobic cleft is available (could accommodate the lipid side chain),
and three variable loops and clusters of positive charges are present (could confer
specificity for the polyphosphate moiety). PH domains which bind $\beta\gamma$ subunits (or
PKC) on the other hand - have been suggested to interact via their C-terminal $\alpha$ helix
which is extended in PH domains that bind $\beta\gamma$ subunits. At present, it is not known how
ligand binding to the PH domain 'activates' the protein possessing it. Possible
mechanisms are conformational changes or localisation to specific areas within the
plasma membrane mediated by ligand-binding. Furthermore, PH domains could confer
constitutive membrane assignment - in particular when the PH domain binds a lipid that
is contained in the plasma membrane, such as phosphatidylinositol 4,5-bisphosphate
(PI($4$,5)P$_2$). The importance of an intact PH domain is illustrated by a missense mutation
in the X-linked $btk$ gene that alters an amino acid (R28C) in the Btk PH domain and
impairs murine B cell development (Thomas et al., 1993).

1.4.2 Adaptor molecules, substrates of TCR-controlled protein tyrosine
kinases and overview on TCR-Induced signalling pathways

Possible functions of adaptor molecules in TCR signalling

One crucial function of TCR signalling - or receptor signalling in general - is
signal amplification at the inner phase of the plasma membrane. One possible
mechanism to achieve this has been mentioned above and involves the activation of
several PTK molecules per TCR. A second wave of amplification could take place by
recruiting more that one downstream effector enzyme to one PTK resulting in the
stimulation of these effectors - directly or indirectly - by tyrosine phosphorylation
events. The TCR, however, only provides a limited number of docking sites - most of
which are likely to be occupied by ZAP-70 - and therefore requires additional means to attract effectors to the PTK. Such means could be provided by adaptor molecules which have the common feature to contain at least two binding modules and therefore can form a bridge between at least two different molecules. At their proximal end, these adaptors could bind to the TCR/CD3 chains or alternatively to ZAP-70 (or a src family PTK) which could also act as a docking protein. At their distal site, however, they could have the capacity to associate with several different downstream effector molecules: the adaptor molecule could bind distinct effector molecules at the same time, an example is IRS-1 (White, 1994); or alternatively, the adaptor molecule could interact with different downstream signalling molecules, in a timely and spatial restriction, an example is Grb2 (see below and chapters 3, 4). A selection of adaptor molecules or enzymes containing binding modules is illustrated in Fig. 1.6 (see pages 24, 25).

**Brief overview of TCR-induced signalling pathways**

The simplest of adaptor molecules do not contain an enzymatic activity. Such adaptor molecules are important for coupling the TCR to downstream signalling pathways and include Grb2, p85 of PI 3-kinase, or Crk (Cantrell, 1996; Reif et al., 1994; Wange and Samelson, 1996) (Fig. 1.6, Fig. 1.7). Others, such as Nck or Shc, have not as yet been allocated a function in TCR signal transmission, although both proteins become tyrosine phosphorylated after TCR triggering (Park and Rhee, 1992; Ravichandran et al., 1993). The role of Crk in TCR signalling will be discussed below. The discovery of the function of Grb2 will be described in this section, however, a discussion of its role in TCR signalling will only follow in chapter 3 and chapter 4. The activation of PI 3-kinase mediated by its adaptor subunit p85 will be introduced in section 1.6, and its regulation by the TCR will be examined in chapter 4. Nck may have a potential role in TCR signalling by connecting the TCR to the Wiscott-Aldrich syndrome protein (WASP) (Rivero-Lezcano et al., 1995), to the proto-oncogene c-cbl (Cbl) (Rivero-Lezcano et al., 1994), or to the serine/threonine kinase Pak1 (Galisteo et
(Galisteo et al., 1996; Lu et al., 1997), that have been shown to associate with Nck in other systems. Although tyrosine phosphorylated by the TCR, Shc phosphorylation is low when compared to IL-2 induced phosphorylation of Shc and thus Shc is thought to play a crucial role in IL-2R signal transmission (see section 1.4.3) (Osman et al., 1995).

**Figure 1.7.** Adaptor proteins and molecules with signalling modules couple the TCR/PTK complex to the activation of downstream effector cascades. P, phosphotyrosine.

At least two major signalling pathways are initiated in response to the TCR-controlled action of PTKs and involve effector enzymes with modular structure (Fig. 1.7): phospholipase-γ1 (PLCγ1) and the GTPase activating protein (GAP) rasGAP (p120 GAP). PLCγ1 is tyrosine phosphorylated after TCR-triggering and is responsible for the hydrolysis of PI(4,5)P2 generating diacylglycerol (DAG) and I(1,4,5)P3 that lead to the activation of the serine/threonine kinase protein kinase C (PKC) and the stimulation of Ca2+-dependent signalling pathways. rasGAP is tyrosine phosphorylated in response to TCR ligation and is thought to couple the TCR to p21ras effector pathways including the activation of the Erk Map kinase pathway (see section 1.5 and chapter 3).
The role of the guanine nucleotide exchange factor (GEF) Vav - a TCR/PTK substrate - for T cell responses will be elucidated in chapter 3. Another substrate of TCR induced PTKs is Cbl, a proto-oncogene that may possibly have a function as a transcription factor and negative regulator of PTK-controlled effector pathways (Hunter, 1997). Recently, the tyrosine-phosphatases SHP-1 and SHP-2 have been shown to be regulated by the TCR (Plas et al., 1996; Tailor et al., 1996). SHP-1 associates via its SH2 domain with tyrosine phosphorylated ZAP-70 (Plas et al., 1996). This association decreases the catalytic activity of ZAP-70 and increases the one of SHP-1. Overexpression of SHP-1 leads to a reduction in IL-2 production delivered by the TCR. Thus, SHP-1 may by responsible for the termination phase of TCR-initiated events.

**Crk**

The product of the v-crk oncogene was the first adaptor-like protein to be identified (Mayer et al., 1988). Three mammalian homologues have since been described, Crk I, Crk II and Crk L (Matsuda et al., 1992; ten Hoeve et al., 1993). Crk I has an SH2 domain and an SH3 domain, whereas Crk II and Crk L consists of one SH2 domain followed by two SH3 domains. Crk L seems to be the predominate Crk protein in haematopoietic cells (ten Hoeve et al., 1993). In T cells, Crk interacts with Cbl via its SH2 domain (see below) and Cbl may be identical with a 116 kDa molecule, previously reported to bind to the Cbl SH2 domain (Sawasdikosol et al., 1995). Via its SH3 domains Crk binds to C3G, an exchange factor for ras family or related proteins (Tanaka et al., 1994), and complex formation between Cbl-Crk-C3G upon TCR stimulation has been reported (Reedquist et al., 1996). Furthermore, Crk SH3 domains may associate with the p21ras exchange factor, Sos (Matsuda et al., 1994). The role of Crk in T cells is not clear, but oncogenic v-Crk can cause differentiation in PC12 cells (Hempstead et al., 1994) and C3G has been suggested to be a GEF for Rap1 (Gotoh et al., 1995).


Cbl

The cbl oncogene was first identified as part of the Cas NS-1 transforming retrovirus which arose in a mouse pre-B cell lymphoma. Its protein product, p120cbl, is cytoplasmic and has several distinctive domains including a highly basic region, a RING finger motif found in transcription factors, a large proline-rich domain and several tyrosine phosphorylation motifs. Cbl is a substrate for TCR-controlled PTKs (Donovan et al., 1994), and has been reported to associate with ZAP-70 (Fournel et al., 1996) which may be mediated by a phosphotyrosine binding domain present in the N-terminal region of Cbl (Lupher Jr. et al., 1996). Cbl is also phosphorylated by many other PTKs and receptor tyrosine kinases (RTKs) including the EGF receptor. In T cells, Cbl apparently can form complexes with several proteins. Tyrosine phosphorylated Cbl may associate with the SH2 domains of p85β (Hartley et al., 1995), or with a region containing the SH3 and N-SH2 domain of p85α (Fukazawa et al., 1995; Meisner et al., 1995). Likewise, Cbl can bind to the SH2 domain of Crk (Buday et al., 1996; Reedquist et al., 1996). In quiescent cells, Cbl is constitutively associated with the SH3 domains of Grb2, but dissociates from the Grb2 SH3 domains upon TCR stimulation and can then bind equally to the Grb2 SH2 domain and the Grb2 SH3 domains (Buday et al., 1996; Meisner et al., 1995). Finally, it may associate with the 14-3-3 protein (Liu et al., 1996). A possible function for Cbl has been revealed during a genetic analysis in Caenorhabditis elegans. These studies identified a Cbl homologue, called Sli-1, that negatively regulates the EGF receptor-related Let23 RTK (Yoon et al., 1995). Cbl may play a similar role in downregulating TCR-induced PTK-responses in T cells. Interestingly, CD38-mediated growth suppression of B cell progenitors requires activation of PI 3-kinase and involves its association with Cbl (Kitanaka et al., 1996).

Grb2

Grb2 contains a central SH2 domain flanked by two SH3 domains. Cloned in mammalian cells due to its association with the EGF receptor (Lowenstein et al., 1992)
(Grb=growth factor receptor-bound protein), its function was revealed by studies of the *Caenorhabditis elegans* vulval development which implicated the Grb2 worm homologue Sem5 as the link between the RTK Let23 and Ras (Let60) (Clark et al., 1992). The final clue of how Grb2 may achieve this task derived from studies of the eye development in *Drosophila melanogaster*, regulated by the Sevenless RTK, which identified a putative nucleotide exchange factor, named Son of Sevenless (Sos), as the missing component between Sevenless and *Drosophila* Ras1 (Downward, 1994). Once the mammalian mouse homologue of Sos was cloned (Bowtell et al., 1992), the race was on to experimentally prove the functional link-up of RTK, Grb2, Sos, and Ras. The result turned out to be quite simple as Grb2 was found in a complex with Sos (Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993), and the interaction was demonstrated to occur directly between the SH3 domains of Grb2 and proline-rich motifs in the C-terminal region of Sos (Egan et al., 1993; Rozakis-Adcock et al., 1993). During the course of my thesis, I investigated whether Grb2 could have pleiotropic functions and play a role in linking the TCR to PI 3-kinase or other signalling pathways (see chapters 3, 4).

### 1.4.3 Adaptor molecules in IL-2R signalling and IL-2 induced signalling pathways

Similar to the TCR, the IL-2R orchestrates effector cascades via adaptor molecules. In contrast to the TCR, however, the adaptor proteins appear to bind directly to the cytoplasmic region in the IL-2R chains. Several proteins have been identified that associate with the phosphotyrosines in the IL-2Rβ chain and link the IL-2R to downstream events (Fig. 1.8). These include Shc, p85 of PI 3-kinase, and STAT5 (see section 1.4.4).
Figure 1.8. IL-2R signalling pathways.
Simplified schematic to illustrate signalling pathways orchestrated by the IL-2R. Adaptor molecules link IL-2R PTK activation to downstream signalling events. P, phosphotyrosine (red) or phosphoserine/threonine (yellow); note: only phosphorylation events which are referred to in the text are indicated.

Shc

Shc is tyrosine phosphorylated upon IL-2 ligation and binds to Tyr338 in the IL-2Rβ chain (Burns, 1993; Ravichandran and Burakoff, 1994). Shc is likely to couple the IL-2R via Grb2 and the GEF Sos to Ras-controlled downstream signalling cascades (Ravichandran and Burakoff, 1994). In this respect, it has been known for long that Ras is activated by IL-2 (Graves et al., 1992; Satoh et al., 1991), however, the precise stimulation mechanism has not been determined - either by GAPs or GEFs. It is an attractive possibility, that the Shc-Grb2-Sos complex formation links the IL-2R to Ras effector pathways. Ras effectors that are activated by IL-2 include Raf (Turner et al., 1991; Zmuidzinas et al., 1991), Mek (Kamitz et al., 1995), and Erk (Fairhurst et al., 1993;
The Ras Map kinase pathway is thought to be important for gene transcription (possibly c-fos) and essential for mitogenesis (Friedmann et al., 1996; Gaffen et al., 1996; Zhu et al., 1994).

**p85 of PI 3-kinase**

After IL-2 stimulation the p85 subunit can directly associate with the phosphotyrosine residue 392 in the IL-2R β chain (Truitt et al., 1994). Alternatively, in response to IL-2, the p85 subunit can bind to the src PTKs Lck or Fyn (Karnitz et al., 1994; Taichman et al., 1993). This interaction is predicted to be mediated by the SH3 domain of the src PTK and proline-rich motifs in the p85 subunit (for further details see section 1.6, chapter 4 and chapter 5). Therefore, due to the direct interaction of PI 3-kinase with the tyrosine phosphorylated IL-2R or src PTKs, PI 3-kinase activity can be recovered from anti-phosphotyrosine immune complexes in IL-2 stimulated cells (Augustine et al., 1991; Kanazawa et al., 1994; Merida et al., 1991; Merida et al., 1993; Remillard et al., 1991; Truitt et al., 1994). The association of PI 3-kinase with the IL-2R (or a src PTK) recruits the p110 catalytic subunit of PI 3-kinase to the plasma which in turn leads to the production of D-3 phosphoinositides. Such an increase in phosphoinositides can be prevented by wortmannin or LY294002 treatment, agents which block the catalytic activity of the p110 subunit. Using these inhibitors, PI 3-kinase activity was shown to be essential for the IL-2 mediated stimulation of the serine/threonine kinase p70 S6 kinase (p70S6k) (Monfar et al., 1995). p70S6k activation by IL-2 is also prevented by the immunosupressant rapamycin, which is known to block entry into S phase of the cell cycle (Kuo et al., 1992). In this respect, both PI 3-kinase and p70S6k are thought to be essential for events leading to proliferation (see section 1.6 and chapter 7).
1.4.4 The SH2 domain in STATs - mediator of dimerisation and nuclear translocation in IL-2R signal transmission

Unlike the TCR, cytokine receptors utilise a group of SH2/SH3 domain containing proteins - the STATs - which upon 'activation' can directly translocate into the nucleus to induce gene transcription. STATs are transcription factors that contain in their C-terminal region a SH3 domain and a SH2 domain followed by a tyrosine phosphorylation site which is crucial for homo- (or hetero)-dimerisation and hence STAT function (see Figs. 1.6, 1.8). The DNA binding domain is located in front of the SH3 domain and the transactivation domain is at the very C-terminus (Moriggl et al., 1996).

At present 7 mammalian STATs have been molecularly cloned: STAT1 (STAT1α, STAT1β), STAT2, STAT3 (STAT3α [90 kDa], STAT3β [83 kDa]), STAT4, STAT5A (92 kDa), STAT5B (80 kDa) and STAT6 (IL-4 STAT) (Caldenhoven et al., 1996; Lin et al., 1996; Liu et al., 1995; Mui et al., 1995; Schaefer et al., 1995; Schindler and Darnell, 1995). The predominant IL-2-induced STATs are STAT5A and STAT5B (Beadling et al., 1994; Beadling et al., 1996; Brunn et al., 1995; Hou et al., 1995; Lin et al., 1995; Lin et al., 1996; Wakao et al., 1995). STAT5 binding has been mapped to the phosphorylated tyrosine residues at position 392 and 510 in the IL-2Rβ chain (Lin et al., 1995).

The current model of action holds that once bound via its SH2 domain to the tyrosine phosphorylated IL-2Rβ chain, STAT5 is phosphorylated on tyrosine in its C-terminal region (Tyr694 in huSTAT5A and Tyr699 in huSTAT5B) by Jak1 or Jak3. This phosphotyrosine allows a second STAT5 molecule - mediated by its SH2 domain - to bind to its partner. The STAT5 homodimer falls off the IL-2R - may be upon tyrosine phosphorylation of the second STAT5 - and then is lured into the nucleus via an as yet unknown mechanism. During this journey further phosphorylation events on serine/threonine residues occur which contribute to the localisation and transcriptional activity of STAT5. The serine/threonine kinase(s) which is responsible for these
phosphorylation events is sensitive to H7 - a broad specificity serine/threonine kinase inhibitor (Beadling et al., 1996). However, the known IL-2 regulated serine/threonine kinases Raf, Erk and p70S6k are not involved in this phosphorylation event. (Other STATs including STAT1α, STAT3α and STAT4 contain a consensus site for Map kinases (XPXSP) in their C-terminus (Ihle, 1996).) The identification of this H7-sensitive serine/threonine kinase thus awaits analysis. Finally, although the consensus binding sequences of STATs are known, in IL-2 induced genes only one gene target of STAT5 containing this sequence has thus far been identified, the IL-2Rα chain (John et al., 1996; Lecine et al., 1996). Studies that have concerned the role of STAT5 in IL-2-controlled proliferation - based on IL-2R truncation / phosphorylation mutants with ablated STAT5 binding - came to the conclusion that STAT5 is not required for mitogenesis, although it may contribute to proliferation (Friedmann et al., 1996; Fujii et al., 1995; Gaffen et al., 1996). These results suggest that STAT5 activity participates in other aspects of IL-2 responses, such as regulation of cell survival or cell differentiation. The first study on STAT5A knockout mice has just been published (although it does not directly address the role of STAT5 in IL-2 signalling) (Liu et al., 1997): STAT5A-deficient mice develop normally, however, mammary gland development during pregnancy, which is controlled by prolactin, is impaired demonstrating a predominant role of STAT5A in prolactin-mediated responses. This defect could not be counterbalanced by the presence of STAT5B indicating that STAT5A and STAT5B may perform unique functions.

In other cytokine receptor systems some of the biological functions of STATs have been elucidated: STAT6 binds to an element in the immunoglobulin locus required for IL-4-induced class switching and is therefore important for the occurrence of Th2-controlled responses (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996). STAT4 is essential for IL-12 signal transmission and the development of Th1-mediated immunity (Kaplan et al., 1996; Thierfelder et al., 1996). Finally, STAT3 in gp130 signal
transduction has recently been implicated in the regulation of bcl-2 and suppression of apoptosis (Fukada et al., 1996).

1.5 GTPases govern Map kinase cascades leading to gene transcription

Mitogen-activated protein (Map) kinase cascades are important intermediates in signal transduction pathways. Originally identified as mediators of mitogenic growth factors, it is now clear that Map kinase signalling cascades regulate cellular responses to a wide range of extracellular stimuli. Moreover, it is becoming evident that each Map kinase pathway has 'its own' GTPase as a molecular gatekeeper between input signal derived from the plasma membrane and the occurrence of the first phosphorylation events that eventually will activate the cellular genetic program (Fig. 1.9). An additional level of complexity derives from the notion that each of these GTPases activates more than one effector cascade (Marshall, 1996; Tapon and Hall, 1997).

1.5.1 The ras superfamily of small GTP-binding proteins

The ras superfamily of GTP-binding proteins are all GTPases and function as molecular switches, cycling between an active GTP-bound and an inactive GDP-bound form. Over 50 members of the ras superfamily are currently known and based on their sequence homologies they have been grouped into seven subfamilies: ras, rho, rab, ARF, sar1, ran and rad/gem. Members of the ras family include H-, Ki-, N-Ras; R-Ras; Rap1, 2; Ral; and TC21. Rho family members are RhoA, B, C; Rac1, 2; Cdc42; RhoG; and TC10. Ras family GTPases are predominantly involved in processes leading to growth and differentiation. The biological functions of rho family proteins comprise the regulation of the actin cytoskeleton (see section 1.7.4), however more recently these GTPases have also been shown to be involved in signalling pathways leading to gene
transcription (see below). The Rab, ARF, Sar1 and Ran GTPases play a role in vesicle transport and nuclear protein import, respectively.

GTPases are regulated by GTPase activating proteins (GAPs) or guanine nucleotide exchange factors (GEFs). Increasing the activity of a GAP protein leads to inactivation, whereas increasing the GEF activity results in stimulation. The rho family of GTPases, in addition, can be regulated by GDP dissociation inhibitors (GDIs) which keep the GTPase in a locked GDP-bound form; upon activation the GDI is released which allows exchange of GDP to GTP. In T cells, Ras is activated by the TCR and the IL-2R but not by CD28 (when a physiological stimulus is used) (Downward et al., 1990; Graves et al., 1992; Nunes et al., 1994). Its regulation by GAPs and GEFs in TCR and IL-2 signalling has been mentioned in the previous section and will be further discussed in chapter 3. The regulation of Cdc42, Rac and Rho proteins by surface receptors in T cells has not been addressed. However, Rac may be regulated by Ras or Vav (see below and chapter 3).

It is now recognised that Ras can activate multiple effector pathways mediated by specific interactions of Ras in its GTP-bound form with distinct downstream signalling molecules (Marshall, 1996). In this respect, mutants of Ras which selectively interact with only one effector have now been identified (Joneson et al., 1996; White et al., 1995). Thus, Ras can interact with Raf and activate the Mek/Erk Map kinase pathways (see below). Studies on the role of Ras in fibroblast transformation have concluded that the Raf/Mek pathway does not mediate all Ras effector functions (White et al., 1995). Similarly, in T lymphocytes, the Raf/Mek pathway has been shown to mediate Ras effects on positive selection of thymocytes but apparently is not required for proliferation (Alberola-Illa et al., 1995). Other molecules that could be involved in mediating the tremendous array of Ras responses include rasGAP (Downward, 1992), PI 3-kinase (Rodriguez-Viciana et al., 1994) and Ral-GDS (GEF for Ral) (Hofer et al., 1994).
Moreover, Ras and Rho are essential for Ras transformation (Khosravi-Far et al., 1995; Qiu et al., 1995; Qiu et al., 1995) and rho family proteins may control Ras effects on the actin cytoskeleton (Ridley et al., 1992). In T cells, Rac may mediate some of the functions of Ras leading to induction of the transcription factor NF-AT (see below).

Similar to Ras, it has now been shown that the rho family proteins Cdc42, Rac and Rho activate at least two effector pathways, one leading to gene transcription and one leading to rearrangement of the actin cytoskeleton (see below, section 1.7.4 and chapter 6). Moreover, these GTPases seem to be required for the progression through G1 of the cell cycle (Olson et al., 1995). These distinct functions are thought to be reflected by the interaction of these GTPases with different effector molecules (Lamarche et al., 1996; Tapon and Hall, 1997; Westwick et al., 1997).

1.5.2 Map kinase modules

Map kinases (MAPK, see Fig. 1.9) are proline-directed kinases which phosphorylate sites containing the core consensus motif Ser/Thr-Pro. Such motifs are found in the transactivation domains of a wide range of transcription factors and one feature of Map kinases is their ability to translocate into the nucleus where they can directly phosphorylate these sites (Fig. 1.9). Alternatively, they can also phosphorylate and hence regulate additional protein kinases such as Mapkapk2 (Cuenda et al., 1995; Rouse et al., 1994; Stokoe et al., 1992) or Rsk (Blenis, 1993). Three mammalian Map kinase groups have been characterised - the Erks (extracellular-signal regulated kinase), the Jnks/Sapks (c-Jun N-terminal kinase / stress-activated protein kinase) and the p38/RK/Mpk2 (kinase homologous to Hog1 of S. cerevisiae; RK, Reactivating kinase) - a fourth group may exist in one that is regulated by the GTPase Rho and impinges on serum response factor (SRF)-mediated gene transcription (Fig. 1.9); reviewed in (Cahill et al., 1996; Cano and Mahadevan, 1995; Davis, 1994; Marais and Marshall, 1996; Marshall, 1994; Treisman, 1996; Woodgett et al., 1996). Map kinases themselves are
activated through phosphorylation of a threonine and tyrosine residue by dual-specificity kinases (MAPKKs). The specific phosphorylation motif (T-X-Y) in the activation domain of each Map kinase is depicted in Fig. 1.9.

**Figure 1.9.** In response to extracellular stimuli GTPases orchestrate Map kinase signalling pathways leading to gene transcription. Three distinct mammalian Map kinase signalling pathways - termed Map kinase modules - have been described. A fourth governed by the GTPase Rho may exist, although not yet characterised; P, phosphoserine/threonine; LPA, lysophosphatic acid; PSI, protein synthesis inhibitors; OS, osmotic shock; LPS, lypopolysaccharide. For further details see text.

MAPKKs in turn are activated through phosphorylation of (two) critical serine/threonine residues by yet another serine/threonine kinase (MAPKKK). An additional layer of kinases (MAPKKKK) exists in some of the Map kinase modules. GTPases which regulate Map kinase modules include Ras, Rac, Cdc42 and possibly
Rho (Fig. 1.9). At present, Raf is the only MAPKKK which is well characterised, the involvement of Pak is still subject to debate. While the events that lead to the activation of MAPKs and MAPKKs appear to be mediated solely by phosphorylation events, the activation mechanism of MAPKKKs (or MAPKKKKs) by GTPases is more complex and may afford, like in the case of Raf by Ras, a combination of several factors: (1) translocation from the cytoplasm to the plasma membrane; (2) phosphorylation on various Ser/Thr residues; (3) phosphorylation by src family tyrosine kinases on Tyr residues; and (4) interaction with other regulatory proteins such as 14-3-3 proteins which lock the enzyme in a certain conformation or protect it from dephosphorylation. Mechanisms leading to Pak activation appear to be complex and may involve autophosphorylation events and membrane-recruitment by Nck or the GTP-bound GTPase (Lu et al., 1997; Manser et al., 1997).

The distinction of the mammalian Map kinase modules originates from the observation that they are activated preferentially by different extracellular stimuli, either by mitogens or alternatively by TNFα, IL-1, or stress-inducing factors such as heat shock, osmotic shock, UV irradiation, and treatment with protein kinase inhibitors. Nevertheless, many stimuli activate simultaneously more than one Map kinase pathway in parallel and distinct Map kinases phosphorylate the same nuclear transcription factors such as Elk-1 or ATF-2 (see below). Moreover, it is possible that there is considerable cross-talk between the pathways, thus Mekk1 is able to activate Mek at least when overexpressed in Cos cells (Lange-Carter et al., 1993; Minden et al., 1994; Yan et al., 1994). There are also alternative mechanisms to stimulate a particular member of one pathway which may involve the serine/threonine kinases of the PKC family (see section 1.6), Mos or Tpl2 (Posada et al., 1993; Salmeron et al., 1996). The interplay of these pathways is likely to play a role in modulating biological responses and 'fine-tune' the genetic program.
One point of convergence has been identified in the transcription factor Elk-1 which is targeted by both the Erks and the Jnks/Sapks (Cavigelli et al., 1995; Gille et al., 1992; Gille et al., 1995; Whitmarsh et al., 1995). Elk-1 is one member of the ternary complex factors (TCFs) which regulate transcription from the c-fos serum response element (SRE) together with the serum response factor (SRF) and hence contribute to c-Fos production (Fig. 1.9). Likewise ATF-2 is a substrate for Jnks/Sapks and p38/Rk Map kinases (Gupta et al., 1995; Livingstone et al., 1995; Raingeaud et al., 1995). ATF family members bind as homo- or heterodimers to 'cAMP response elements' (CREs). Finally, Map kinase cascades can also synergise to induce gene transcription. One example is transcription from the 'TPA response element' (TRE) which binds members of the c-Jun and c-Fos family in a complex termed AP1 (Karin and Hunter, 1995). Phosphorylation of c-Jun by Jnks is essential for its transcriptional activity and Erks contribute to increase the cellular levels of c-Fos. In T cells, AP1 activity is important for induction of early activation genes such as cytokine genes including the IL-2 gene.

1.5.3 Map kinase pathways in T cells

In T cells the Ras-Raf-Mek-Erk pathways is activated by the TCR (and the IL-2R, see section 1.4.3) and is essential for IL-2 gene transcription (Cantrell, 1996; Izquierdo et al., 1994; Izquierdo et al., 1993). Transcription factors that bind to elements in the enhancer region of the IL-2 gene include AP1, NFκB, Oct-1 and in particular NF-AT ('nuclear factor of activated T cells') (Serfling et al., 1995). The major target in the IL-2 gene for Ras/Raf/Erk signalling pathways is the nuclear component of NF-AT, NF-ATn, which contains c-Jun and c-Fos family proteins (often termed AP1) (Cantrell, 1996; Genot et al., 1996; Jain et al., 1992; Woodrow et al., 1993). However, Ras signalling pathways are not sufficient for NF-AT induction and require the synergistic effects of Ca^{2+} signals leading to the activation of the phosphatase calcineurin (Clipstone and Crabtree, 1992; Woodrow et al., 1993) (Fig. 1.10). Calcineurin signals lead to
the translocation of the pre-existing cytosolic component of NF-AT, NF-ATc, into the nucleus. The combined NF-ATn/NF-ATc complex is transcriptionally active. The importance of the Ras effector pathway was shown in experiments where an active form of Ras, Ha-v-ras, could synergise with Ca^{2+}/Calcineurin signals to induce NF-AT transcriptional activity (Woodrow et al., 1993; Woodrow et al., 1993). The involvement of the Raf/Mek/Erk Map kinase module was demonstrated in studies where dominant negative Mek inhibited NF-AT induction by the TCR, although active mutants of Mek
cannot synergise with Ca\(^{2+}\)/calcineurin signals to induce NF-AT transcriptional activity (Genot et al., 1996). Nevertheless, active Ras can induce several effector pathways including the activation of the GTPase Rac, and a dominant negative form of Rac, N17Rac, prevented TCR- or Ha-v-ras-controlled NF-AT transcriptional activity. The Rac effector pathways is not known. In this respect, it should be noted that Rac is likely to be regulated by several different upstream regulators such as Vav.

Little is known about the Jnk/Sapk or p38/Rk Map kinase pathways in T cells. However, in Jurkat T cells Jnk can be activated by costimulation of the TCR and CD28 (Su et al., 1994). The signalling pathways that lead to Jnk stimulation are not well defined but may involve PKC and Ca\(^{2+}\) signalling pathways, as Jnk activation can be achieved by combinatorial treatment with phorbol esters and calcium ionophore. Whether the TCR and CD28 regulate Jnk activity via Rac has not yet been determined.
1.6 Phosphoinositide kinases and other enzymes involved in phosphoinositide metabolism

The three main lipids in the plasma membrane lipid bilayer are phospholipids, glycolipids and cholesterol, the phospholipids being the most abundant. The phospholipids include phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine and sphingomyelin. I will describe some aspects of PI metabolism and introduce the enzymes involved in its regulation, in particular I will concentrate on the role of phosphatidylinositol 3-kinases (PI 3-kinases).

![Figure 1.11. Structure of phosphatidylinositol.](image)

1.6.1 PI metabolism: D-3 phosphoinositides are generated by three distinct classes of PI 3-kinases

Three different groups of phosphoinositide kinases can phosphorylate the D-3, the D-4 and D-5 positions of PI (Fig. 1.11), to generate PI(4)P, PI(4,5)P₂, PI(3)P, PI(3,4)P₂ and PI(3,4,5)P₃ as illustrated in Fig. 1.12. The levels of these lipids in subcompartments of the cellular membranes is regulated by extracellular stimuli that control the enzymatic activity of the kinases, lipases and phosphatases involved in PI metabolism. However, PI(4)P, PI(4,5)P₂, and PI(3)P are constitutively present in cells,
while PI(3,4)P$_2$ and PI(3,4,5)P$_3$ are almost absent in quiescent cells. One of the best studied reactions is the hydrolysis of PI(4,5)P$_2$ by members of the phospholipase C (PLC) family giving rise to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (I(1,4,5)P$_3$ or IP3), which in turn release intracellular Ca$^{2+}$ and activate protein kinase C (PKC). The kinases which phosphorylate the hydroxyl group at position 3 of the inositol ring are less well characterised, but it is now becoming evident that there are at least three classes of D-3 phosphoinositide kinases based on their in vivo substrate specificity and their structural features. PI 3-kinases of the class I can phosphorylate PI, PI(4)P and PI(4,5)P$_2$ in vitro, however based on detailed kinetic studies of the changes of cellular levels of the different D-3 phosphoinositides in response to agonist stimulation, it is thought that their preferred in vivo substrate is PI(4,5)P$_2$ (Stephens et al., 1993). Members of the class II of PI 3-kinases were originally identified in Drosophila melanogaster (MacDougall et al., 1995), but now mouse homologues have been found (Molz et al., 1996; Virbasius et al., 1996). Class II PI 3-kinases phosphorylate PI and PI(4)P in vitro, but do not recognise PI(4,5)P$_2$ as a substrate (MacDougall et al., 1995). Their in vivo specificity is unknown. Finally, the PI 3-kinases of class III can only phosphorylate PI in vitro and in vivo (Stephens et al., 1994; Volinia et al., 1995).
kinases are related to the PI 3-kinase family (see below) whereas PI(4)P 5-kinases belong to a non-related group of phosphoinositide kinases.

In T cells, the production of D-3 phosphoinositides has been measured after stimulation of the TCR, CD28 and the IL-2R (Remillard et al., 1991; Ward et al., 1993; Ward et al., 1992). The fold induction of these lipids after receptor stimulation is shown in Table 1.2. Signals and receptors which trigger class I PI 3-kinases are listed in (Fry, 1994; Stephens et al., 1993).

Table 1.2. Fold increases in D-3 polyphosphoinositides in T cells after receptor stimulation.

<table>
<thead>
<tr>
<th></th>
<th>PI(3,4)P₂</th>
<th>PI(3,4,5)P₃</th>
</tr>
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<tbody>
<tr>
<td>TCR 2 (5 min)</td>
<td>2</td>
<td>3 (10 min)</td>
</tr>
<tr>
<td>CD28 (5 min)</td>
<td>3-4</td>
<td>2-4</td>
</tr>
<tr>
<td>TCR + CD28</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>IL-2R (12 min)</td>
<td>2.7</td>
<td>4.2</td>
</tr>
</tbody>
</table>

1.6.2 Structural features of the D-3 phosphoinositide kinases and their regulation

The three classes of PI 3-kinases can be distinguished based on their structural features and their mode of regulation. Many PI 3-kinases are heterodimeric complexes and comprise a catalytic subunit and a regulatory adaptor subunit. The members of the PI 3-kinase family cloned to date are summarised in Table 1.3 and the main structural properties of some members are shown in Fig. 1.13 and for the adaptor subunits in Fig. 1.14.
Class I PI 3-kinases and general features of the PI 3-kinase family

The class I PI 3-kinase are heterodimeric complexes and comprise a catalytic subunit of 120 to 130 kDa predicted molecular mass and are generally referred to as p110 subunit. It is likely that all the catalytic subunits from this class interact with an adaptor subunit of 50 to 100 kDa, albeit some of the adaptor subunits have only been biochemically characterised. In addition, they contain a domain which allows them to bind to Ras, an interaction which may complement other regulation/activation mechanisms (Klinghofer et al., 1996; Kodaki et al., 1994; Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1996). All catalytic subunits contain the kinase domain in their C-terminus and a conserved region termed PIK-domain (Fig. 1.13). The PIK and kinase domains are conserved in all PI 3-kinase family members, and are related to similar regions found in PI 4-kinases (Flanagan et al., 1993). Moreover, these domains are also found in a wide range of related protein kinases termed PIK-related kinases (Fig. 1.13) (Keith and Schreiber, 1995). The function of the PIK-domain is not known but its conservation suggests that it may serve a role in lipid (or protein) substrate recognition/presentation. The kinase domains of PI 3-kinases retain some motifs present in the catalytic domains of protein kinases, and indeed members of the PI 3-kinase class I group are dual specificity kinases and can phosphorylate a limited number of substrates, in particular their own adaptor subunit (Dhand et al., 1994). In contrast, members of the PIK-related kinase family have not been shown to phosphorylate lipid substrates, but rather contain protein kinase activity (Hunter, 1995).

Members of the class I of PI 3-kinases are activated by two principal receptor-mediated activation mechanisms. The first is thought to integrate signals from heterotrimeric guanine nucleotide-binding (G) protein-coupled receptors, most of which belong to the seven transmembrane receptor family, such as the thrombin receptor or the receptor for f-Met-Leu-Phe. This mode of activation has been ascribed to the class IB (see Table 1.3) and one mammalian member has been cloned to date, p110γ. The
activation mechanism of p110γ is likely to involve the Gβγ subunit (Lopez-Illasaca et al., 1997; Stephens et al., 1994). The cloning of its putative adaptor subunit has not yet been reported.

<table>
<thead>
<tr>
<th>Table 1.3. The members of the PI 3-kinase family.</th>
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<tbody>
<tr>
<td>Class</td>
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</tr>
<tr>
<td>IA</td>
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<td>IB</td>
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<td>II</td>
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<td>III</td>
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Based on their in vitro substrate specificity and their overall structural features (see Fig. 1.13) the D-3 phosphoinositide kinases can be classified into 3 distinct groups. The cDNAs of the present enzymes have been cloned except p117/p120 and the adaptors p60 or p101 which have been biochemically characterised. The different clones of the mammalian p85/p55 adaptors have not been specified (see Fig. 1.14).

Hs-Homo sapiens, Bt-Bos taurus, Mm-Mus musculus, Dm-Drosophila melanogaster, Ce-Caenorhabditis elegans, Dd-Dictyostelium discoideum, Sc-Saccharomyces cerevisiae, Sp-Schizosaccharomyces pombe, At- Arabidopsis thaliana

Figure 1.13. Structural features of PIK-related kinases, PI 4-kinases and PI 3-kinases. All the kinases depicted are homologues in their kinase domain. Furthermore they contain an additional region of homology common to all kinases, termed PIK-domain for phosphatidylinositol kinase domain, first described by Flanagan and coworkers for yeast PI 4-kinase (Flanagan et al., 1993). The PI 3-kinase family can be further divided into 3 classes, for details see Table 1.3 and text.

References: Tor1 (Helliwell et al., 1994); Tor2 (Kunz et al., 1993); Frap (Brown et al., 1994); Raft1 (Sabatini et al., 1994); Rapt1 (Chiu et al., 1994); mTor (Sabers et al., 1995); ATM (Savitsky et al., 1995); Tel1 (Greenwell et al., 1995; Morrow et al., 1995); Frp1 (Cimprich et al., 1996); Rad3 (Jimenez et al., 1992; Seaton et al., 1992); Mei-41 (Hari et al., 1995); Mec1 (Paulovich and Hartwell, 1995); DNA-PK (Hartley et al., 1995); hPI4K\(\alpha\) (Wong and Cantley, 1994); STT4 (Yoshida et al., 1994); (Sc)PI4K (Flanagan et al., 1993; Garcia-Bustos et al., 1994); for references of the PI 3-kinase family see Table 1.3.
The second group of class I PI 3-kinases, class IA, includes the first member of
the PI 3-kinase family to be characterised. PI 3-kinase was initially identified as a PI
kinase activity which associated tightly with the transforming protein tyrosine kinases v-src and v-ros (Macara et al., 1984; Sugimoto et al., 1984), and was present in polyoma
middle-T transformed cells (activates c-Src) (Whitman et al., 1985). This PI activity was
shown to be distinct from the previously characterised PI 4-kinase activity by its
sensitivity to nonionic detergent such as Nonidet P-40 (Whitman et al., 1987), and
subsequently characterised to catalyse the phosphorylation of inositides at the D-3
hydroxyl of the \textit{myo} inositol ring (Whitman et al., 1988). Finally, it was demonstrated
that D-3 phosphoinositides are generated in cells in response to PDGF receptor
stimulation (Auger et al., 1989). It is now well established that this class of PI 3-kinases
(class IA) is activated by receptor tyrosine kinases (RTKs) and by receptors with
associated cytoplasmic PTK activity such as the TCR or the IL-2R (Remillard et al.,
1991; Ward et al., 1992). One property of PI 3-kinase activity observed during these
early studies was its tight association with activated RTKs/PTKs, an interaction which
did not occur in quiescent/non-transformed cells and that the PI kinase activity may
reside in a 81-85 kDa protein (Courtneidge and Heber, 1987; Kaplan et al., 1987). The
tight association of PI 3-kinase activity with a tyrosine-phosphorylated residues,
Tyr751, in the PDGF receptor cytoplasmic tail (Kazlauskas and Cooper, 1989;
Kazlauskas and Cooper, 1990), finally allowed purification and cloning of two p85
adaptor subunits of PI 3-kinase, p85α and p85β (Escobedo et al., 1991; Otsu et al.,
1991; Skolnik et al., 1991) and subsequently of its catalytic p110 subunit (termed
p110α) (Hiles et al., 1992). An additional catalytic subunit encoded by a different gene,
p110β, was cloned subsequently (Hu et al., 1993) and recently a p110δ protein has
been cloned which is exclusively expressed in leukocytes (Bart Vanhaesebroeck,
personal communication). p110α/p110β homologous proteins have been identified in
\textit{Drosophila melanogaster}, \textit{Caenorhabditis elegans} and \textit{Dictyostelium discoideum} (Table
1.3). Furthermore, a third adaptor subunit encoding a protein of 55 kDa (p55γ) and
various splice variants of the p85α regulatory subunit have been identified (Fig. 1.14) (Vanhaesebroeck et al., 1997). An adaptor subunit for Drosophila Dp110 has been biochemically characterised.

Figure 1.14. Adaptors of PI 3-kinases.
P1, P2, proline-rich regions; BH, domain homologous to the rhoGAP domain in Bcr; α, β, γ suffixes indicate whether the protein is encoded by the same or a different gene; 'arrow', indicates a splice variant; M, myristoylation site; S/T-PK, serine/threonine protein kinase activity; p65-PP2A, region homologous to the 65 kDa regulatory subunit of protein phosphatase 2A; WD repeat, protein-protein interaction motif (Neer et al., 1994). The p55α and the p55γ adaptor subunit contain an unique region in their N-terminus.

References: p85α (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1991); p55α/AS53/p50α (Antonetti et al., 1996; Fruman et al., 1996; Inukai et al., 1996); p85β (Otsu et al., 1991); p55γ/p55PIK (Pons et al., 1995); p60 (Leevers et al., 1996); p150 of hVps34 (Panaretou et al., 1997); Vps15 of yeast Vps34 (Herman et al., 1991).
The p85α and p85β regulatory subunit of PI 3-kinase contain two proline-rich regions, two SH2 domains and one SH3 domain, as well as a region homologous to the rhoGAP domain in Bcr (Dieckmann et al., 1991; Heisterkamp et al., 1985) (Fig. 1.14). The region between the two SH2 domains is responsible for binding to the N-terminus of the catalytic p110 subunit which contains the p85 binding domain (Figs. 1.13, 1.14). The inter-SH2 domain also carries the major phosphorylation site of p85 by p110, Ser608. The SH2 domains bind to phosphorylated tyrosine residues in a wide range of RTKs, e.g. in the PDGF receptor to Tyr740 and Tyr751 (Kashishian et al., 1992) and specifically recognise 'TyrP-Xxx-Xxx-Met' motifs with high affinity. The p85 is therefore thought to act as an adaptor that mediates the recruitment of the catalytic p110 subunit to the plasma membrane. An additional function for p85 may derive from the notion that p85/p110 is often found in multi-enzyme complexes which contain signalling molecules such as Raf-1, PLCγ1, rasGAP, Nck, Cbl or src family PTKs (Cantley et al., 1991; Pawson, 1992; Wange and Samelson, 1996). Therefore, the p85 adaptor subunit may position p110 into the proximity of putative effector molecules. The role of p85 in regulating and positioning the p110 catalytic activity of PI 3-kinase in T cells, will be discussed in chapter 4.

Bovine p85α and p85β are highly homologous proteins with an overall identity of 62%. The region with the lowest homology (42%) is the Bcr homology (BH) domain containing a rhoGAP domain. No function has yet been found for this domain. The p85 subunits also contain two proline-rich motifs which may contribute to p85 interaction with regulatory proteins (see chapter 4). Whether p85α and p85β serve divergent functions is currently not clear, however in T cells they are differentially phosphorylated in response to TCR engagement (Reif, 1992; Reif et al., 1993) and Cbl interact preferentially with p85β (Hartley et al., 1995). Likewise, the role of the low molecular weight p50-p55 adaptors is not known. The different adaptors show no specificity for binding either p110α, p110β or p110δ (Bart Vanhaesebroeck, personal communication).
Tissue-specific expression of some of the adaptors suggests possible differences in function and regulation.

**Class II PI 3-kinases**

The most striking feature of class II PI 3-kinases is that they contain a C2 domain in their C-terminus, which is also present in PKCs (Fig. 1.13). C2 domains bind to phospholipids / inositol phosphates in a Ca^{2+}-dependent or -independent manner (Newton, 1995; Ponting and Parker, 1996). The C2 domain in class II PI 3-kinases lack critical Asp which confer Ca^{2+}-binding and have been reported to bind lipids in a Ca^{2+}-independent manner (MacDougall et al., 1995). The class II PI 3-kinases also contain proline-rich regions possibly mediating SH3 binding. The regulation and function of these PI 3-kinases is not known.

**Class III PI 3-kinases**

These PI 3-kinases are homologous to a PI 3-kinase originally found in *S. cerevisiae*, Vsp34. Vsp34 is the only PI 3-kinase present in yeast and is essential for the trafficking of newly formed proteins from the Golgi to the vacuole, the equivalent of the mammalian lysosome (Herman et al., 1992; Shepherd et al., 1996). Both yeast Vsp34 and hVsp34 tightly associate with a serine/threonine kinases termed Vsp15 or p150, respectively, which recruits the lipid kinase to membranes. Based on the established function of yeast Vsp34 and on the observation of the relative invariant cellular levels of PI(3)P, it can be speculated that class III PI 3-kinases and PI(3)P have a role in maintaining membrane trafficking and vesicle morphogenesis on a constitutive basis. Interestingly, the lipid product of class I PI 3-kinases also play a role in vesicular trafficking and endocytosis, including glucose transport (Cheatham et al., 1994; Joly et al., 1995; Joly et al., 1994; Li et al., 1995; Martys et al., 1996; Okada et al., 1994; Shpetner et al., 1996) - in contrast to PI(3)P lipid function, however, some of these responses appear to be regulated by agonists.
**PIK-related kinases**

The catalytic domain of PIK-related kinases and PI 3-kinases is about 20% homologous. In addition, PIK-related kinases can be identified based on an unique region at their C-terminal end (Keith and Schreiber, 1995). As mentioned above, lipid kinase activity has not been demonstrated for these proteins. PIK-related kinases play a role in DNA repair, V(D)J recombination, and cell cycle control. Members include the targets of rapamycin, Tor/Frap kinases, the catalytic subunit of DNA-dependent protein kinase (DNA-PK), or ataxia telangiectesia mutated (ATM) gene product and Mec1, the latter two being important for cell arrest / checkpoint control at various transition stages of the cell cycle (Abraham, 1996; Paulovich et al., 1997).

1.6.3 Phospholipases

Similar to the PI 3-kinase family three different classes of PLCs exist, PLCs, PLCγ, PLCβ and PLCδ (Rhee and Choi, 1992). All three classes share two regions of homology, referred to as catalytic domain I and II, and a N-terminal PH domain. Members of the PLCγ group contain in addition two SH2 domains and one SH3 domain (see Fig. 1.6, pp. 24, 25). PLCs can be distinguished based on their mode of activation. PLCβs are activated by G protein coupled receptors, whereas PLCγs associate with and/or are activated by RTKs or PTKs such as PLCγ1 in TCR signal transduction. It is not clear how PLCδ is regulated in vivo, however in vitro activity can be induced by free fatty acids in the presence of micromolar amounts of Ca²⁺ (Liscovitch and Cantley, 1994). Other phospholipases include phospholipase A₂ (hydrolyses PC releasing lyso-phosphatidylcholine and cis-unsaturated fatty acids), sphingomyelinase, and phospholipase D (hydrolyses PC to phosphatidic acid which can then be converted into DAG by removal of its phosphate).
1.6.4 Inositol / phosphatidylinositol polyphosphate 5-phosphatases

Inositol / phosphatidylinositol polyphosphate (IP) 5-phosphatases are likely to act in concert with PI 3-kinases and may hence be important in modulating PI 3-kinase-controlled responses; in particular in the light that PI(3,4,5)P₃ is the predominant product of class I PI 3-kinases in vivo. The regulation and function of IP 5-phosphatase is not yet well defined, however, novel members forming a third class of IP 5-phosphatases have recently been cloned due to their association with Grb2 and Shc (Fig. 1.15 and Table 1.3). Three major classes of IP 5-phosphatases can be defined based on their
substrate specificity, summarised in Table 1.3; it should be noted that the substrate specificity of class III IP 5-phosphatases is not yet well established. For review see (Majerus, 1996). A possible functions for p145/p150Ship may exist in FcγRIIb1 signalling, a receptor which attenuates BCR signal transmission (Ono et al., 1996).

### Table 1.4. Substrate specificities of (phosphatidyl)-inositol polyphosphate 5-phosphatases.

<table>
<thead>
<tr>
<th>5-Phosphatase</th>
<th>I(1,4,5)P₃</th>
<th>I(1,3,4,5)P₄</th>
<th>PI(4,5)P₂</th>
<th>PI(3,4,5)P₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 43 kDa Type I</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II 75 kDa Type II</td>
<td>+</td>
<td>+</td>
<td>+ [9]</td>
<td>+ [8]</td>
</tr>
<tr>
<td>OCRL1</td>
<td>+</td>
<td>+</td>
<td>+ [10]</td>
<td>na</td>
</tr>
<tr>
<td>Synaptojanin</td>
<td>+</td>
<td>+</td>
<td>+ [11]</td>
<td></td>
</tr>
<tr>
<td>III p145/p150 Ship</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>SIP-110</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SIP-130</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SIP-145</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PI3K assoc. 5-PP</td>
<td>- [7,8]</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

According to their substrate specificity the 5-phosphatases can be divided into at least 3 groups. The SIP-110/SIP-130/SIP-145 proteins are likely to be products of the same gene and appear to be the mouse homologue of the human p145/p150 Ship SH2-containing inositol 5-phosphatase. From SIP-145 only a partial cDNA clone has been characterised. The PI3K associated 5-phosphatase (5-PP) has only been biochemically described.

OCRL1 - Lowe’s oculocerebrorenal syndrome; na - not analysed.

1.7 Potential effectors of PI 3-kinase action

Several potential targets of PI 3-kinase have been identified based on studies using chemical inhibitors of PI 3-kinase such as wortmannin and LY294002, receptor mutants which prevent binding of PI 3-kinase to its cognate binding site, or the PI 3-kinase inhibitory mutant, p85Δ, that lacks the p110 binding site (see introduction to chapter 5). In addition, in vitro binding studies with 'purified/synthetic' D-3 phosphoinositides attempted to find direct targets of PI 3-kinase. In this section, I will introduce some of the possible mediators of PI 3-kinase action in general terms in that I will give their family background, however, how they relate to PI 3-kinase will be mainly discussed in the result chapters 6, 7 and 8.

1.7.1 Protein kinase C

In vitro activation studies using purified D-3 phosphoinositides have implicated protein kinase C (PKC) family members as potential targets for these lipids. PKC comprises a family of phospholipid dependent serine/threonine protein kinases that have important roles in cell proliferation, differentiation, development, secretion and tumour promotion (Nishizuka, 1992). PKCs are the major cellular receptor protein for phorbol esters substances which promote oncogenic transformation of cells. On the basis of their sequence and biochemical properties, PKCs are divided into three groups (Fig. 1.16): the conventional PKCs (cPKC) α, β1, β2 and γ that are activated in a calcium-dependent manner, the calcium-independent novel PKCs (nPKC) δ, ε, η and θ and a third group of atypical PKCs (aPKC) ζ, τ or λ. The latter group of isotypes is unresponsive to DAG, phorbol esters and Ca\(^{2+}\) and the exact activation mechanism is subject to debate. In addition, an eleventh family member, Protein kinase D (PKD or PKCμ), shares moderate homology with the PKC family, however it resembles PKC regarding its phorbol ester and DAG binding (Johannes et al., 1994; Valverde et al., 1994; Zugaza et al., 1996). PKD contains a PH domain and a putative
transmembrane domain which are not found in any of the other PKCs. PKCs which may be involved in mediating PI 3-kinase function include the atypical PKC_\zeta which seems to be activated by PS or Pl(3,4,5)P_3 (Nakanishi et al., 1993). Likewise nPKC have been reported to be stimulated by D-3 phosphoinositides (Toker et al., 1994). However, no defined D-3 phosphoinositide-binding motif has been found in any of these PKCs. In addition, a PKC-related kinase (PRK) also termed protein kinase N (PKN) has been shown to be activated by Pl(4,5)P_2 and Pl(3,4,5)P_3 in vitro (Palmer et al., 1995).

1.7.2 Protein kinase B / Akt

Protein kinase B / Akt was first cloned in 1991 due to its homology to PKC (73% similarity to the kinase domain of PKC_\gamma) and PKA (68% similarity to the kinase domain of PKA), and hence was termed protein kinase B (PKB) (Coffer and Woodgett,
1991) or RAC-PK (related to the A and C kinases) (Jones et al., 1991). At the same time, this kinase was identified as the product of the oncogene \textit{v-akt} of the acutely transforming retrovirus AKT8 found in a rodent T cell lymphoma (Bellacosa et al., 1991). The retroviral oncogene encoded a fusion of the cellular Akt protein to the viral Gag structural protein. PKB/Akt has a molecular mass of 60 kDa (480 aa) and in addition to its serine/threonine kinase domain (aa 148-411), it contains a PH domain at its amino-terminal end (aa 1-106) (see Fig. 1.6; pp. 24, 25). Three PKB closely related gene products have been identified: huPKB\(_\alpha\)=Akt1=Rac-PK\(_\alpha\) (see above); huPKB\(_\beta\)=Akt2=huRac-PK\(_\beta\), and ratPKB\(_\gamma\).

PKB is activated by a wide range of growth stimuli including PDGF, EGF, insulin, thrombin, and NGF. The positioning of Akt downstream of PI 3-kinase is based on the following observations. Treatment of cells with wortmannin/LY294002 (Burgering and Coffer, 1995; Franke et al., 1995; Kohn et al., 1995) or the overexpression of p85\(_\Delta\) (Burgering and Coffer, 1995) inhibit PKB stimulation by PDGF, EGF or insulin. In addition, PDGF receptor mutants that fail to recruit PI 3-kinase also fail to activate PKB (Burgering and Coffer, 1995; Franke et al., 1995). Under certain conditions Akt may be a downstream target of Ras: dominant negative Ras, N17Ras, could prevent PDGF stimulation of PKB in one study (Franke et al., 1995) but did not in another (Burgering and Coffer, 1995). Finally, PKB may also be regulated by PI 3-kinase independent pathways, as stress induced activation of PKB by heat shock or hyperosmolarity is insensitive to wortmannin (Konishi et al., 1996). Whether cytokine receptors or the TCR regulate PKB and whether this involves PI 3-kinase will be investigated in chapter 7 or chapter 8, respectively.

The regulation of PKB appears to be complex and is subject to debate. The integrity of the PH domain has been found to be essential for activation of PKB in intact cells in response to PDGF, serum or the phosphatase inhibitor vanadate (Andjelkovic et
In this respect, direct \textit{in vitro} activation of PKB by PI(3,4)P$_2$ has been reported (Franke et al., 1997; Klippel et al., 1997), however it is equally possible that D-3 phosphoinositides play a spatial/structural role but do not directly contribute to an increase in the catalytic activity of PKB (James et al., 1996). Other lines of evidence indicate that PKB can be activated independently of its PH domain by growth factors such as insulin (Kohn et al., 1995). This could reflect the involvement of intermediate protein kinases which are activated by a PI 3-kinase dependent mechanism: in response to insulin or IGF-1, PKB becomes phosphorylated at two major sites in a wortmannin-sensitive manner, Thr308 in the kinase domain and Ser473 in the C-terminal tail (Alessi et al., 1996). The sequences surrounding these residues are very different, suggesting that two distinct kinases are involved in phosphorylating these sites. Phosphorylation of PKB appears to be a general regulatory mechanism as it undergoes an electrophoretic mobility shift in response to growth factor activation and phosphatase treatment of 'activated' PKB reverses its activation (Andjelkovic et al., 1996; Burgering and Coffer, 1995).

The only direct \textit{in vivo} substrate of PKB identified so far is glycogen-synthetase 3 (GSK3) (Cross et al., 1995). Phosphorylation of GSK3 by PKB results in its inactivation and consequent stimulation of glycogen synthesis. GSK3 contributes to the regulation of several intracellular pathways including control of transcription factors (c-Jun/AP1, CREB, c-Myc) or translation initiation factors (elF-2B) (Welsh et al., 1996). GSK3 is regulated by several different mechanisms (e.g. the Raf/Erk pathway). Another target of PKB is p70S6k (see below), although there is no evidence that it is a direct substrate. Recent reports have revealed a role for PKB in the protection from apoptosis, for further details see \textit{chapters 7-9}. 
1.7.3 p70 S6 kinase

The serine/threonine kinase p70 S6 kinase (p70S6k) is thought to play an important role in altering the pattern of protein synthesis during G1 of the cell cycle, reviewed in (Brown and Schreiber, 1996; Chou and Blenis, 1995; Kozma and Thomas, 1994; Proud, 1996). So far one gene for p70S6k has been identified encoding two different splice variants, p70S6k and p85S6k. p85S6k contains an N-terminal extension of 23 amino acid residues including a hexa-Arg sequence typical of nuclear localisation signals and is hence found in the nucleus (Fig. 1.17). Whether p85S6k has a specific role is not known, albeit its presence in the nucleus may be important for proliferation (Reinhard et al., 1994). Both isoforms appear to be regulated in a coordinate fashion (therefore, from now on, p85S6k regulation is not specified). p70S6k is regulated by...
multiple interdependent phosphorylation events in response to mitogen. Much of the understanding of the involvement of these sites in p70S6k regulation stems from studies using truncation/phosphorylation mutants and the immunosuppressant rapamycin. Two sets of phosphorylation sites can be distinguished (Fig. 1.17): one set is rapamycin-insensitive and can modulate kinase activity; in contrast, the other set is rapamycin-sensitive and Thr229/Thr389 are essential for kinase activity (Pearson et al., 1995). Thus a kinase pathway inhibited by rapamycin is required for events leading to activation of p70S6k. Indeed, the kinase which controls this pathway has been identified, first in yeast and later in mammalian cells, and was termed '(m)Tor' - (mammalian) target of rapamycin (Kunz et al., 1993). Its second name Frap (FKBP12-rapamycin-associated protein) derives from the observation that rapamycin does not bind Tor directly but in a complex with a cellular protein named FKBP12 (12 kDa FK506-binding protein). Frap/Tor is a PIK-related kinase and hence related to PI 3-kinase. The inhibitory effect of rapamycin on p70S6k can be overcome by expression of mutated Frap which no longer binds the FKBP12/rapamycin complex, demonstrating that Frap is an essential regulator of p70S6k in vivo (Brown et al., 1995). However, Frap cannot phosphorylate p70S6k and at present, extracellular signals linked to Frap are unknown. Moreover, the identity of the proline-directed kinases responsible for phosphorylating the 'other' set of Ser/Thr residues remains elusive. The involvement of PI 3-kinase in the regulation of these putative upstream p70S6k kinases will be introduced in chapter 6 and elaborated on in chapter 7. A detailed analysis of the significance of the different phosphorylation sites including their putative upstream regulatory kinases can be found in reference (Dennis et al., 1996).

Rapamycin has been used to study downstream events of Frap and p70S6k. p70S6k phosphorylates the S6 protein of the 40S ribosomal subunit, an event also occurring in response to mitogenic stimuli. S6 phosphorylation has been correlated with the translational upregulation of a family of mRNA which contain a polypyrimidine tract at
their 5′ transcriptional start sites (Brown and Schreiber, 1996). Rapamycin blocks the phosphorylation of components required for the translation initiation of these mRNAs, such as 4E-BP1 (eIF-4E binding protein-1). Polypyrimidine-cap mRNAs have been found to encode predominantly ribosomal proteins and elongation factors both being important for the translational apparatus. Increases in the rate of translation (2-3 fold) in response to mitogen are essential for transit through the cell cycle and even partial inhibition of protein synthesis causes fibroblasts to accumulate in the G1 phase (Norbury and Nurse, 1992). This indicates the importance of these events for proliferation. Finally, another potential link of (p70S6k)/Frap to processes leading to mitogenesis have been identified in T cells. Thus, the IL-2 induced downregulation of the cyclin-dependent kinase inhibitor p27\(^{kip1}\) is prevented by rapamycin (Nourse et al., 1994).

### 1.7.4 Rac, Rho, and Cdc42 GTPases- regulators of the actin cytoskeleton

Many growth factors in addition to regulating proliferation, also cause rapid changes in cell shape. Cdc42, Rac1 and RhoA are essential in directing actin reorganisation pathways that result in these changes in cell morphology. In Swiss 3T3 cells Rac1 induces membrane ruffles and lamellipodia formation (Ridley et al., 1992), RhoA induces focal adhesion assembly and stress fibre formation (Ridley and Hall, 1992), and human Cdc42, Cdc42Hs, controls the formation of microspikes and filopodia (Nobes and Hall, 1995). Focal adhesions are clusters of integrin receptors binding to the extracellular matrix proteins such as fibrinogen and collagen. A number of proteins are found to be associated with focal adhesions at the intracellular face of the plasma membrane: these include vinculin, talin, tensin, and α-actinin (Yamada and Geiger, 1997). Cdc42-like proteins are also required for the establishment and maintenance of cell polarity in budding and fission yeast. In T cells, Cdc42 may play an important role during the regulation of polarisation towards antigen-presenting cells (Stowers et al., 1995). Rho has been implicated in LFA-1-mediated aggregation of lymphocytes.
(Laudanna et al., 1996; Tominaga et al., 1993) and is required for lymphocyte-mediated cytotoxicity (Lang et al., 1996; Lang et al., 1992). The molecular details of how these rho family GTPases are regulated are complex and not well understood, although several candidate regulatory proteins have been identified. These include GDIs, about 15 GEFs containing a Dbl homology domain (Fig. 1.18) (Cerione and Zheng, 1996) and 10 rhoGAPs (Lamarche and Hall, 1994). For details on the involvement of PI 3-kinase in Rac/Rho regulation, see chapter 6.

Several targets of rho family GTPases have now been identified in yeast two-hybrid screens, genetic analyses, or affinity column purifications using recombinant GTP-loaded Rac/Rho, for review see (Tapon and Hall, 1997). While there is no apparent homology in the (putative) binding domain of proteins that interact with Rho, some molecules that associate with Cdc42/Rac contain a sequence motif which may confer binding. The minimal consensus Cdc42/Rac-binding motif stretches over 18 amino acids and is referred to as CRIB motif (Cdc42/Rac interactive binding) (Burbelo et al., 1995). CRIB motif containing proteins include the serine/threonine kinase Pak and Mlk2,3, the tyrosine kinase p120ACK, and WASP. Early work on Pak implicated the kinase in mediating Rac-effects on Jnk activation, now the story appears to be more complex and activated Pak has also been shown to induce cytoskeletal changes (Manser et al., 1997). WASP may be a good candidate for mediating effects of Cdc42 on T cell morphology (Symons et al., 1996). However, there are also quite a few proteins that associate with Cdc42/Rac but lack a CRIB motif. Examples are POR1 (partner of Rac1), p67phox or PI(4)P 5-kinase. POR1 has a leucine-zipper region but no catalytic activity and can synergise with Ras to induce membrane ruffles (Van Aelst et al., 1996). p67phox contains two SH3 domains and a proline-rich region and binds to Rac in the activated phagocytic NADPH oxidase complex (Diekmann et al., 1994). It will be interesting to know how each putative effector fits into the picture.
Figure 1.18. Schematic representation of proteins containing a Dbl homology (DH) domain. Proteins which contain a Dbl homology domain are thought to have exchange activity towards GTPases of the rho family. The first GEF for Rho-like proteins to be identified was Cdc24. Subsequently, it was noticed that the human oncoprotein Dbl contains a region with similarity to Cdc24 and Dbl was later shown to activate nucleotide exchange on human Cdc42 and Rho in vitro. A family of proteins with a Dbl homology domain has now been identified and for some of these proteins exchange activity has been demonstrated in vitro. The domains are drawn roughly to scale. The predicted molecular mass for each gene product is indicated to the right. Except Cdc24 (S. cerevisiae) all proteins depicted are of mammalian origin. The DH domain in Sos shows moderate homology to the one in Dbl. PH, pleckstrin homology domain; PEST, putative protein instability domain; SH, Src homology domain; Cys, cysteine rich / Zinc-butterfly motif providing putative diacylglycerol-binding site; EF, putative Ca2+ binding EF-hand motif; proline, proline-rich region providing SH3 domain binding site; Rho GAP, GTPase activating protein domain homologous to the one of rho family proteins; IQ, calmodulin-binding motif; Ras GEF (=Cdc25), Ras guanine nucleotide exchange factor domain.
Chapter 2

Materials and methods

Reagents

The following reagents were purchased from the sources indicated: ionomycin (Ca\(^{2+}\) salt), phorbol-12,13-dibutyrate (PdBu), and wortmannin were from Calbiochem; LY294002 was from Zeneca; PD098059 was from New England Biolabs; rapamycin was a gift from George Thomas, FMI, Basel; phytohemagglutinin was from Wellcome Diagnostics; human recombinant IL-2 (rIL-2) was from Eurocetus; foetal calf serum (FCS) was from GIBCO; Ficoll-Hypaque (Lymphoprep) was from Nycomed Pharma; soybean phosphatidylinositol and phosphatidylserine were from Sigma; \[^{\alpha-35}\text{S}]\text{deoxy adenosine 5'\text{-thiotriphosphate (dATP)} (1,000 Ci/mmol), \[^{\gamma-32}\text{P}]\text{ATP (5,000 Ci/mmol), \[^{32}\text{P}]\text{orthophosphate (10 mCi/ml) L-}[^{35}\text{S}]\text{methionine (1,000 Ci/mmol), \[^{14}\text{C}]\text{acetyl coenzyme A (50 mCi/mmol), and }^{125}\text{I}-\text{conjugated protein A (30 mCi/mg) were from Amersham; }[^{3}\text{H}]\text{phosphatidylinositol 4-phosphate (2-10 Ci/mmol), }[^{3}\text{H}]\text{phosphatidylinositol 4,5-bisphosphate (2-10 Ci/mmol), and }[^{3}\text{H}]\text{inositol 1,3,4,5-tetrakisphosphate (21 Ci/mmol) was from DuPont. }[^{32}\text{P}]\text{phosphatidylinositol 3,4-bisphosphate and }[^{32}\text{P}]\text{phosphatidylinositol 3,4,5-trisphosphate were kindly provided by Barbara Marte, ICRF, London.}

Antibodies

Monoclonal antibodies (mAb) against the bcr domain of p85\(\alpha\) (U5) (End et al., 1993), phosphotyrosine (4G10) (Druker et al., 1989), CD3\(\varepsilon\) (UCHT-1) (Beverley and Callard, 1981), rat CD2 (rCD2) (Ox34) (He et al., 1988), the 9E10 Myc epitope (9E10) (Evan et al., 1985), and haemagglutinin (HA) (12CA5) (Kolodziej and Young, 1991) were purified from hybridoma supernatants by protein A affinity chromatography and supplied by the ICRF Hybridoma Development Unit.
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The mAb against Grb2 was purchased from Affiniti. The CD3 mAb OKT3 was purchased from Ortho Pharmaceuticals. The F(ab')_2 fragment of OKT3 was a gift from Narin Osman. The RNGS antiserum reactive with Lck was a gift from Mark Marsh, UCL, London, and the CST-1 antiserum reactive with Fyn and Yes was kindly provided by Sara Courtneidge, Sugen, Redwood City, CA. Anti-human S6 kinase antibody (Ab) was from Santa Cruz (referred to as M5, (Lane et al., 1993)). The M1 antiserum reactive with p70S6k (Lane et al., 1993) was a gift from George Thomas, FMI, Basel. The polyclonal mSos1 Ab (Sos1) and the Rac-PK-CT Ab reactive with PKB were from Upstate Biotechnology Incorporated. The Sos3 Ab was raised against a peptide from mSos1 (aa 100-120) (Buday et al., 1994). The p110 Ab (PW38) was raised against a peptide from bovine p110α of PI 3-kinase (Hiles et al., 1992). (KRPLWLNWENPDIMSE, aa776-791). The Sos3 and p110 Abs were kindly provided by Julian Downward, ICRF, London. The JNK2 (FL) Ab reactive with p54α Sapk and p54β Sapk was from Santa Cruz. The pan-ras (OP41) mAb was from Oncogene Science.

Mouse immunoglobulins G (mlgG) purified from serum was purchased from Sigma and rabbit anti-mouse immunoglobulins (Igs) from DAKO. Goat anti-mouse IgG (γ chain specific) conjugated to fluorescein isothiocyanate (FITC) (Sigma) and FITC-conjugated rabbit anti-mouse Igs (DAKO) were used for cell staining procedures.

**Peptides**

The PDGFR-Y(751)-P phosphotyrosine peptide had the sequence DYVPML(G), the EGFR-Y(1068)-P phosphotyrosine peptide had the sequence PVPEYINQS and the HaMT-Y(324)-P phosphotyrosine peptide had the sequence EEEPQYEEIPI. The CD28-Y(173)-P phosphotyrosine peptide corresponds to amino acid residues 166 to 200 of human CD28 (Aruffo and Seed, 1987) and had the sequence SRLLHSQDYMNMTPRR (Pages et al., 1996). (Residues which are phosphorylated are in boldface.) The Sos-PRO peptide had the sequence SKGTVGVPVPVPPVPR which corresponds to the amino acid residues 1144 to 1159 in
the C-terminus of mSos1 (Bowtell et al., 1992). The p85-PRO peptide had the sequence QPAPALPPPKPPKPTTV which corresponds to amino acid residues 302-317 of bovine p85α (Otsu et al., 1991).

**Fusion proteins**

Fusion proteins encoding GST alone (GST), full length GST-Grb2 (Grb2), full length myc tagged GST-Grb2 (Grb2myc), myc tagged double SH3 mutant GST-Grb2 49L/203R (named Grb2mycμSH3 or Grb2μSH3), as well as C-terminal GST-mSos1 (aa 1135-1336) (GST-C-Sos) have been described (Egan et al., 1993). The isolated N-terminal GST-huGrb2 SH3 domain (aa 1-58) (N-SH3) and the C-terminal GST-huGrb2 SH3 domain (aa 159-217) (C-SH3) have been described (Gout et al., 1993).

**Plasmids and reporter constructs**

HA-p70S6k (pBJ5) (Brown et al., 1995); HA-PKB (pSG5) and gagPKB (pSG5) (Burgering and Coffer, 1995); HA-Erk1 (pcDNAneo) (Ming et al., 1994); HA-Erk2 (pCEP4) (Frost et al., 1996); HA-p54β Sapk (pMT2) (here termed Jnk) (Yan et al., 1994) (a gift from J. R. Woodgett, OCI, Toronto); p85 (pcD-SRa) and p85A (pcD-SRa) (Dhand et al., 1994); Ha-v-ras (S12V, A59T) (Izquierdo et al., 1993); Myc-V14Rho (pEF), Myc-V12Rac (pEF), Myc-V12Cdc42 (pEF) and C3 transferase (pEF) (Hill et al., 1995) vector constructs have been described. For the microinjection experiments V12Rac was expressed as myc-tagged derivative in the pRK5 vector (Ferrari et al., 1993). Human Dbl (aa 495-826; EMBL accession number X12556) (pRK5) was kindly provided by M. F. Olson, Chester Beatty Laboratories, London. The reporter plasmids have been described: Nlex.elk-1 (pMLV or pEF) and 2lexoptk.CAT (Marais et al., 1993); Nlex.jun (aa 1-193) (pMLV) was a gift from R. Treisman, ICRF, London; lacZ encoding β-galactosidase (pMLV or pEF) (Price et al., 1995); AP1.CAT was used to assess AP1 activity in adherent cells consisted of one copy of the AP1 binding site from the collagenase promotor/enhancer linked to the CAT
gene (Angel et al., 1987); AP1.CAT used for expression in T cells consisted of three copies of the AP1 binding site from the IL-2 promoter/enhancer linked to the CAT gene (Williams et al., 1992); wild type SRE.CAT (Hill et al., 1993); c-fos.CAT (Treisman, 1985) and NF-AT.CAT (three copies of the IL-2.NF-AT binding site: TAAGGAGGAAAACGTGTTCATACAGAAGCG) (Woodrow et al., 1993).

**Cells and cell culture**

Human blood was obtained from the blood transfusion service, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque discontinuous gradient centrifugation. T lymphoblasts were prepared as described (Cantrell and Smith, 1984). Briefly, PBMC (10^6/ml) were resuspended in complete media consisting of RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated (56 °C for 30min) FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin and stimulated with 2 μg of phytohemagglutinin per ml for 72 h at 37 °C. After being washed, cells were maintained in exponential growth phase in RPMI/10% FCS supplemented with 20 ng of rIL-2 per ml. After 10 to 15 days, the T lymphoblasts (>99% T cells, represented by CD4^+ and CD8^+ subpopulations) were cultured without rIL-2 for 16 to 48 hours to ensure their reaccumulation in the early G1 phase of the cell cycle (Cantrell and Smith, 1983).

The Kit225 T leukaemic cell line (Hori et al., 1987), the JHTAg subclone (Clipstone and Crabtree, 1992) or the JKHML 2.2 subclone (Goldsmith et al., 1989) of the Jurkat T leukaemic cell line were maintained in RPMI 1640 medium containing 10% heat-inactivated FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C. Kit225 cells were supplemented with 20 ng/ml (1 nM) of rIL-2 during normal growth conditions. For IL-2 activation assays of endogenous proteins, Kit225 cells were washed three times with PBSA to remove the IL-2 and cultured further in RPMI supplemented with 5% FCS in the absence of rIL-2 for 48h to 72h prior to IL-2
activation assays. When Kit225 cells were transfected, cells were treated as above but only deprived of rIL-2 for 24h prior to transfection.

Rat-1 fibroblasts, simian Cos7 kidney cells (Gluzman, 1981), human embryonic kidney 293 cells (epithelial) (Graham et al., 1977), Swiss 3T3 cells and NIH 3T3 cells (referred to as adherent cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C. For microinjection experiments (into sub-confluent cells), Swiss 3T3 cells were plated at a density of 1 x 10^5 onto 6 cm dishes or 3 x 10^5 onto 80 cm² flasks and grown to confluence. The cells were left without medium change to become quiescent (usually 7-10 days after seeding) and then serum-starved overnight in DMEM containing 2 g/l NaHCO₃. After washing with PBSA cells were trypsinised briefly, resuspended in serum-free medium containing 0.5 mg/ml soy bean trypsin inhibitor (Sigma), pelleted and resuspended in serum-free medium and allowed to attach for 30 min before injection.

**Metabolic labelling**

**T cells:** The labelling of cells with [³⁵S]methionine was in methionine free RPMI medium and for the [³²P]orthophosphate labelling in Pi free DMEM. For both labelling procedures the T cells were washed three times in labelling media including 10 min incubation in these media at 37 °C between each wash and finally resuspended in media supplemented with 4% dialysed FCS and 10 mM Hepes, pH7.4. The [³⁵S]-labelling was performed for 6 h at 37 °C with 1mCi [³⁵S]methionine (14 x 10⁷ cells/14 ml) and the [³²P]-labelling was carried out for 5 h at 37 °C with 2.5 mCi [³²P]orthophosphate (1.7 x 10⁸ cells/18 ml). The cells were washed twice in labelling media, and incubated for 10 min at 37 °C prior to stimulation with UCHT-1 (10 μg/ml) prior to cell lysis and purification procedures as described above. The [³⁵S]-metabolically labelled proteins or the [³²P]-phosphoproteins were separated by SDS-
polyacrylamide gel electrophoresis (PAGE) and visualised by fluorography or autoradiography, respectively at -70 °C on XAR-5 film (Kodak).

**Cos7 cells:** For phosphoinositide analysis Cos7 cells were metabolically labelled 36 h (to 40 h) after transfection. Cells were washed extensively in P_1 free DMEM and labelled with [^{32}P]orthophosphate (1mCi per 10 cm dish) in P_1 free DMEM containing 0.8% dialysed FCS and 20 mM Hepes, pH7.4 for 8 h. Phospholipids were extracted as described below.

**Transient transfections**

**Cos7 cells:** Cos7 cells were transfected by electroporation with 5 to 10 μg of plasmid DNA. Amounts of plasmid DNA were kept constant per cuvette by adding vector DNA. Cos7 cells (4 x 10^6/0.8 ml) were pulsed at 450 V and 250 μF using a Gene Pulser (Bio-Rad). After 42 h to 48 h cells were collected.

**293 cells:** 293 cells were seeded at 3 x 10^6 cells per 10-cm dish in DMEM containing 10% FCS the day before transfection. Cells were washed and further cultured in serum-free DMEM when cells were transfected using lipofectamine reagent (GibcoBRL). Total amount of plasmid DNA was adjusted to 11 μg per dish with vector DNA. After 32 h cells were stimulated by addition of FCS as indicated and cells were collected. (In some experiments, 293 cells were transfected using a CaPO_4-based method and N-N-Bis(2-hydroxy-ethyl)-2-amino-ethanesulfonic acid (pH6.95) as transfection buffer (Chen and Okayama, 1988)).

**NIH 3T3 cells:** NIH 3T3 cells were transfected by the diethylaminoethyl (DEAE)-Dextran method (Hill et al., 1993). DNA concentrations in each experiment were constant (generally 8 μg) by adding vector DNA. Transfected cells were serum deprived for 36 h, stimulated by UV irradiation or addition of FCS as indicated and harvested after 8 h to 10 h. (In some experiments, NIH 3T3 cells were transfected using a CaPO_4-based method and Hepes buffered saline (pH7.05) as transfection buffer.)
Jurkat T cells: Jurkat T cells were transfected by electroporation with 20 to 40 \( \mu \text{g} \) of plasmid DNA. Amounts of plasmid DNA were kept constant per cuvette by adding vector DNA. Jurkat cells (1.0 to 1.2 \( \times 10^7 \) cells/0.5 ml) were pulsed at 310 V and 960 \( \mu \text{F} \). For gene reporter assays cells were stimulated 3h to 6h after transfection. Fourteen hours to 20 h after transfections Jurkat cells were harvested.

Kit225 cells: Kit225 were transfected by electroporation with 20 to 40 \( \mu \text{g} \) of plasmid DNA. Amounts of plasmid DNA were kept constant per cuvette by adding vector DNA. Kit225 cells (1.5 \( \times 10^7 \) cells/0.625 ml) were pulsed at 320 V and 960 \( \mu \text{F} \). For gene reporter assays cells were stimulated as indicated 2 h to 4 h after transfection. Cells were collected 14 h to 18 h after transfection.

**Amounts of plasmid DNA used for transfections**

The following amounts of plasmid were used unless indicated otherwise:

**Cos7 cells:** 5 \( \mu \text{g} \) of rCD2-PI 3-kinase chimeras (pcDNA3)

**293 cells:** 2 \( \mu \text{g} \) of HA-p70S6k, 2 \( \mu \text{g} \) of HA-Erk1, 1 \( \mu \text{g} \) of vector plasmid (control), 1 \( \mu \text{g} \) of rCD2p85 (pRK5), 0.5 \( \mu \text{g} \) of rCD2p110 (pRK5), 0.5 \( \mu \text{g} \) of rCD2p110-R/P (pRK5) or 1 \( \mu \text{g} \) of Ha-v-ras.

**NIH 3T3 cells:** 0.5 \( \mu \text{g} \) rCD2p110 (pRK5), 0.5 \( \mu \text{g} \) rCD2p110-R/P (pRK5), 1 \( \mu \text{g} \) V14Rho, 1 \( \mu \text{g} \) V12Rac, 1 \( \mu \text{g} \) Dbl or 2 \( \mu \text{g} \) C3 transferase. Four \( \mu \text{g} \) of CAT reporter constructs, 1 \( \mu \text{g} \) of lacZ plasmids and 0.25 \( \mu \text{g} \) of Nlex.elk-1 (pMLV) were used.

**Kit225 cells:** 7.5 \( \mu \text{g} \) of HA-p70S6k; 12.5 \( \mu \text{g} \) of HA-PKB; 10 \( \mu \text{g} \) of HA-Erk2; 20 \( \mu \text{g} \) of the following plasmids: pEF empty, rCD2p110 (pEF), rCD2p110-R/P (pEF), Ha-v-ras, V12Rac, V12Cdc42, gagPKB, or rCD2p85 (pEF); 7.5 \( \mu \text{g} \) of 2lexoptk.CAT and 15 \( \mu \text{g} \) of Nlex.elk-1 (pEF).

**Jurkat cells:** 7.5 \( \mu \text{g} \) of HA-p54\( \beta \) Sapk; 15 \( \mu \text{g} \) of the following plasmids: pEF empty, rCD2p110 (pEF), rCD2p110-R/P (pEF), rCD2p85 (pEF), rCD2 (pEF), Ha-v-ras, V12Cdc42 or gagPKB; 10 \( \mu \text{g} \) of the following plasmids: HA-PKB, p85\( \Delta \), V12Rac, or Dbl;
3 μg of 2lexoptk.CAT; 6 μg of Nlex.elk-1 (pMLV); 6 μg of Nlex.jun (pMLV); 2 μg of AP1.CAT; 15 μg of NF-AT.CAT; and 5 μg of SRE.CAT.

Figure 2.1 Gradient profile used to elute deacylated phosphoinositides during anion-exchange high-pressure liquid chromatography.

Extraction of phospholipids from Cos7 cells and HPLC analysis of inositol-phosphates

Phospholipid extractions were based on the method described previously (Carter and Downes, 1992). Briefly, cells were washed with PBSA, collected in 1 ml 2.4 M HCl and transferred to a polypropylene test tube. CHCl₃/MeOH (1:2 v/v) (1.5 ml) containing 10 μg phosphoinositides (Sigma) to act as carrier lipids were added followed by 1 ml CHCl₃. After vortexing and centrifugation the lower phase was removed to a fresh tube, the upper phase was re-extracted twice with 1.5 ml of CHCl₃ and the CHCl₃ phases were combined. Dry lipid films were deacylated with 0.5 ml methylamine at 50 °C for 1 h in the presence of 50 μl n-butanol. The resulting glycerophosphoinositol esters were re-dissolved in 0.5 ml H₂O and once extracted with an equal volume of n-butanol. The aqueous phase was mixed with $^3$H-labelled Ins(1,3,4,5)P₄ as an internal standard (Stephens et al., 1991) and resolved by anion-exchange high-pressure liquid chromatography (HPLC) using a S5 SAX column (Phase Separations). The column was eluted at 1 ml min⁻¹ with a linear gradient of Milli Q water versus 2.5 M NaH₂PO₄.
(pH 3.8 with NaOH (%B)); times were t=0, %B=0; t=10, %B=0; t=60, %B=12; t=70, %B=19; t=110, %B=50, t=130, %B=50; t=140, %B=0 (Fig. 2.1); fractions were collected every 0.5 min. The data were collected as values of $^{32}$P and $^3$H radioactivity measured using a calibrated $^3$H/$^{32}$P dual-label program on a Beckman LS6000 series scintillation counter.

**Immunofluorescent staining and flow cytometry**

Cell populations were washed twice in RPMI 1640 or DMEM medium containing 2% FCS (wash medium, WM) and resuspended at a concentration of $1 \times 10^7$ cells per ml in WM at 4°C. All antibody staining procedures were carried out on ice. Cells ($5 \times 10^5$) were incubated with either 6 µg/ml rCD2 mAb OX34 or for control staining mIgG (Sigma) in 50 µl WM. After 30 to 60 min incubation, the cells were washed twice in WM, then 50 µl of a dilution (1:40) of FITC-conjugated rabbit anti-mouse IgG (DAKO) in WM was added. After 30 to 60 min, the cells were washed three times in WM. The cells were analysed by flow cytometry on a FACScan (Becton Dickinson & Co.) using CellQuest™ software.

**Microinjection experiments**

Serum-starved sub-confluent Swiss 3T3 cells were prepared for microinjection as described above. Plasmid DNAs were microinjected at 200 µg/ml in PBS into the nucleus. For vinculin-staining cells were co-injected with Texas Red-lysinated dextran (2 mg/ml) as an injection marker. After 3 h to 4 h cells were fixed with 4% paraformaldehyde (w/v). To prevent activation of endogenous Rac and Rho proteins, rCD2p110 microinjected cells were re-injected with recombinant N17Rac proteins (0.7 mg/ml) and C3 transferase (100 µg/ml), respectively together with rat IgG (0.5 mg/ml) as an injection marker and after 30 min cells were fixed. Recombinant N17Rac proteins and C3 transferase were prepared as described (Ridley et al., 1992). Vinculin or actin was visualised by immunofluorescence as detailed below.
Immunofluorescence microscopy

Cell staining of Swiss 3T3 and Cos7 cells and immunofluorescence microscopy was carried out as described (Dieckmann et al., 1995). Briefly, cells were permeabilised for 5 min at room temperature with 0.2% Triton X-100 in PBSA. Permeabilised cells were labelled for protein expression (Cos7 cells) or double-labelled for actin and protein expression by first incubating with Oxa3 mAb (2.4 μg/ml) to detect rCD2-PI 3-kinase chimeras. Then cells were incubated with FITC-conjugated goat anti-mouse Ab, and for detection of actin, with rhodamine-phalloidin (0.1 μg/ml). Permeabilised cells were labelled for vinculin by incubating with anti-vinculin mAb (Sigma, VIN 11-5) for 60 min in PBS, then with FITC-conjugated goat anti-mouse Ab for 30 minutes. Finally, an additional antibody layer (FITC-conjugated donkey anti-goat) was used for enhancement of the vinculin signal. Injected cells were visualised via the Texas Red-lysinated dextran injection marker. For re-injection experiments with recombinant proteins, co-injected cells was detected via the injection marker rat IgG which was visualised using a cascade blue-conjugated goat anti-rat IgG.

The coverslips were mounted by inverting them onto 5 μl moviol mountant containing p-phenylenediamine (1 mg/ml) as an antibleach agent and cells were examined on a Zeiss Axioskop microscope using Zeiss 63 x 1.4 (Cos7 cells), 40 x 1.3, and 100 x 1.3 oil immersion objectives. Fluorescence images were recorded on Kodak T-max 400 ASA film or Kodak Ectachrome 200 ASA film (Cos7 cells).

Inductions

The following concentration of agonists were used unless stated otherwise:

- 10 μg/ml UCHT-1 for activation of the TCR/CD3 complex (< 1 h)
- 5 μg/ml UCHT-1 for activation of the TCR/CD3 complex (> 1 h)
- 1.6 μg/ml OKT3F(ab')2 for activation of the TCR/CD3 complex
- 50 ng/ml PdBu for activation of PKC
- 500 ng/ml ionomycin for activation of Ca\(^{2+}\)-regulated pathways
- 20 ng/ml rIL-2 for activation of the IL-2R
- 20% FCS for Erk assays and gene reporter assays (adh. cells)
Chapter 2  Materials and methods

10% FCS for p70S6k assays in adherent cells
40 J/m² UV-C for activation of Sapks in adherent cells

**General induction times**

- 2 min UCHT-1 for adaptor association assays in Jurkat cells
- 5 min UCHT-1 for PKB assays in Jurkat cells
- 15 min PdBu + Iono for Jnk assays in Jurkat cells
- 15 min rIL-2 for p70S6k assays in Kit225 cells
- 15 min PdBu for p70S6k assays in Kit225 cells
- 5 min PdBu for Erk assays in Kit225 cells
- 5 min rIL-2 for PKB assays in Kit225 cells
- 60 min FCS for p70S6k assays in 293 cells
- 5 min FCS for Erk assays in 293 cells

**Cell lysis**

When not stated otherwise cells were lysed in the following buffers:

**T cells:** T cells (1 x 10⁷ to 2 x 10⁷ cells per ml) were lysed in 0.5 ml *lysis buffer 1:* 1% NP40, 150 mM NaCl, 50 mM HEPES pH7.4, 10 mM NaF, 10 mM iodoacetamide, 100 μM sodium orthovanadate (Na₃VO₄), 1 mM phenylmethylsulfonyl fluoride (PMSF), and the small peptide protease inhibitors leupeptin, pepstatin A, and chymostatin, all at 1 μg/ml.

**Rat-1 cells:** Rat-1 cells plated onto 9 cm cell culture dishes (Nunc) were lysed in 1 ml *lysis buffer 1.*

**Cos7 cells:** Cos7 cells plated onto 10 cm cell culture dishes (Falcon) were lysed in 0.5 ml *Cos7 lysis buffer:* 1%(w/v) Triton X-100, 20 mM Tris pH7.5, 137 mM NaCl, 15%(v/v) glycerol, 2 mM EDTA, 2 mM PMSF, 20 μg/ml aprotinin, 2 mM benzamidine, 1 mM Na₃VO₄, and the small peptide inhibitors leupeptin, pepstatin A, and chymostatin all at 2 μg/ml.

**293 cells:** 293 cells plated onto 10 cm cell culture dishes (Falcon) were lysed in 0.4 ml *lysis buffer 2:* 120 mM NaCl, 50 mM Tris pH8.0, 20 mM NaF, 1 mM benzamidine, 1 mM EDTA, 6 mM EGTA, 7.5 mM PPI, 15 mM p-Nitrophenylphosphate (pNPP), 1% NP40,
0.1 mM PMSF, and 0.1 mM Na$_3$VO$_4$. Extracts were prepared as described (Ferrari et al., 1993).

**Determination of protein concentration**

Aliquots of the supernatants were taken for a Bradford protein assay (Bradford, 1976), using the Coomassie Blue G-250 reagent solution from Pierce and bovine serum albumin as a standard.

**Expression of recombinant proteins in *E. coli***

Desired recombinant proteins were expressed in *E. coli* as glutathione-S-transferase (GST) fusion proteins to allow purification from bacterial lysates. An overnight culture of *E. coli* (XL1-Blue strain) carrying the pGEX expression plasmid which contains the desired cDNA were diluted in 400 ml L-broth/50 µg/ml ampicillin (see below). The culture was grown until an OD$_{550}$=0.5 to 0.6 was reached and expression of fusion protein was induced by adding isopropyl β-D-thiogalactopyranoside (Sigma) to a final concentration of 0.4 mM. The culture was left growing for an additional 2 h to 5 h at 37 °C. A shorter incubation times yields less protein but the protein obtained may be more soluble and stable. *E. coli* were harvested by centrifugation at 4,000 rpm for 15 min at 4 °C, the pellet was resuspended in 10 ml of ice-cold GST lysis buffer (1% Triton X-100, 2 mM EDTA, 1 mM PMSF, in PBSA) and stored at -20 °C.

**Purification of recombinant proteins from *E. coli***

The bacteria were lysed by sonication on ice (4 to 6 x 1 min) and cell debris were removed by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatant was incubated with 1 ml of 50%(w/v) suspension of glutathione agarose beads (Sigma) which had been washed thoroughly in PBSA containing 2 mM EDTA. After 1 h to 2 h at 4 °C, the suspension was centrifuged at room temperature in a bench top centrifuge and the beads were washed 2 times in GST lysis buffer and 3 times in PBSA containing 2
mM EDTA to remove unbound proteins. As an additional purification step the GST fusion proteins were eluted from the beads by competition with 25 mM reduced glutathione in 50 mM Tris pH8. Proteins were dialysed against PBSA containing 2 mM EDTA at 4 °C and the protein concentration was determined. GST fusion proteins were either re-coupled to glutathione agarose beads or left uncoupled and stored in 50% glycerol containing 50 mM Hepes pH7.4 and 50 mM NaCl at -20 °C.

**Protein isolation from cell lysates**

Postnuclear cell lysates were pre-cleared with protein A cell suspension (Sigma) prior to purification of cellular proteins with affinity resins. Protein A cell suspension (Staph A) (Sigma) (Kessler, 1975) was equilibrated, by washing four times in the appropriate lysis buffer. 1/10 volume of the washed Staph A suspension (10%) was added to the cell lysate (final conc. 1%) and lysates were pre-cleared by rotating for 20 min at 4 °C and subsequent spinning at 15,000 x g for 5 min at 4 °C. The supernatant was transferred into a separate Eppendorf microcentrifuge tube and the pre-cleared cell lysates were subjected to protein isolation procedures. Precipitation procedures were carried out for two to four hours by gently rotating the tubes on a wheel at 4 °C as described below. Precipitated protein complexes were processed as detailed in following sections.

**Affinity purification of cellular proteins:** Proteins were affinity purified from pre-cleared postnuclear lysates with either glutathione-agarose beads (Sigma) preloaded with approximately 5 μg of GST fusion proteins or 25 μM of the various synthetic peptides precoupled to 'Affigel 10' activated ester agarose beads (Bio-Rad). 1 x 10^7 cell equivalents (0.5 ml volume of cell lysate) were used per sample.

**Immunoprecipitations:** To immunoprecipitate rCD2-PI 3-kinase chimeras 0.5 ml of pre-cleared postnuclear lysates from transfected cells were incubated with 5 μg (to 10 μg) OX34 anti-rCD2 mAbs. After 30 min 30 μl protein G-Sepharose beads (34% suspension of beads) (Sigma) were added and incubations were continued for 90 min.
The p85 subunit of PI 3-kinase was immunoprecipitated with 10 μg p85α (U5) mAb followed by protein G-Sepharose beads (see above). Lysates containing HA- or Myc-epitope tagged proteins (0.3 ml volume) were incubated with 2 μg 12CA5 mAbs or 2 μg 9E10 mAbs, respectively. After 90 min 30 μl of 34%(w/v) protein G-Sepharose beads were added and the immunocomplexes were precipitated after further 30 to 45 min. Endogenous p70S6k or endogenous PKB were isolated with 1 μg of M5 Abs or 2 μg of Rac-PK-CT Abs, respectively (150 μl volume and 90 min incubation). Immune complexes were precipitated with protein A-Sepharose beads (Pharmacia) (after an additional 45 min incubation).

**In vitro protein kinase assay**

The immunoprecipitates were washed three times in the appropriate lysis buffer and two times in **protein kinase assay buffer** (25 mM HEPES, pH7.4, 100 mM NaCl, 5 mM MnCl₂, 10 mM MgCl₂, 100 μM Na₃VO₄). With the final wash the immunoprecipitates were transferred into a separate tube and the rest of the solution was removed with a Hamilton syringe. The protein kinase assay was initiated by adding 100 nM [γ-³²P]ATP (10 μCi) and 30 μM ATP in 50 μl protein kinase assay buffer. After 20 min incubation at 25 °C in vitro phosphorylation was stopped by diluting the reaction mixture with 1 ml appropriate lysis buffer containing 20 mM EDTA. The immunoprecipitates were then washed two times in this buffer and the rest of the solution was removed with a Hamilton syringe. The immunoprecipitated proteins were eluted from the beads by boiling for 8 min in 60 to 90 μl reducing sample buffer and separated by SDS-PAGE as detailed below. The gels were fixed in 30%(v/v) methanol/10%(v/v) acetic acid for 30 min, dried and ³²P-labelled proteins were visualised by autoradiography at -70 °C.
PI kinase assays

The immunoprecipitates were washed once in the appropriate lysis buffer, twice in washing buffer 1 (100 mM Tris pH 7.5, 0.5 M LiCl, 100 μM Na₃VO₄) and twice in washing buffer 2 (50 mM Hepes pH 7.4, 100 mM NaCl, 1 mM EDTA, 100 μM Na₃VO₄). With the final wash the immunoprecipitates were transferred into a separate tube (Sarstedt, with a rubber sealed lid) and the rest of the washing buffer 2 was removed with a Hamilton syringe. The immunoprecipitates were assayed for inositol lipid kinase activity using unilamellar liposomes composed of a 1:1 mixture of L-α-phosphatidylinositol (PI) and L-α-phosphatidyl-L-serine (PS), which were prepared by sonication (30 s, 30 s break, 1 min, 30 s break, 30 s; 50% level) of the lipid mix (PI and PS, both at a concentration of 1 mg per ml) in 25 mM HEPES buffer, pH 7.4 containing 1 mM EDTA on ice. The washed immunoprecipitates were resuspended in 10 μl lipid mixture and the reaction was initiated by the addition of 40 μl PI kinase reaction buffer (25 nM [γ⁻³²P]ATP (5 μCi), 125 μM ATP, 12.5 mM MgCl₂, 125 mM NaCl, 25 mM Hepes pH 7.4, 250 μM adenosine). The final concentrations in the assay were: 20 nM [γ⁻³²P]ATP (5 μCi), 100 μM ATP, 10 mM MgCl₂, 100 mM NaCl, 25 mM Hepes pH 7.4, 200 μM EDTA, and 200 μM adenosine (to inhibit PI 4-kinase activity, (Whitman et al., 1988)). After 15 min at 25 °C the reaction was terminated by the addition of 500 μl chloroform : methanol (1:2) in 1% conc. HCl plus 125 μl chloroform and 125 μl HCl (10 mM). The mixture was vigorously mixed and then centrifuged to separate the phases. 200 μl of the organic (lower) layer was removed (with a chloroform sealed tip) and washed once with 400 μl methanol : 100 mM HCl plus 2 mM EDTA (1:1). The organic phase was extracted, dried in vacuo, and resuspended in 25 μl chloroform. The phospholipids were separated by thin layer chromatography (TLC) on 1% potassium oxalate pre-treated Silica gel 60 plates (Whatman) in propanol-1 : 2 M acetic acid (65:35 (v/v)) developing solvents and visualised by exposure to iodine vapour and autoradiography at -70 °C. Phospholipids were identified by comparison with non-labelled standards. Incorporation of ³²P into PI was quantified using a PhosphorImager (Molecular Dynamics).
Materials and methods

[\text{\textsuperscript{32}P}]\text{PIP} counts obtained for each sample were normalised to the specific activity of the [\text{\textsuperscript{\gamma-32}P}]\text{ATP} used for the assay, and are expressed as fmol of PIP per min.

\textit{p70S6k assays and processing of kinase assays}

After stimulations as indicated cells from every cell line used were lysed in lysis buffer 2. For 293 cells 100 \(\mu\)g total protein extract, and for Kit225 cells cell extracts corresponding to 3 x 10\(^6\) cell equivalents (non-transfected cells) or 1.5 x 10\(^6\) live cell equivalents (transfected cells) were used per point.

The immunoprecipitates were washed three times in lysis buffer 2, and once in \textit{p70S6k assay buffer} (50 mM Mops pH7.2, 5 mM MgCl\(_2\), 0.1% Triton X-100) and assayed as described (Ming et al., 1994). Briefly, the reaction was initiated by the addition of 10 \(\mu\)l \textit{p70S6k reaction buffer} containing 3 \(\mu\)Ci [\text{\textsuperscript{\gamma-32}P}]\text{ATP}, 100 \(\mu\)M ATP, 50 mM Mops pH7.2, 5 mM MgCl\(_2\), 10 mM pNPP, 1 mM dithiothreitol (DTT), 500 nM Protein Kinase Inhibitor (PKI) (Sigma), 0.05% Triton X-100 and 20 \(\mu\)g 40S ribosomes as a source for S6 substrate (a gift from George Thomas, FMI, Basel). After 30 min at 37 \(^\circ\)C the reaction was terminated by adding reducing SDS-PAGE sample buffer and boiling. Proteins were resolved by SDS-PAGE. The lower part of the gel was stained with Coomassie Blue, destained in 30%(v/v) methanol/10%(v/v) acetic acid, dried and \textsuperscript{32}P-labelled S6 proteins were detected by autoradiography. The levels of p70S6k protein in each immunoprecipitate were assessed by transferring the proteins in the upper part of the gel onto PVDF membranes and Western blot analysis with 12CA5 mAbs or M1 Abs using the ECL detection system (Amersham). If the p70S6k protein levels in the immunoprecipitate were not equal, activities were normalised for p70S6k expression levels by quantitation of Western blots probed with M1 Abs followed by \textsuperscript{125}I-conjugated protein A (Amersham). Quantitation of incorporated \textsuperscript{32}P into S6 or of bound \textsuperscript{125}I-conjugated protein A was performed using a PhosphorImager.
PKB assays

After stimulations as indicated cells from every cell line used were lysed in lysis buffer 3 (120 mM NaCl, 50 mM Hepes pH7.4, 10 mM NaF, 1 mM EDTA, 40 mM β-glycerophosphate pH7.5, 1% NP40, 0.1 mM PMSF, 0.1 mM Na₃VO₄). For Kit225 cells cell extracts corresponding to 3 x 10⁶ cell equivalents (non-transfected cells) or 1.5 x 10⁶ live cell equivalents (transfected cells) were used per point. For Jurkat cells cell extracts corresponding to 1 x 10⁶ live cell equivalents were used per point.

The immunoprecipitates were washed twice in lysis buffer 3, twice in high salt wash buffer (500 mM LiCl, 100 mM Tris pH7.5, 1 mM EDTA pH7.5) and once in PKB assay buffer (50 mM Tris pH7.5, 10 mM MgCl₂, 1 mM DTT). The reaction was initiated by the addition of 15 μl PKB reaction buffer containing 3 μCi [γ-³²P]ATP, 50 μM ATP, 7.3 mM MgCl₂, 730 μM DTT, 500 nM PKI, 40 mM Tris pH 7.5, and 2.5 μg histone 2B (H2B) (Boehringer Mannheim). After 30 min at 25 °C the reaction was terminated by adding reducing SDS-PAGE sample buffer and boiling. Proteins were resolved by SDS-PAGE and the gel was treated as for p70S6k assays. To detect PKB proteins Western blot analysis was performed with Rac-PK-CT Abs.

Erk assays

Cells and cell extracts were processed as for p70S6k assays except that for 293 cells 250 μg total protein extract was used.

Precipitated immune complexes were washed three times with lysis buffer 2 and once with Erk wash buffer (30 mM Tris pH8.0, 20 mM MgCl₂, 2 mM MnCl₂). The reaction was initiated by the addition of 10 μl Erk reaction buffer containing 4 μCi [γ-³²P]ATP, 20 μM ATP, 20 mM MgCl₂, 2 mM MnCl₂, 5 mM pNPP, 500 nM PKI, 30 mM Tris pH8.0, and 15 μg Myelin Basic Protein (MBP) (Sigma). After 30 min at 37 °C the reaction was terminated by adding reducing SDS-PAGE sample buffer and boiling. Proteins were resolved by SDS-PAGE and the gel was treated as for p70S6k assays. To detect Erk proteins Western blot analysis was performed with 12CA5 mAbs as
primary Ab, rabbit anti-mouse IgG as secondary Ab (DAKO) and \(^{125}\)I-conjugated protein A.

**Jnk assays**

Cells and cell extracts were processed as for p70S6k assays. For Jurkat cells cell extracts corresponding to \(1 \times 10^6\) live cell equivalents were used per point.

Precipitated immune complexes were washed three times with lysis buffer 2 and once with *Jnk assay buffer* (25 mM Hapes pH7.4, 20 mM \(\beta\)-glycerophosphate pH7.0, 10 mM MgCl\(_2\), 0.5 mM DTT, 0.1 mM Na\(_3\)VO\(_4\)). The reaction was initiated by the addition of 15 \(\mu\)l Jnk assay buffer containing 4 \(\mu\)Ci \(\gamma^{32}\)P]ATP, 20 \(\mu\)M ATP, 333 nM PKI and 2.5 \(\mu\)g GST-Jun (aa 1-135) (Kyriakis et al., 1994). After 20 min at 30 °C the reaction was terminated by adding reducing SDS-PAGE sample buffer and boiling. Proteins were resolved by SDS-PAGE and the gel was treated as for p70S6k assays. To detect Jnk proteins Western blot analysis was performed with JNK2 (FL) Abs.

**Protein expression analysis**

To test for effector protein expression in transfected cells, postnuclear cell extracts corresponding to 2 to 4 \(\times\) \(10^6\) cell equivalents were analysed. Postnuclear cell lysates were acetone-precipitated for Western blot analysis as follows: 700 \(\mu\)l pre-cooled (-20 °C) acetone was added to 500 \(\mu\)l lysate and samples were incubated at -20 °C for 2 h. Precipitated proteins were pelleted in an Eppendorf microcentrifuge at 15,000 x g for 30 min at 4 °C. The supernatant was discarded and the protein pellets were washed once with 400 \(\mu\)l pre-cooled (-20 °C) ethanol. The proteins were dried and rehydrated by adding 20 \(\mu\)l to 40 \(\mu\)l distilled water and incubating for 15 min at 37 °C. An equal volume of two times reducing sample buffer was added and samples were boiled for 8 min prior to SDS-PAGE as detailed in a following section.

To test for effector protein expression in transfected 293 cells, 50 \(\mu\)g postnuclear cell extract were mixed with reducing sample buffer and analysed.
To test for the presence of proteins when proteins were affinity purified, the protein precipitates were washed four times in lysis buffer. With the final wash the immunoprecipitates were transferred into a separate tube and the rest of the solution was removed using a Hamilton syringe. Samples were boiled for 8 min in 60 µl to 90 µl reducing sample buffer prior to SDS-PAGE as described below.

**SDS-polyacrylamide gel electrophoresis (PAGE)**

For a comprehensive description of SDS-PAGE see 'Gel electrophoresis of Proteins' (Hames et al., 1981). The principal of discontinuous SDS-PAGE used here was first described by Laemmli for acrylamide capillary tube gels (Laemmli, 1970).

The **reducing sample buffer** contained: 3%(w/v) SDS, 10%(v/v) glycerol, 62.5 mM Tris-HCl pH6.8, 5%(v/v) β-mercaptoethanol, 0.005%(w/v) bromophenol blue. For normal gels a readily made stock solution of 30%(w/v) acrylamide/0.8%(w/v) N,N'-methylene-bis-acrylamide (National Diagnostics) was used for 7-17% gradient, 7%, 8%, 10% or 12.5% SDS-polyacrylamide gels (percentage acrylamide for running gel (pH8.8)). For shift gels a Cold Spring Harbour gel mix containing a lower concentration of N,N'-methylene-bis-acrylamide was used (10% acrylamide/0.13% N,N'-methylene-bis-acrylamide or 15% acrylamide/0.075% N,N'-methylene-bis-acrylamide). The stacking gel generally had a concentration of 5% acrylamide (pH6.8). The **running buffer** used contained: 25 mM Tris, pH8.3, 190 mM glycine, 3.5 mM SDS. Prestained protein molecular weight standards had a molecular weight range of 14,300 to 200,000 and were purchased from GIBCO BRL.

**Western blot analysis**

After SDS-PAGE, samples for Western blot analysis, either immunoprecipitates or acetone-precipitated cell lysates, were transferred by wet electroblotting onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) (Kamps and Sefton, 1989). The **transfer buffer** used was 10 mM CAPS buffer (Sigma), pH11, and
blotting was carried out for 8 h to 14 h at 0.3 A. The filters were blocked using 5% dried skinned milk (DSM) in PBSA. The membranes were then incubated overnight at 4 °C with the first layer antibody in a sealed plastic bag on a rocker. The antibodies were applied typically in PBSA containing 0.5% DSM, 0.05% Tween-20, 0.1% NaN₃ in the following concentration or dilution:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Antibody Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7 μg/ml</td>
<td>anti-p85α, U5 (mouse); 5% DSM, 0.5% Tween-20, 0.1% NaN₃</td>
</tr>
<tr>
<td>1 μg/ml</td>
<td>anti-rCD2, OX34 (mouse)</td>
</tr>
<tr>
<td>0.5 μg/ml</td>
<td>anti-Myc epitope, 9E10 (mouse)</td>
</tr>
<tr>
<td>0.5 μg/ml</td>
<td>anti-HA epitope, 12CA5 (mouse)</td>
</tr>
<tr>
<td>1 μg/ml</td>
<td>anti-phosphotyrosine, 4G10 (mouse)</td>
</tr>
<tr>
<td>0.25 μg/ml</td>
<td>anti-Grb2, Grb2 (mouse)</td>
</tr>
<tr>
<td>1 μg/ml</td>
<td>anti-Ras, pan-ras (OP41) (mouse)</td>
</tr>
<tr>
<td>2.5 μg/ml</td>
<td>anti-mSos1, Sos1 (rabbit)</td>
</tr>
<tr>
<td>1/100</td>
<td>anti-mSos1, Sos3 (rabbit)</td>
</tr>
<tr>
<td>1/1,000</td>
<td>anti-Ick, RNGS (rabbit)</td>
</tr>
<tr>
<td>1/500</td>
<td>anti-fyn, anti-yes, CST-1 (rabbit)</td>
</tr>
<tr>
<td>1/500</td>
<td>anti-p110, p110 (PW38) (rabbit)</td>
</tr>
<tr>
<td>1/5,000</td>
<td>anti-p70S6k, M5 (rabbit)</td>
</tr>
<tr>
<td>0.5 μg/ml</td>
<td>anti-PKB, Rac-PK-CT (rabbit)</td>
</tr>
<tr>
<td>0.1 μg/ml</td>
<td>anti-Jnk2, JNK2 FL (rabbit)</td>
</tr>
</tbody>
</table>

For far-Western blotting, 1 μg/ml Myc-tagged fusion proteins were incubated overnight in PBSA containing 1% DSM, 0.1% Tween-20, 0.1% NaN₃. Membranes were washed three times for 15 min at room temperature in PBSA containing 1% DSM, 0.1% Tween-20 and incubated with 9E10 mAbs for 2 h at room temperature in this buffer.

The membranes were washed three times in PBSA containing 0.05% Tween-20 and proteins were visualised using a chemiluminescence detection system (ECL, Amersham) with a sheep anti-mouse Ig or donkey anti-rabbit, horseradish peroxidase as secondary antibody (Amersham). Secondary antibodies were applied in a 1/5,000 to 1/10,000 dilution in PBSA containing 0.05% Tween-20.
Identification of unknown proteins by mass spectrometry

The identification of unknown proteins by mass spectrometric analysis is based on a combination of protease digestion, matrix-assisted laser-desorption ionisation mass spectrometry (MALDIMS) and screening of peptide-mass databases, e.g. the molecular weight search (MOWSE) peptide-mass database at the SERC Daresbury Laboratory, UK (Pappin et al., 1993). This method generates molecular weight fingerprint maps and allows an identification of proteins from as few as three or four experimentally determined peptide masses when these are screened against a fragment database that is derived from over 160,000 proteins (Pappin et al., 1993; Pappin et al., 1996). The analysis requires very low amounts of protein and the sensitivity extends into the low femtomole range. Additional information about the identity of a protein can be gained by specific chemical modification (e.g. esterification of acidic residues) of the sample followed by a comparative search with these parameters in the MOWSE database that includes search options for these criteria.

Proteins isolated from T cell lysates were separated by SDS-PAGE and transferred onto PVDF membrane. The membrane was washed thoroughly in deionised water to remove buffer salts and dried in vacuo for 20 min. Proteins were visualised by staining with sulforhodamine B (Kodak Ltd.) as described (Coull and Pappin, 1990).

Digestion of proteins: The apparent protein bands were excised from the membrane, dried, placed in 0.5 ml eppendorf tubes and wet with 2-4 μl of 50 mM ammonium bicarbonate solution containing 1%(w/v) octyl glucoside and 40 ng/ml trypsin protease (modified grade, Promega). Digestion and processing of samples were performed as described (Sutton et al., 1995). Aliquots of the sample (0.3-0.5 μl) were applied to sample slides, and dried under high vacuum for 30 min for mass spectrometry analysis.

Esterification of peptides: Aliquots of the sample (0.5-2 μl) from the digested protein were dried in vacuo and treated with 10 μl of 1%(w/v) thionyl chloride in dry methanol for 30 min at 50 °C to effect esterification (methyl esters) of peptide acidic residues (E=Glu,
D=Asp). Samples were processed as described (Pappin et al., 1996) and aliquots were dried onto target slides for mass spectrometry analysis.

**Mass spectrometry analysis of samples:** Dry samples on target slides were re-wet with 0.5 μl matrix solution (1%(w/v) alpha cyano-4-hydroxycinnamic acid in 50% aq. acetonitrile containing 0.1% TFA and 200 fmol/μl insulin B chain as internal standard), allowed to air dry and analysed by MALDIMS using a Finnigan MAT LaserMat 2000 mass spectrometer (Mock et al., 1992). Spectra were calibrated using the insulin B chain as an internal standard. Observed proteolytic fragment masses were screened against the MOWSE peptide-mass database (established by Darryl Pappin in collaboration with the SERC Daresbury Laboratory, Warrington, UK) as described (Pappin et al., 1993; Pappin et al., 1996).

**Gene expression analysis**

Determination of chloramphenicol acetyltransferase (CAT) activity was determined according to the method of Sleigh (Sleigh, 1986).

**NIH 3T3 cells:** Eight to 10 hours after inductions as indicated transfected serum-starved NIH 3T3 cells were washed with PBSA and cells were lysed in 150 μl CAT lysis buffer 1 (0.65% Triton-X100, 10 mM Tris pH8, 1 mM EDTA, 150 mM NaCl). The relative protein concentration of each postnuclear lysate was measured. An equivalent amount of protein was used per CAT assay, sample volumes were kept constant (typically 75 μl). Samples were heat-inactivated for 10 min at 68 °C and cooled on ice. The CAT reaction was initiated by adding 40 μl of CAT assay reaction buffer (21 mM [14C]acetyl coenzyme A (0.05 μCi), 0.5 mM acetyl coenzyme A, 5 mM chloramphenicol, 500 mM Tris pH8.0). After 2 h to 6 h at 37 °C reactions were terminated by freezing. Acetylated chloramphenicol was extracted with 200 μl ethylacetate and vortexing vigorously. The phases were separated by centrifugation in an Eppendorf microcentrifuge at 15,000 x g for 5 min and 100 μl of the upper, organic phase was removed to a scintillation vial containing 3 ml EcoLume™ scintillant (ICN) and counted.
using a calibrated $^{14}$C-label program on a Beckman LS6000 series scintillation counter. Transfection efficiency was monitored with appropriate β-galactosidase (lacZ) expression vectors as described (Price et al., 1995). The data are presented as the ratio of CAT activity (percentage conversion) to β-galactosidase optical density (OD) units.

**T cells:** Fourteen to 16 hours after inductions as indicated Kit225 or Jurkat T cells were harvested and cells were lysed in 200 μl CAT lysis buffer 2 (0.65% NP40, 10 mM Tris pH 8, 1 mM EDTA, 150 mM NaCl). Similar cell equivalents were tested for enzymatic activity able to transfer $^{14}$C-labelled acetyl groups onto chloramphenicol as described above. The data are presented as percentage conversion.

**Minipreparation of plasmid DNA**

Single colonies were used to inoculate 5 ml of *Luria broth* (L-broth) (10 g bacto tryptone, 5 g yeast extract, 10 g NaCl, ad 1 l) containing 50 μg/ml ampicillin (or other appropriate antibiotics). Minipreps were carried out using 3 ml of an *E. coli* culture grown for 8 h to 15 h. Cells were pelleted by centrifugation and the pellet was resuspended in 200 μl solution I (25 mM Tris pH 8, 50 mM Glucose, 10 mM EDTA) on ice. 400 μl of solution II (0.2 M NaOH, 1% SDS) were added and the tubes were inverted 3 to 4 times. After addition of 300 μl of solution III (60%(v/v) 5 M potassium acetate, 11.5%(v/v) glacial acetic acid, in water), the suspension was mixed and left on ice for 15 min. The sample was spun in a microcentrifuge for 5 min and the supernatant was transferred to a new tube. The plasmid DNA was precipitated with 600 μl isopropanol (2-propanol) at 4 °C. After 10 min centrifugation, the dried pellet was resuspended in 40 μl H$_2$O and 5 μl to 10 μl were used in restriction digest analysis. In some experiments, the DNA solution was further purified with an equal volume (typically 100 μl) of 5 M LiCl to precipitate high molecular weight RNA. After incubation on ice for 5 min, RNA was pelleted and the plasmid DNA in the supernatant was precipitated with 2 to 3 volumes ethanol at -20 °C.
Maxipreparation of plasmid DNA

Milligram quantities of very pure plasmid DNA for cell transfections and microinjections were obtained by an alkaline/SDS lysis method (Birnboim and Doly, 1979) followed by isopycnic centrifugation in a caesium chloride solution (Radloff et al., 1967). Briefly, 400 ml culture of E. coli carrying the desired plasmid, grown overnight in brain heart infusion (BHI) broth (37 g BHI powder in 1 l), were pelleted at 4,000 rpm, and resuspended in 20 ml solution I. Bacterial cells were lysed by adding 40 ml of solution II. Denatured proteins, chromosomal DNA and cellular debris were precipitated by adding 30 ml of solution III. After 15 min on ice, precipitates were removed by centrifugation, the DNA in the supernatant was recovered with 70 ml isopropanol at 4 °C and the pellet was washed with 10 ml of cold 75% ethanol. The DNA pellet was resuspended in 4.5 ml of TE buffer (10 mM Tris pH 8, 1 mM EDTA) and 4.9 g CsCl was added. After the CsCl was dissolved 0.4 ml of 10% (w/v) ethidium bromide solution was added, the tubes were covered with aluminium foil and the solution was incubated for 30 min at room temperature to precipitate proteins. The proteins were pelleted by centrifugation at 10,000 rpm (Beckman JA-20 rotor) for 20 min at 20 °C. The supernatant was transferred to a heat-sealable ultracentrifugation tube (Beckman) and centrifuged in a vertical rotor (VTi 65.2, Beckman) at 65,000 rpm for 4 h at 20 °C to allow density gradient formation. The plasmid band was harvested with a syringe, transferred to a new heat-sealable ultracentrifugation tube and the plasmid DNA was further purified on a second CsCl gradient. The plasmid DNA was removed and the ethidium bromide extracted with water saturated n-butanol. The DNA was precipitated with 2 to 3 volumes of ethanol and resuspended in TE buffer.

Restriction enzyme analysis of DNA

Plasmid DNA (1 μg) was digested in a volume of 10 μl in the buffer supplied by the manufacturer at 37 °C for 30 min to 60 min using ~10 units (1 μl) of restriction enzyme. Fragments were separated on an agarose gel.
Partial digest of DNA

For cloning purposes restriction sites had to be used that occurred more than once in the cDNA. Hence digest were carried out under limiting conditions in the presence of 0.05 μg/μl ethidium bromide, less than 1 unit of restriction enzyme (< 0.1 μl enzyme) per 1 μg DNA (10 μl volume), short incubation times or a combination of these conditions.

Phosphatase treatment of DNA

To avoid religation of linerised vector, 5'-terminal monophosphate groups of DNA were hydrolysed with 1 unit of alkaline phosphatase (New England Biolabs) per pmol DNA ends in phosphatase buffer (50 mM NaCl, 10 mM Tris pH8, 10 mM MgCl₂, 1 mM DTT) for 30 min at 37 °C (typically 0.5 μg DNA/10 μl). EDTA (pH8) was added to 5 mM and the enzyme was heat-inactivated for 10 min at 75 °C. The solution was extracted with an equal volume of phenol/chloroform and the DNA precipitated with 0.1 volume 3 M sodium acetate pH 5.5 and 2 volumes ethanol and resuspended in TE buffer.

Agarose gel electrophoresis

For fragments between 500 and 1,500 basepairs, an agarose gel of 1% was used. Fragments larger than 1,500 basepairs were separated on a 0.7% gel. Ultra pure agarose (GIBCO) was boiled in 1 x TAE buffer (0.04 M Tris-acetate, 2 mM EDTA pH8; 50 x TAE buffer: 242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 mM EDTA pH8, ad 1 l), ethidium bromide (0.5 μg/ml) was added and the solution was left to solidify in a gel plate. The DNA was loaded with 0.1 volume of 10 x DNA sample buffer (40% glycerol, 0.4% bromophenol blue) and electrophoresed at 120 to 150 mAmp.
Chapter 2  Materials and methods

Isolation of DNA fragments

DNA fragments were excised from agarose gels and DNA was isolated using the Geneclean II® kit (BIO 101 Inc., CA) according to the manufacturer's instructions.

Ligation of DNA fragments

To ligate DNA fragments into a cut plasmid T4 DNA ligase was used which catalyses the formation of phosphodiester bonds between neighbouring 3'-hydroxyl and 5'-phosphate ends in double stranded DNA. Typically a 3 molar excess of cDNA fragment compared to linear plasmid was used, keeping the total amount of DNA to less than 50 ng. Ligation was performed in a volume of 10 µl in 1 x T4 DNA ligase buffer (GIBCO) using 1 U T4 ligase (GIBCO) for 1 h to 2 h at room temperature or alternatively for 8 h to 15 h at 16 °C.

DNA transformation of E. coli

The ligation reaction (less than 50 ng DNA in 10 µl volume) or 0.5 ng to 1 ng of other circular plasmid DNA was added to 20 µl to 100 µl of competent E. coli cells (see below) on ice and mixed gently. After 30 min on ice, the E. coli cells were heat shocked by placing for 5 min at 37 °C (or for 2 min at 42 °C) and then returned to ice. 200 µl of L-broth was added and cells were left to recover for 30 min to 60 min at 37 °C. Subsequently cells were plated onto L-broth agar plate containing typically 50 µg/ml ampicillin.

Preparation of competent bacteria cells

E. coli strains used were JM101 or DH5 for amplification of plasmid DNA and XL1-Blue for expression of recombinant proteins. Best results for preparation of plasmid DNA have been observed with the DH5 E. coli strain. A colony was picked from a minimal plate and grown overnight in 25 ml L-broth. The culture was diluted 1/200 in 400 ml L-broth and E. coli were grown to logarithmic phase (OD550=0.45). The cells were
chilled on ice, spun at 4,000 rpm for 10 min at 4 °C and resuspended in ice-cold 160 ml 
transformation buffer I (30 mM potassium acetate, 100 mM RbCl₂, 10 mM CaCl₂, 50 mM 
MnCl₂, 15% glycerol; final pH 5.8). After 30 min on ice, bacteria cells were pelleted at 
4,000 rpm for 10 min at 4 °C, the cells were resuspended in 32 ml transformation buffer II 
(10 mM PIPES, 75 mM CaCl₂, 10 mM RbCl₂, 15% glycerol; final pH 6.5) and incubated 
for 30 min on ice. Aliquots of 400 µl were snap frozen on cardice and stored at -70 °C.

Mutagenesis of DNA using polymerase chain reaction

Polymerase chain reaction (PCR) was employed to change sequences close to 
the ends of a cDNA fragment using the Taq polymerase which is a highly processive 5'-
3' DNA polymerase lacking 3'-5' exonuclease activity, originally from Thermus aquaticus 
strains. An oligonucleotide in sense orientation corresponding to the 5' end of the cDNA 
fragment was used together with an oligonucleotide in the antisense orientation 
corresponding to the 3' end. Reactions were performed in a total volume of 100 µl 
containing 100 ng DNA template, 2 µl dNTP mix (dATP, dCTP, dGTP, dTTP; each 10 
mM), 10 µl 10 x PCR reaction buffer (100 mM Tris pH 8.3, 25 mM MgCl₂, 500 mM KCl, 
0.01% gelatine), and 5 µl 3' and 5' primer each (stock conc. each 5 µM). 2 units (0.4 µl) 
of Taq polymerase (Perkin Elmer) were used per reaction and the sample was overlaid 
with mineral oil (Sigma) to prevent evaporation. Typically an initial 96 °C melting step 
was followed by 35 cycles of 94 °C 1 min, 2 min annealing at 60 °C and 2.5 min 
extension at 72 °C. A final 9.9 min chase step at 72 °C was included. To test for the 
size of the PCR product 10 µl sample were loaded on an analytical agarose gel. The 
PCR products in the remaining sample were removed from primers and unincorporated 
nucleotides using the Geneclean ll® kit (BIO 101 Inc., CA). The PCR products were 
used for cloning after phenol/chloroform extraction and ethanol precipitation of the 
sample.
Sequencing of DNA

A standard single stranded DNA sequencing protocol was applied which is based on the dideoxy chain termination principle of Sanger using the Sequenase® version 2.0 T7 DNA polymerase kit (United States Biochemical). The radioactive labelling of the DNA was accomplished by adding $^{35}$S-labelled dATP (Amersham). To denature the double stranded plasmid DNA, 2 μg plasmid DNA resuspended in 18 μl water were mixed with 2 μl of 2 M NaOH and incubated for 15 min at 37 °C. The denatured DNA was precipitated quickly with 100 μl ethanol in the presence of 4 μl 10 M NH₄Ac. After 20 min on ice the DNA was pelleted in a microcentrifuge at 15,000 x g for 10 min at 4 °C, the DNA pellet was washed with 75% ethanol, dried and resuspended in 7 μl water. 2 μl of 5 x Sequenase reaction buffer and 1 μl of primer (stock conc. 5 pmol) was added and the primer was allowed to anneal to the DNA template for 20 min at 37 °C. The sample was chilled on ice and sequencing reactions were carried out according to the manufacturers instructions.

Samples were loaded onto a 6% or 8% polyacrylamide gel, containing 7 M urea in 1 x TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2.5 mM EDTA; final pH8.3; 10 x stock: 108 g Tris, 55 g boric acid, 10 ml of 0.5 M EDTA pH8, ad 1l) and electrophoresis was carried out for about 2 h at 38 W. The gel was fixed in 10% methanol/10% acetic acid for 10 to 15 min and dried under vacuum at 80 °C. Autoradiography was performed overnight at room temperature.
Chapter 3

The Grb2 adaptor protein associates via its SH3 domains with a novel 75 kDa tyrosine phosphoprotein in T lymphocytes

3.1 Introduction

Signals generated by TCR engagement are integrated by an array of cytoplasmic protein tyrosine kinases (PTKs) (Klausner and Samelson, 1991; Weiss, 1993). One signalling pathway which requires the activity of PTKs is the activation of PI 3-kinase (Carrera et al., 1994; Von Willebrand et al., 1994) although PI 3-kinase activity is not regulated by direct tyrosine phosphorylation (Reif, 1992; Reif et al., 1993; Ward et al., 1992). It is currently not clear how PI 3-kinase is coupled to and regulated by the TCR. I therefore was interested to study possible mechanisms that link the TCR to PI 3-kinase.

Another PTK controlled signalling pathway to originate from the TCR regulates the activity of the GTPase Ras (Downward et al., 1992; Izquierdo et al., 1992). The PTK link between receptors and Ras in many cells involves guanine nucleotide exchange proteins such as Sos, the homologue of a Drosophila Ras GDP/GTP exchange protein, the son of sevenless gene product (Bowtell et al., 1992). Sos complexes to the adapter protein Grb2/Sem5 which is composed of one SH2 domain and two SH3 domains. The SH3 domains of Grb2 bind to the C-terminal proline rich domain of Sos whereas the SH2 domain binds to tyrosine phosphorylation sites in a number of proteins such as the EGF receptor, Shc, IRS-1 and SHP-2 (Buday and Downward, 1993; Buday et al., 1994; Chardin et al., 1993; Egan et al., 1993; Li et al., 1994; McCormick, 1993; Rozakis-Adcock et al., 1993; Skolnik et al., 1993). The
interactions between the Grb2 SH2 domain and these tyrosine phosphoproteins is proposed to be important in determining the cell localisation/function of Sos.

It was described that the Grb2 SH2 domain interacts with at least two substrates for TCR activated PTKs, Shc and a 36 kDa membrane localised protein (Buday et al., 1994; Ravichandran et al., 1993; Tailor et al., 1996) and therefore appears to play a role in TCR signal transduction. Sos has been identified as one molecule that interacts with the Grb2 SH3 domains in fibroblasts. However, studies of the in vitro binding characteristics of Grb2 have revealed that the Grb2 SH3 domains can interact and regulate another protein, the cytoskeletal GTPase dynamin (Gout et al., 1993; Herskovits et al., 1993). These data raise the possibility that Grb2 SH3 domains can couple to effector molecules other than Sos and thus may function as an adaptor molecule in more than one signalling pathway. Grb2 SH3 domains bind proline-rich motifs. The p85 regulatory subunit of PI 3-kinase contains proline-rich regions. It therefore seemed possible that Grb2 may provide a link between the TCR and PI 3-kinase. This will be addressed in chapter 4. In the present chapter, I will describe that a novel protein, p75/SLP-76, and Sos can complex to Grb2 SH3 domains in T lymphocytes.

3.2 Results

Identification of proteins that complex to the Grb2 SH3 domains in T cells

To isolate endogenous proteins from cellular lysates the most widely used technique is to immunoprecipitate the protein of interest with specific antibodies. However, this method did not appear to be ideal to purify Grb2 from lysates in order to identify its SH3 domain associated partners as Grb2 is small (26 kDa) compared to an immunoglobulin (IgG = 150 kDa). Thus, it seemed very likely that antibody binding may
interfere with effector protein binding. Moreover, Grb2 co-migrates with the light chains of antibodies which hampers a quantitative analysis of Grb2 protein levels in the immune complexes. Grb2 can bind via its SH2 domain to phosphorylated tyrosine at residue 1068 in the EGF receptor cytoplasmic tail. Further, Grb2 associates via its SH3 domains with proline-rich regions in the C-terminus of Sos. I therefore tested the possibility to use synthetic peptides corresponding to the specific Grb2 binding sequence in the EGF receptor around residue 1068 or the Sos C-terminal proline-rich region to affinity purify Grb2 and its associated proteins from T cell lysates. To isolate Grb2 via its SH3 domains, I used the synthetic proline peptide SKGTDEVVPVPPVPRR corresponding to aa 1144-1159 in the C-terminal tail of mSos1 (Sos-PRO) that binds to the SH3 domains of Grb2 (Bowtell et al., 1992; Gout et al., 1993; Rozakis-Adcock et al., 1993). Grb2 was also precipitated from T cell lysates using the tyrosine phosphorylated peptide PVPEYINQS corresponding to aa 1064-

**Figure 3.1.** Schematic representation of peptide affinity resins used to isolate Grb2 from T cell lysates.

The EGFR-Y(1068)-P peptide binds Grb2 via its SH2 domain, hence blocks other SH2 binding proteins from associating with Grb2 and therefore allows to specifically isolate proline-rich motif containing proteins that bind to Grb2 SH3 domains. *Vice versa* a peptide corresponding to a proline-rich region in the C-terminus of Sos, Sos-PRO, purifies Grb2, prevents the interaction of other SH3 domain binding proteins and allows to separate tyrosine phosphorylated proteins which associate with the Grb2 SH2 domain.
1072 in the cytoplasmic tail of the EGF receptor (EGFR-Y(1068)-P) (Fig. 3.1). The EGFR-Y(1068)-P and the Sos-PRO peptide reagents have the clear advantage that their binding sites on Grb2 are known and therefore they are likely to allow the specific co-purification of proteins that associate with Grb2 SH3 domains or the Grb2 SH2 domain, respectively (Fig. 3.1).

The data in Fig. 3.2A show a Grb2 Western blot of the cellular proteins purified with the EGFR-Y(1068)-P peptide or the Sos derived proline peptide, SOS-PRO, and illustrate that these two peptides isolate equivalent levels of Grb2 from T cell lysates. The specificity of the interaction of the EGFR-Y(1068)-P peptide with Grb2 is indicated by the failure to precipitate Grb2 with the PDGFR-Y(751)-P phosphotyrosine peptide, DYVPML(G), derived from the cytoplasmic domain of the PDGF receptor. The EGFR-Y(1068)-P motif binds to the Grb2 SH2 domain and thus allows the co-purification of Grb2 and proteins that complex to the Grb2 SH3 domains. Western blot analysis with a Sos antiserum demonstrate that Sos co-purifies with Grb2 from T cell lysates when the EGFR-Y(1068)-P motif is used as an affinity matrix (Fig. 3.2B). This interaction was

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**Figure 3.2.** The EGFR-Y(1068)-P affinity resin co-purifies endogenous Grb2, Sos and other cellular proteins from T cell lysates. (A, B) Immunoblot analysis of proteins affinity-purified from quiescent (-) or T lymphocytes activated with 10 μg/ml of the TCR agonist UCHT-1 for 2 min (+) with EGFR-Y(1068)-P, SOS-PRO, and PDGFR-Y(751)-P peptides coupled to Affi-Gel 10 beads or beads alone (control). Precipitated proteins (isolated from 2 x 10^7 cells per lane) were analysed by 10% SDS-PAGE, immunoblotted with Grb2 mAb (A), or Sos1 antiserum (B) and visualised by ECL detection. The migration of the molecular mass standards is indicated to the left in kilodaltons. (C) [3^S]Methionine-labelled T lymphocytes were untreated (-) or stimulated with 10 μg/ml UCHT-1 for 3 min (+) and lysed (50 mM NaCl, 50 mM Hepes pH 7.4, 1% Brij 96, 10 mM NaF, 10 mM iodoacacetamide, 5 mM sodium orthovanadate, 1 mM PMSF, and the small peptide inhibitors leupeptin, pepstatin A, and chymostatin all at 1 μg/ml). Cellular proteins (1.0 x 10^7 cell equivalents per lane) were precipitated with EGFR-Y(1068)-P or Sos-PRO peptide coupled beads or beads alone (control). (D) T lymphocytes were labelled with [32P]orthophosphate as described in Methods. T cells were incubated in medium (0) or stimulated with 10 μg/ml of the TCR agonist UCHT-1 for the times indicated. Cell lysates were incubated with either Affi-Gel 10 beads alone (control) or EGFR-Y(1068)-P peptide coupled beads. (C, D) Affinity purified proteins were subjected to SDS-PAGE in 7-17% polyacrylamide gels and visualised by autoradiography. The migration of the molecular mass standards is indicated to the left in kilodaltons.
Chapter 3

Results: p75 associates with Grb2-SH3 domains

A

B

EGF-P SOS-PRO PDGF-P control

Grb2

Sos1
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Results: p75 associates with Grb2-SH3 domains

**A**

![Image A]

**B**

![Image B]

**C**

![Image C]

**D**

![Image D]
specific as the PDGFR-Y(751)-P motif did not precipitate Sos (Fig. 3.2B). Moreover, the Sos derived proline peptide that isolates Grb2 competed with endogenous proteins for Grb2 SH3 domains and failed to purify Sos (Fig. 3.2B). These data confirm that the EGFR-Y(1068)-P motif can be employed to isolate Grb2 and proteins associated with Grb2 SH3 domains, as the interaction of SH2 binding proteins with Grb2 is prevented. The Sos-PRO peptide also affinity purifies Grb2 but blocks the association of Grb2 SH3 binding proteins.

To assess whether Sos is the only intracellular protein that can associate with Grb2 SH3 domains, metabolic labelling experiments were performed in which EGFR-Y(1068)-P peptide complexes were isolated from \[^{35}\text{S} \text{methionine-labelled}\] (Fig. 3.2C) or \[^{32}\text{P} \text{l-labelled}\] (Fig. 3.2D) T lymphocytes. The relative molecular mass of the proteins found in EGFR-Y(1068)-P precipitates from \[^{35}\text{S} \text{metabolically labelled}\] T cells was 150, 75, 38, and 26 kDa (Fig. 3.2C). As well, a spectrum of phosphoproteins with the molecular mass of 150, 125, 75, and 38 kDa was present in EGFR-Y(1068)-P complexes purified from \[^{32}\text{P} \text{l-labelled}\] T lymphocytes (Fig. 3.2D). Proteins isolated with the Sos-PRO peptide beads from \[^{35}\text{S} \text{methionine-labelled}\] T cell lysates corresponded to the approximate molecular mass of 160, 130, 120, 80, 70, 50, 36, and 26 kDa (Fig. 3.2C). The most abundant protein present in EGFR-Y(1068)-P and Sos-PRO complexes of the \[^{35}\text{S} \text{methionine-} \text{labelling study}\] was a 26 kDa protein that on the basis of its electrophoretic mobility is likely to be Grb2. The pattern of proteins isolated with the EGFR-Y(1068)-P and Sos-PRO peptides were distinct and non-overlapping.

The relative molecular mass of the 150 kDa \[^{35}\text{S} \text{methionine-labelled}\] protein in the EGFR-Y(1068)-P protein complexes corresponds in size to Sos which is known to associate with the Grb2 SH3 domains. Sos is posttranslationally modified in TCR activated T cells which results in a reduction in its electrophoretic mobility (Fig. 3.2B). Similarly, the 150 kDa \[^{35}\text{S} \text{methionine-labelled}\] protein undergoes as well a reduction in
its electrophoretic mobility in TCR activated cells (Fig. 3.2C). Such gel shift behaviour can be explained with hyperphosphorylation of the protein although no increase in $^{32}\text{P}_1$ incorporation into the proteins corresponding to Sos was observed in the $^{32}\text{P}_1$-labelling experiment in Fig. 3.2D. The additional proteins that co-purify with Sos (Figs. 3.2C, 3.2D) could also bind to the Grb2 SH3 domain and hence be affinity purified with the EGFR-Y(1068)-P motif because of their association with Grb2. Alternatively, these

Figure 3.3. A 75 kDa protein binds to Grb2 SH3 domains in T lymphocytes. (A) Endogenous proteins from unstimulated (-) or 1.6 μg/ml OKT3 Fab' stimulated (2 min) (+) T lymphocytes were purified as indicated with EGFR-Y(1068)-P and Sos-PRO affinity resins or with Affi-Gel 10 beads alone (control). Precipitated proteins were analysed by 7-17% SDS-PAGE and replicate PVDF membranes were subjected to far-Western blot analysis with Grb2myc or Grb2mycSH3 fusion proteins as indicated. The migration of the molecular mass standards is indicated to the left in kilodaltons. (B) Far-Western blot analysis of Grb2myc binding to cellular proteins purified from either Rat-1 fibroblasts or T cells with EGFR-Y(1068)-P and Sos-PRO affinity resins or Affi-Gel 10 beads alone (control) as indicated. The migration of the molecular mass standards is indicated to the left in kilodaltons.
proteins could bind directly to the EGFR-Y(1068)-P phosphopeptide or indeed be associated with Sos. To establish whether the proteins detected in Grb2 complexes from these *in vivo* labelling experiments directly bind to Grb2, overlay assays were performed on EGFR-Y(1068)-P/Grb2 complexes isolated from quiescent or TCR activated T cells using either the intact Grb2myc or the Grb2myc|xSH3 fusion protein (Fig. 3.3). The data show that the EGFR-Y(1068)-P peptide precipitates contain 75 kDa and 150 kDa proteins that bind to intact Grb2myc but not to the SH3 mutant Grb2myc|xSH3 protein (Fig. 3.3A). The 150 kDa protein is almost certainly Sos as it co-migrates with the protein reactive with the Sos specific antisera. The interaction between Grb2 and Sos or p75 was not dependent on cell activation (Fig. 3.3A).
p75, like Sos, appeared to be a protein that binds to Grb2 SH3 domains. Firstly, p75 was only present in the Grb2 complexes isolated with the EGFR-Y(1068)-P motif and not in the ones purified with the Sos-PRO peptides (Fig. 3.3A). Secondly, p75 was only seen when the PVDF membranes were probed with intact Grb2myc protein but not with mutated Grb2myc\(^{iSH3}\) protein (Fig. 3.3A). Sos binds to Grb2 SH3 domains in fibroblasts. The data in Fig. 3.3B compare the Grb2 binding proteins in \(T\) cells and in Rat-1 fibroblasts. Grb2 complexes purified from fibroblasts with the EGFR-Y(1068)-P affinity matrix contain a 150 kDa protein reactive with Sos in the Western blot analyses but do not contain a protein of 75 kDa (Fig. 3.3B).

In the biosynthetic labelling experiments, multiple proteins could be co-purified with the Grb2/Sos-PRO peptide complex (Fig. 3.3C). These proteins did not apparently bind directly to Grb2 as they did not interact with the Grb2 fusion protein in the far-Western blot analyses (Fig. 3.3). Accordingly, it was concluded that although the Sos-PRO proline peptide appears to have a preferential affinity for Grb2 it also binds to additional proteins.

A 75 kDa protein that binds to the Grb2 SH3 domain is a substrate for TCR activated PTKs

In the \(^{32}\text{P}\)-labelling experiments in Fig. 3.2D a 75 kDa protein was seen in the EGFR-Y(1068)-P/Grb2 complexes which suggested that the 75 kDa protein that binds to Grb2 SH3 domains might be a phosphoprotein. Previous studies have shown that the Grb2 SH2 domain interacts with two proteins that become tyrosine phosphorylated in response to engagement of the TCR, Shc and a 36 kDa membrane associated protein (Buday et al., 1994; Ravichandran et al., 1993). To determine whether the Grb2 SH3 domains can interact with substrates for TCR activated PTKs anti-phosphotyrosine Western blot analyses were carried out on proteins purified from \(T\) cell lysates with a panel of full length or truncated Grb2 GST fusion proteins (as described above). The data in Fig. 3.4A show that recombinant Grb2 can precipitate multiple proteins that are...
Figure 3.4. A 75 kDa protein that binds to the Grb2 SH3 domains is a substrate for TCR activated PTKs. T lymphocytes were untreated (-) or stimulated with 10 μg/ml UCHT-1 mAbs for 2 min (+) or the times indicated. Cellular proteins were purified as described below and resolved by 10% SDS-PAGE. Protein complexes were analysed by immunoblotting with the anti-phosphotyrosine mAb 4G10 (A-C) or Sos3 antiserum (D). The migration of the molecular mass standards is indicated to the left in kilodaltons.

(A) Proteins were isolated from 2 x 10^7 T cells with the fusion protein of full length GST-Grb2 or GST alone (control) immobilised on glutathione-agarose beads.

(C) Proteins were purified from 2 x 10^7 T cells (per lane) with EGFR-Y(1068)-P or Sos-PRO affinity resins or Affi-Gel 10 beads alone (control) as indicated.

(B, D) Proteins were precipitated from T cell lysates (1.6 x 10^7 cell equivalents per lane) with fusion proteins encoding GST alone (GST), full length GST-Grb2 (Grb2), a double SH3 mutant GST-Grb2 49L/203R (Grb2pSH3), the isolated N-terminal GST-huGrb2 SH3 domain (aa 1-58) (N-SH3) and the C-terminal GST-huGrb2 SH3 domain (aa 159-217) (C-SH3).
Chapter 3

Results: p75 associates with Grb2-SH3 domains

Rapidly (within 2 min) and transiently tyrosine phosphorylated in TCR stimulated cells. Proteins of the following relative molecular mass were detected: 130, 110, 100, 75, 50, 36, and 34 kDa. The reactivity of many of these tyrosine phosphoproteins is abrogated when the SH3 domains of Grb2 are mutated (Fig. 3.4C). Thus, in TCR activated T cells the major TCR induced PTK substrate isolated with the Grb2

\[ \text{Grb2} \]

fusion protein complex must bind to Grb2 via its SH3 domains. This conclusion is supported by the observation that these proteins can be precipitated with the single N-terminal and C-terminal Grb2 SH3 domains (Fig. 3.4C).

One of the major tyrosine phosphoproteins that associates with full length Grb2 or the C-terminal Grb2 SH3 domain was a 75 kDa protein that was of similar size as the Grb2 SH3 domain binding protein shown to co-precipitate with endogenous Grb2 in the EGFR-Y(1068)-P/Grb2 complexes. Therefore examined whether the 75 kDa protein purified with endogenous Grb2 becomes tyrosine phosphorylated in TCR activated T
lymphocytes. As shown in Fig. 3.4B, the EGFR-Y(1068)-P peptide precipitates a 75 kDa tyrosyl phosphoprotein from TCR stimulated but not from quiescent T cells. This tyrosine phosphoprotein co-migrated with the Grb2 binding protein described in Figs. 3.2 and 3.3 and bound to the Grb2 SH3 domains as it did not co-precipitate with Grb2 isolated with the Sos-PRO peptide. When isolated recombinant Grb2 SH3 domains were used to purify cellular proteins the p75 tyrosine phosphoprotein preferentially recognised the C-terminal SH3 domain of Grb2 (Fig. 3.4C). The data in Fig. 3.4D show that Sos has an \textit{in vitro} preference for the N-terminal SH3 domain of Grb2.

The 36 kDa PTK substrate associates with the Grb2-SH2 domain only when both SH3 domains of Grb2 are engaged

It has been demonstrated that a 36 kDa tyrosine phosphorylated protein can complex to the Grb2 SH2 domain in TCR activated cells. This protein was first detected in anti-phosphotyrosine Western blots of anti-Sos immunoprecipitates and in Grb2 complexes affinity purified with a GST fusion protein of a proline rich fragment from the C-terminus of mSos (Buday et al., 1994). In the present series of experiments the p36 tyrosine phosphoprotein was detected in the proteins purified with recombinant Grb2\(\mu\)SH3 proteins (Fig. 3.4C) but were not observed in the Grb2 complexes isolated with the Sos-PRO proline peptide (Fig. 3.4B). I therefore compared the binding properties of the Grb2 proteins isolated with the Sos-PRO peptide to the ones purified with the recombinant C-terminal fragment of Sos (C-Sos) (Fig. 3.5). In these experiments the Sos based affinity reagents purify identical levels of Grb2 from T cell lysates (Fig. 3.5, \textit{lower panel}). However, the 36 kDa TCR induced tyrosine phosphoprotein only associated with Grb2 when the C-terminal fragment of Sos and not the Sos-PRO proline peptide were used to isolate Grb2 (Fig. 3.5, \textit{upper panel}).
Figure 3.5. A 36 kDa tyrosine phosphoprotein can be co-purified with the Grb2 complexes isolated with GST-C-Sos fusion protein but not the Sos-PRO peptide. T lymphocytes were untreated (-) or stimulated with 10 μg/ml UCHT-1 mAbs for 2 min (+). Grb2 was purified from cell lysates with either the Sos-PRO peptide or the GST fusion protein of the C-terminal fragment of Sos. The affinity-purified proteins were resolved by 10% SDS-PAGE. Protein complexes were analysed by immunoblotting with the phosphotyrosine mAb 4G10 (upper panel) or the Grb2 mAb (lower panel). The migration of the molecular mass standards is indicated to the left in kilodaltons.

Purification of Grb2 SH3 domains associated proteins and identification of isolated proteins by peptide-mass fingerprinting

In an attempt to purify and identify proteins, in particular the p75 protein, that associate with Grb2 SH3 domains in T lymphocytes, proteins were precipitated from human peripheral blood T cell lysates with the EGFR-Y(1068)-P peptide beads which
allows purification of Grb2-SH3 domains associated proteins. T blasts (440 x 10^6 cells) were lysed and proteins were isolated from lysates with either EGFR-Y(1068)-P peptide or as a control with PDGFR-Y(751)-P peptide. The proteins purified with these peptide complexes were separated by SDS-PAGE and transferred onto PVDF membrane. The membrane was stained with sulforhodamine B solution to detect purified proteins (Fig. 3.6). The apparent protein bands (depicted by arrows) were excised and digested with trypsin (trypsin cleaves C-terminal to K=Lys or R=Arg). Derived peptides were subjected to matrix-assisted laser-desorption ionisation mass spectrometry (MALDIMS) as described in Materials and methods (performed by Darryl Pappin, Protein Sequencing Laboratory, ICRF, London). In addition, some of the sample from the trypsin-digest was chemically modified by esterification (methyl esters) to measure the content of acidic residues (E=Asp, D=Glu) in potentially matching peptide sequences. The esterified peptides were then also analysed by MALDIMS and the peptide spectra compared with the ones of underivatised material. Cross-correlation of observed peptide masses allows for assignment of acidic residue content, as masses must increase by integer multiples of 14 kDa (methyl esters). This derivatisation gives an additional search parameter (see below) and increases the search discrimination by almost two orders of magnitude (D. J. C. Pappin, unpublished data).

The obtained peptide mass-fingerprints (Fig. 3.7 depicts the mass spectra for the 75 kDa protein) were analysed using the molecular weight search (MOWSE) peptide-mass database at the SERC Daresbury Laboratory, UK (Pappin et al., 1993). The MOWSE database comprises calculated weights of all peptide fragments derived from a set of specific enzyme or reagent cleavage rules, derived from at the time over 60,000 proteins (Pappin et al., 1993). The fragment database is generally searched with the following parameters: peptide mass-fingerprint, the cleavage reagent used and the molecular weight of the intact protein (optional). The search program outputs a
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Results: p75 associates with Grb2-SH3 domains

Figure 3.6. Large scale purification of Grb2 SH3 domains associated proteins using the EGFR-Y(1068)-P peptide as affinity resin.

Rhodamine-stain of proteins isolated with EGFR-Y(1068)-P or PDGFR-Y(751)-P peptide beads from T cell lysates. T blasts (440 x 10^6 cells) were lysed in 10 ml lysis buffer 1. As control 40 x 10^6 cells each were left untreated or stimulated with 10 μg/ml UCHT-1 mAb for 2 min and lysed in 1 ml lysis buffer 1. Postnuclear lysates were precleared once and proteins were isolated from lysates (200 x 10^6 cells in 5 ml volume) with either 200 μl packed EGFR-Y(1068)-P peptide beads (peptide conc. on packed beads was 6.2 μg/μl) or with 100 μl PDGFR-Y(751)-P peptide beads (peptide conc. 5 μg/μl). Proteins (20 x 10^6 cell equivalents) from untreated or stimulated control lysates were purified with either 20 μl EGFR-Y(1068)-P peptide or 10 μl PDGFR-Y(751)-P peptide beads (in 0.5 ml volume). After 3 h peptide complexes were washed 4 times in lysis buffer 1, pooled where appropriate, separated on a reducing 10% SDS-polyacrylamide gel and transferred onto PVDF membrane. The part of the membrane containing the proteins purified from stimulated/non-stimulated cell lysates was subjected to Western blot analysis with anti-phosphotyrosine mAbs (4G10) for detection of the 75 kDa phosphotyrosine protein followed by Western blot analysis with p85α (U5) mAbs to localise p85α proteins. The other half of the membrane containing the large scale purification of proteins was stained with sulforhodamine B solution to detect isolated proteins and a photograph was taken. The 85 kDa band identified with the rhodamine stain co-migrated with the p85α subunit of PI 3-kinase detected in the anti-p85α (U5) Western blot. The 75 kDa band detected with the rhodamine stain co-migrated with the 75 kDa protein apparent in the anti-phosphotyrosine (4G10) Western blot. The migration of the molecular mass standards is indicated to the left in kilodaltons. Protein bands which were subjected to MALDIMS analysis are marked by arrows and the initial results of the MOWSE database search with the obtained peptide-mass fingerprints are indicated to the right.
ranked hit list comprising the 50 best matching protein entries. The results of these searches is shown in Fig. 3.6: The 150 kDa band, the two 145 kDa bands and the 26 kDa band were unequivocally identified as Sos, PLCγ1 and Grb2, respectively. The 38 kDa band and the 75 kDa band which co-migrated with the p75 phosphotyrosine protein detected in the anti-phosphotyrosine Western blot analysis that was performed in parallel (see figure legend for Fig. 3.6) did not yield any matches and hence were likely to be novel proteins. Moreover, it was noted that the 75 kDa protein appeared to have a smaller predicted mass, as it did not yield the number of peptides expected for the mass of a 75 kDa protein (Darryl Pappin, personal communication).

The 75 kDa protein that binds Grb2 SH3 domains is identical to the Grb2-associated phosphoprotein SLP-76

After the publication of my report (Reif et al., 1994) and the peptide-mass fingerprint analysis of the proteins isolated with the EGFR-Y(1068)-P peptide from human T cell lysates, Jackman, Motto and colleagues reported the molecular cloning of a 76 kDa tyrosine phosphoprotein associated with Grb2 in T cells, termed SLP-76 (SH2 domain-containing Leukocyte Protein of 76 kDa) (Jackman et al., 1995). This protein was isolated from TCR stimulated Jurkat T cells by co-precipitation with a GST Grb2 fusion proteins and detected with anti-phosphotyrosine (4G10) Western blot analysis. Microsequencing and cDNA cloning revealed a novel protein of 533-amino acid, human

Figure 3.7. Matrix assisted laser-desorption ionisation time-of-flight mass spectra of tryptic peptides of the 75 kDa protein isolated with EGFR-Y(1068)-P peptides from human T cell lysates.

(A) Native tryptic peptide map, 0.5 μl sampled from 25 μl digested supernatant.
(B) Tryptic peptide map following esterification of a separate 2 μl aliquot of the digested supernatant with thionyl chloride in methanol.

Peptide masses denoted by * or an arrow (A) could be matched with masses calculated from the expected tryptic peptide sequence of human tyrosine phosphoprotein SLP-76 (GenBank™/EMBL Data Bank accession number U20158). In addition, the arrow indicates tryptic peptide masses from (A) that could be assigned to its esterified 'partner' in (B) following esterification with thionyl chloride in methanol. A more detailed analysis of the peptide masses and sequences is shown in Table 3.1.
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[Graph A]

[Graph B]
SLP-76, which has a predicted mass of 60 kDa and contains one SH2 domain at its C-terminus. Northern analysis showed that SLP-76 mRNA is expressed exclusively in peripheral blood leukocytes, thymus, and spleen; and in human T cell, B cell and monocyte cell lines. As the SLP-76 protein showed very similar features to what I had found for the 75 kDa protein associated with Grb2 SH3 domains in human peripheral blood T lymphocytes, I was interested to investigate whether the two proteins are identical. After the sequence of the SLP-76 protein had been entered in the MOWSE peptide-mass database, and hence its calculated molecular weight tryptic peptide map was available in this source, the search was repeated with the peptide-mass fingerprint of p75 (see Fig. 3.6) (performed with the help of Darryl Pappin, ICRF). The search program scored SLP-76 as the top ranking match for p75, MOWSE database entries scanned were 160,636 entries. The results of this screen are summarised in Table 3.1 (and Fig. 3.6).

Table 3.1. Summary of the MALDIMS and MOWSE database analysis of p75.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Residue Position</th>
<th>Calcul. Mass (Da)</th>
<th>Measured Mass (Da)</th>
<th>Δ Mass (Da)</th>
<th>Measured Esterified Mass (Da)</th>
<th>No. E or D Measured</th>
<th>Esterified Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPQQPPVPQRMAALPPPPAGR</td>
<td>182-204</td>
<td>2416.8</td>
<td>2418.9</td>
<td>+2.1</td>
<td>n/o</td>
<td>n/o</td>
<td></td>
</tr>
<tr>
<td>NHSPLLPPQNHEEPSR</td>
<td>205-221</td>
<td>1937.1</td>
<td>1939.1</td>
<td>+2.0</td>
<td>n/o</td>
<td>n/o</td>
<td></td>
</tr>
<tr>
<td>GKEFGLSVSDIYDYPFR</td>
<td>491-506</td>
<td>1904.1</td>
<td>1906.6</td>
<td>+2.5</td>
<td>1976.3</td>
<td>3.98 (4)</td>
<td></td>
</tr>
<tr>
<td>SEVLGWDPSLADYFK</td>
<td>10-25</td>
<td>1842.0</td>
<td>1843.9</td>
<td>+1.9</td>
<td>n/o</td>
<td>n/o</td>
<td></td>
</tr>
<tr>
<td>TTTNPYVLVLYK</td>
<td>454-466</td>
<td>1558.8</td>
<td>1558.5</td>
<td>-0.3</td>
<td>n/o</td>
<td>n/o</td>
<td></td>
</tr>
<tr>
<td>KPPFSDKPSIPAGR</td>
<td>260-273</td>
<td>1496.7</td>
<td>1498.2</td>
<td>+1.5</td>
<td>1524.7</td>
<td>0.89 (1)</td>
<td></td>
</tr>
<tr>
<td>KPPVPKHGWGPD</td>
<td>304-316</td>
<td>1470.7</td>
<td>1468.2</td>
<td>-2.5</td>
<td>n/o</td>
<td>n/o</td>
<td></td>
</tr>
<tr>
<td>ESQVYLLGTGLR</td>
<td>479-490</td>
<td>1335.5</td>
<td>1335.0</td>
<td>-0.5</td>
<td>n/o</td>
<td>n/o</td>
<td></td>
</tr>
</tbody>
</table>

Assignment of peptide sequence matches from SLP-76 to tryptic peptide-mass fingerprints from the 75 kDa protein purified with EGFR-Y(1068)-P peptides from human T cell lysates, including assignment of acidic residue composition of tryptic peptides (75 kDa protein) following esterification. Number of acidic residues (E, D) was calculated by subtracting measured masses of underivatised peptides from those following esterification, subtracting 14 Da to account for the C-terminal ω-carboxyl, then dividing by 14 to yield the number of internal methyl esters. As can be seen in the last column, the difference between measured acidic residue composition and expected integer values (parentheses) is within 11%, allowing accurate matching of native to esterified masses. Mass errors (Δ) between measured and calculated masses were within -/+ 2.5 Da. n/o; not observed.
3.3 Discussion

Grb2 complexes via its SH3 domains to a novel 75 kDa protein that is a substrate for TCR controlled PTKs

In the present study, I have demonstrated that the adapter protein Grb2 can complex to at least two proteins via its SH3 domains in T cells, the Ras guanine nucleotide exchange factor Sos and a 75 kDa protein. In contrast to Sos the 75 kDa protein was not expressed in fibroblasts and therefore appears to be a haematopoietic cell lineage-specific protein. The association of p75 with Grb2 is constitutive like the Grb2/Sos association and apparently not controlled by TCR stimulation. Interestingly, the 75 kDa protein was regulated by TCR induced PTKs as it becomes rapidly tyrosine phosphorylated after TCR engagement. Two lines of evidence implicate the p75 protein as a Grb2 SH3 domains binding protein and hence as a novel Grb2 effector: p75 could bind to recombinant Grb2 SH3 domains in in vitro binding assays and importantly p75 could be isolated with endogenous Grb2 proteins of which the SH2 binding partners were competed out. The latter was achieved by using a tyrosine phosphopeptide that binds to the SH2 domain of Grb2, EGFR-Y(1068)-P peptide, to affinity purify endogenous Grb2 and thus allows to isolate Grb2 SH3 domains associated proteins from T cell lysates. Accordingly, Sos and p75 could be precipitated with the EGFR-Y(1068)-P peptide. Concordant with this, neither p75 nor Sos co-precipitate with Grb2 when the SH3 domains are bound to a Sos-derived proline peptide (Sos-PRO). The 75 kDa binds to the Grb2 SH3 domains directly as concluded from the following data: In far-Western blot analysis the p75 protein can bind an intact but not a SH3 mutated Grb2 fusion protein. Moreover, p75 precipitates from T cell lysates with recombinant Grb2 SH3 domains but not with recombinant Grb2 mutated in its SH3 domains. Interestingly, Sos and p75 display differential binding to Grb2 SH3 domains: Sos binds preferentially to the N-terminal SH3 domain whereas p75 has a preference for the C-terminal SH3 domain. Previous studies have indicated that in vivo
binding of Sos to Grb2 requires both SH3 domains of the molecule (Egan et al., 1993). Accordingly, the in vitro binding reactivities of Sos and p75 with isolated Grb2 SH3 domains do not necessarily imply that Sos and p75 bind to different SH3 domains of Grb2 in vivo. Nevertheless, the data do reveal that the binding characteristics of Sos and p75 for the Grb2 SH3 domains are distinct.

**Peptide affinity resins are a highly specific tool to isolate endogenous proteins and their binding partners**

During the past years a plethora of papers was published identifying protein-protein associations mediated by SH2 or SH3 domains. Many of these studies only assess the interaction of such molecules in in vitro binding assays using µg quantities of recombinant proteins, mainly GST fusion proteins. However, in many of these investigations no in vivo interaction between the endogenous proteins such as co-precipitation of the specific molecules in immune complexes were shown. The data presented here clearly demonstrates that Grb2 can interact with endogenous Sos and p75 using synthetic peptides which allow affinity purification of endogenous Grb2 and its SH2 or SH3 domains associated proteins. The difference in affinities between endogenous Grb2 and a GST fusion protein of Grb2 is demonstrated in Fig. 3.4A/C versus Fig. 3.4B where about 10 phosphotyrosine proteins can associate with GST-Grb2 and about 4 with the single Grb2 SH3 domains, GST-SH3, as compared to only one 75 kDa phosphotyrosine protein associated with the SH3 domains of endogenous Grb2. Therefore associations detected with GST fusion proteins have to be tested for their validity in intact cells. However, once an interaction between endogenous proteins has been identified recombinant proteins can be useful in analysing which domain of a certain protein is involved in mediating the association or whether binding is direct.
Sos as a regulator of Ras function in T cells

After TCR stimulation the proportion of Ras in the activated GTP bound state increases. This involves a mechanism where the GAP activity which ensures in quiescent cells that Ras is in the GDP bound state is negatively regulated by the TCR (Downward et al., 1990; Graves et al., 1991). Here I demonstrate that Sos can bind constitutively to Grb2 SH3 domains in T lymphocytes. Upon T cell activation the Grb2/Sos can translocate to the plasma membrane due to the association of the Grb2 SH2 domain with a 36 kDa phosphotyrosine protein (Buday et al., 1994; Sieh et al., 1994). This interaction and translocation event could bring Sos into the proximity of Ras where Sos could catalyse the exchange of GDP to GTP on Ras. However, in a detailed analysis of the exchange rate mechanism in permeabilised T lymphocytes no alteration in the GDP/GTP exchange rate on Ras in quiescent versus activated cells was detected (Downward et al., 1990; Graves et al., 1991). This could be explained by a constitutively high GEF activity and at the same time constitutively high GAP activity in quiescent cells whereby the GAP activity is negatively regulated in response to TCR engagement but the GEF cannot be further increased by T cell activation events. Thus, the GEF activity appears to be optimal in quiescent cells. (One recently published report can detect an increase in the in vitro exchange activity of Sos after T cell activation (Li et al., 1996); however, such an increase in the specific activity of Sos is not generally observed and it is thought that Sos is regulated by its recruitment to the plasma membrane (Buday and Downward, 1993; Buday and Downward, 1993).)

Therefore, although Sos is bound to Grb2 in quiescent cells and the Grb2/Sos complex can translocate to the plasma membrane this complex might not be of any functional relevance during T cell activation. This is supported by experiments showing that dephosphorylation of the 36 kDa protein associated with Grb2 in activated T cells does not affect Ras activation by the TCR (Motto et al., 1996).
The 75 kDa protein is identical with a recently described protein - SLP-76

Affinity purification with the EGFR-Y(1068)-P peptide which isolates Grb2-SH3 domains associated proteins was used to separate Grb2-SH3 domains binding proteins from T cell lysates. Six protein bands of 150, 145, 75, 45, 38 and 26 kDa were detected (Fig. 3.6). The identity of these protein bands was assessed by MALDIMS and revealed that the 26 kDa and the 150 kDa band correspond to Grb2 and Sos, respectively which confirms that the EGFR-Y(1068)-P peptide binds Grb2 and can co-purify Sos. The 145 kDa was identified as PLCγ1. The PLCγ1 SH2 domain has been reported to bind to the 36 kDa PTK substrate that has been shown to interact with the Grb2 SH2 domain (Sieh et al., 1994). Thus, the PLCγ1 SH2 domain and the Grb2 SH2 domain may recognise similar phosphotyrosine motifs and it is possible that PLCγ1 can bind directly to the EGFR-Y(1068)-P peptide motif. Interestingly, upon TCR ligation a phosphotyrosine protein of 75/76 kDa has been detected in anti-PLCγ1 immune complexes and thus it should not be excluded that these two proteins are identical and p75/p76 co-purifies PLCγ1 (Gilliland et al., 1992; Jackman et al., 1995). The identity of the 38 kDa protein remains unknown. Affinity purification with a Grb2 fusion protein led to the identification of a 76 kDa protein, SLP-76, which binds to the C-terminal SH3 domain of Grb2 (Jackman et al., 1995). The MOWSE database search revealed that p75 and SLP-76 are almost certainly identical proteins. The MALDIMS is very reliable in identifying proteins as shown previously for a variety of proteins and hence is a valuable method to reveal the identity of biochemically purified proteins (Pappin et al., 1993; Pappin et al., 1996). Moreover, comparison of the biochemical features of p75 and SLP-76 indicates that the two proteins are likely to be identical: Both proteins have been purified due to their association with Grb2. p75 and SLP-76, both bind preferentially to the C-terminal Grb2-SH3 domain. As well, p75 and SLP-76 are substrates for TCR-activated PTKs and both are expressed in the haematopoietic system. Hence, although a final confirmation is missing showing e.g. by Western blot
analysis of EGFR-Y(1068)-P precipitates with SLP-76 antisera that SLP-76 is present in this affinity complex, it is almost beyond any doubt that the two proteins are identical.

**Figure 3.8.** Schematic representation of SLP-76 / p75. The numbers refer to the amino acid residues of human SLP-76. The N-terminal region of SLP-76 (aa 109-157) contains three 17-amino acid repeats with a conserved tyrosine motif, DYE(S/P)P (Y113, Y128, Y145), and acidic residues. The centre of the protein consist of a proline-rich region, the sequence which is required for Grb2 binding (aa 224-244) is depicted. The C-terminus contains one SH2 domain with a typical FLVR tyrosine binding sequence (aa 445-448).

**p75 / SLP-76 is a novel SH2 domain containing protein expressed in haematopoietic cells and one of the major substrates for TCR controlled PTKs**

Molecular cloning of SLP-76 demonstrated that it encodes a novel haematopoietic cell-specific protein which contains a SH2 domain and hence its name SLP-76 for SH2 domain leukocyte protein of 76 kDa (Jackman et al., 1995) (Fig. 3.8). SLP-76 comprises an N-terminal region with numerous potential tyrosine phosphorylation sites, a central region rich in proline residues, and a single C-terminal SH2 domain (Jackman et al., 1995; Motto et al., 1996). Mapping of the Grb2 binding site on SLP-76 using recombinant SLP-76 proteins showed that the amino acid residues 224 to 244 of SLP-76 mediate the interaction with Grb2 (Motto et al., 1996). After TCR engagement SLP-76 undergoes tyrosine phosphorylation within 30 s, which peaks at 1 min, and returns to basal levels by 1 h of stimulation. This tyrosine phosphorylation
requires ZAP-70 activity and it has been suggested that it may be mediated by ZAP-70 itself or Syk (consensus site in SLP-76: Asp-Tyr-Glu) (Wardenburg et al., 1996).

**The recruitment of p75 / SLP-76 into the activated TCR-PTK complex is likely to be mediated by the association with Grb2 which interacts via its SH2 domain with the TCR controlled PTK substrate p36**

The SH2 domain of Grb2 can interact with a 36 kDa TCR induced tyrosine phosphoprotein that is located at the plasma membrane in T cells (Buday et al., 1994; Sieh et al., 1994). The interaction between the Grb2 SH2 domain and this tyrosine phosphoprotein is proposed to recruit Grb2 and its SH3 domains associated proteins to the plasma membrane. In anti-SLP-76 immune complexes a 36 kDa tyrosine phosphorylated protein is present which co-migrates with the p36 protein that interacts with the Grb2 SH2 domain (Motto et al., 1996). Thus, it is likely that the 36 kDa PTK substrate seen in the anti-Grb2 or anti-SLP-76 immune complexes are identical and that p36 associates with SLP-76 indirectly via Grb2. As p36 is a substrate for TCR controlled PTKs it could play a role in locating SLP-76 at the cell membrane where it can encounter TCR stimulated PTKs (see Fig. 3.9). Moreover, this translocation event could bring SLP-76 into proximity with its effector targets (see below).

**The 36 kDa PTK substrate associates with the Grb2-SH2 domain only when both SH3 domains of Grb2 are engaged**

The Sos-PRO proline peptide, SKGTDEVPVPPPPVPPRR, is equivalent to one of three proline rich sequences contained in the C-terminus of Sos that can compete for Grb2 binding (Rozakis-Adcock et al., 1993). One intriguing observation is that the Sos-PRO peptide or the C-Sos GST fusion protein can interact with Grb2 SH3 domains and purify equivalent levels of endogenous Grb2 from cells (Fig. 3.5). However, the p36 TCR induced tyrosine phosphoprotein that binds to the Grb2 SH2 domain was only seen in the Grb2 complexes isolated with the recombinant C-Sos
protein but not in the Grb2 complexes purified with the Sos-derived proline peptide. It is possible that the additional proline-rich sequence interacts with the Grb2 SH3 domains in a manner that lead to conformational changes of the Grb2 molecule and its SH2 domain which ultimately alters the affinity of the Grb2 SH2 domain for its tyrosine phosphorylated binding partners. This double-interaction with Grb2 SH3 domains could secure that only one effector protein can bind to the same Grb2 molecule and is recruited into the activated TCR complex. In this respect, in an analysis of the tyrosine phosphorylated proteins associated with Sos/Grb2 complexes isolated with Sos antisera only the p36 PTK substrate and not a p75/p76 tyrosine phosphoprotein was detected (Buday et al., 1994). These data support the notion that Sos and SLP-76 may be in mutually exclusive Grb2 complexes. Preclearing experiment with Sos or SLP-76 antisera that would allow quantitation of the relative stoichiometry of the Grb2/Sos versus the Grb2/SLP-76 complexes have not been reported or performed. As well, it has not been assessed whether Sos is present in anti-SLP-76 immune complexes.

Several phosphotyrosine-proteins and a serine/threonine kinase associate with SLP-76 upon TCR engagement

Further to the 36 kDa at least three other tyrosine phosphorylated proteins of 62 kDa, 95 kDa and 130 kDa are present in anti-SLP-76 immune complexes in response to TCR triggering (Motto et al., 1996; Tuosto et al., 1996; Wu et al., 1996) (Fig. 3.9). The identity of the phosphotyrosine proteins p62 and p130 is unknown (they are not p62/SAM68, Cbl, GAP, Fak or p130CAS) but both are likely to interact directly with the SLP-76 SH2 domain as mutation of the predicted-phosphate-binding arginine residue of the SLP-76 SH2 domain to lysine (R448K) abrogates binding of these tyrosine-phosphoproteins to SLP-76 (Motto et al., 1996). Additionally, SLP-76 associates via its SH2 domain with a serine/threonine kinase after TCR ligation that can phosphorylate an
uncharacterised 100 kDa protein \textit{in vitro} (Motto et al., 1996). The 95 kDa phosphotyrosine protein was identified as Vav (Tuosto et al., 1996; Wu et al., 1996).

\textbf{A few comments on the role of Vav during T cell activation}

Vav is a haematopoietic lineage specific protooncogene which contains one C-terminal SH2 domain flanked by two SH3 domains, a PH domain and a cysteine-rich domain which may form zinc-fingers (Boguski and McCormick, 1993; Katzav et al., 1989). Moreover, Vav possesses guanine nucleotide exchange factor activity for Rho family GTPases (in particular for Rac) (Crespo et al., 1996; Crespo et al., 1997; Olson et al., 1996), although this exchange activity for Rho family proteins has not been formally proven to exist in T cells. Vav is tyrosine phosphorylated in response to TCR stimulation (Bustelo et al., 1992; Margolis et al., 1992) and has been reported to associate directly \textit{via} its SH2 domain with the ZAP-70 tyrosine kinase (Katzav et al., 1994) which contains a tyrosine motif \textit{YESP} at residue 315 that is a predicted binding site for the Vav SH2 domain (Songyang et al., 1994). Point mutation in the Vav SH2 domain inhibits its tyrosine phosphorylation and thus it can be reasoned that the association with ZAP-70 is responsible for Vav tyrosine phosphorylation. In addition, it has been reported that recombinant ZAP-70 can phosphorylate Vav on tyrosine Y174 \textit{in vitro} (Deckert et al., 1996).

Overexpression of Vav in T cells has been shown to enhance the transactivation capacity of the transcription factor NF-AT ('nuclear factor of activated T cells') (Wu et al., 1995) which binds to the IL-2 enhancer and appears to determine the T cell specificity and inducibility of IL-2 gene expression (Cantrell, 1996; Cantrell et al., 1994; Cantrell et al., 1995; Cantrell et al., 1993; Crabtree and Clipstone, 1994; Izquierdo Pastor et al., 1995; Rao, 1994; Serfling et al., 1995); thus, Vav overexpression also leads to an increase in transcriptional activity from the entire IL-2 promotor/enhancer which can be further potentiated by TCR stimulation (Wu et al., 1995). NF-AT is a
heterodimer comprising a fos/jun-containing nuclear component, NF-AT\textsubscript{n}, that is induced in response to antigen stimulation and a pre-existing cytoplasmic component, NF-AT\textsubscript{c}, that translocates to the nucleus upon activation (see Fig. 3.9). The induction of NF-AT requires the concerted action of at least two signalling pathways: NF-AT\textsubscript{n} activity is controlled by signals delivered by the Ras signalling cascades whereas Ca\textsuperscript{2+}-dependent signals involving the phosphatase calcineurin mediate the translocation of NF-AT\textsubscript{c} into the nucleus. Where Vav fits into the picture in regulating IL-2 gene and NF-AT activity is currently not clear, in particular the field is open for speculation on how Vav overexpression leads to an increase in the basal transcriptional activity of NF-AT and IL-2 gene expression.

**Tyrosine phosphorylated SLP-76 associates with the Vav SH2 domain**

Similar to the association of Vav with ZAP-70, the interaction of SLP-76 with Vav is mediated by the Vav SH2 domain and hence SLP-76 may be important for Vav signal transduction (Tuosto et al., 1996; Wu et al., 1996) (see Fig. 3.9). A current model predicts that Vav first associates with ZAP-70, becomes tyrosine phosphorylated and subsequently interacts with tyrosine phosphorylated SLP-76 (Wu et al., 1996) which contains two canonical Vav SH2 domain binding sites (YESP motif) at the amino acid residues Y113 and Y128 (and one closely related motif (YEPP) at residue Y145). The significance of such Vav SH2 domain controlled interactions is emphasised by the notion that overexpression of a truncated form of Vav containing its C-terminal SH2 and two SH3 domains blocks TCR-mediated NF-AT induction. This inhibitory effect is dependent on a functional SH2 domain suggesting that phosphotyrosine-SH2 interactions such as the SLP-76-Vav association are essential for Vav function/activation controlled by the TCR. In this respect, overexpression of SLP-76 also leads to an increase in TCR-induced NF-AT and IL-2 gene transcriptional activity, however without a raise in basal levels of activation (Motto et al., 1996). Augmented transcriptional activity induced by SLP-76 overexpression does not occur when the
tyrosine residues Y113, Y128 and Y145 in SLP-76 are mutated to phenylalanine (Fang et al., 1996) further supporting the notion that tyrosine phosphorylation of SLP-76 is important for SLP-76/Vav function and that Vav may mediate the effect of SLP-76 on NF-AT activity and IL-2 gene expression. However, mutation of the SLP-76 SH2 domain partially abrogates the enhancing effect of SLP-76 on TCR mediated induction of NF-AT activity indicating that the molecules which bind to the SLP-76 SH2 domain such as p62 and p130 may contribute to SLP-76/NF-AT responses (Motto et al., 1996). In this respect, Vav and SLP-76 can synergise to induce basal or TCR-stimulated NF-AT and IL-2 promotor activity (Wu et al., 1996). To further delineate the importance of Vav for SLP-76 function on NF-AT/IL-2 promotor transcriptional activity it would be interesting to assess whether the oncogenic version of Vav, which lacks the first 67 N-terminal
amino acids and fails to enhance NF-AT transactivation, can synergise with SLP-76 to augment NF-AT transcriptional activity.

**Summary on the function of SLP-76 (see Fig. 3.9)**

SLP-76/p75 is a principal TCR controlled PTK substrate that undergoes tyrosine phosphorylation during antigen stimulation and contains at least three protein binding modules: N-terminal tyrosine phosphorylation motifs which direct SH2 domain binding, proline rich regions which allow SH3 domain binding and an SH2 domain which enables tyrosine phosphoproteins to associate with SLP-76. Thus, SLP-76 has the structure of a classical adaptor molecule. Indeed, during T cell activation the constitutive Grb2/SLP-76 complex assembles via the Grb2 SH2 domain with a 36 kDa phosphotyrosine protein forming a complex which may enable tyrosine phosphorylation of SLP-76. SLP-76 may then function to integrate signals delivered by TCR engagement to downstream effector pathways as it associates with at least four proteins, Vav, the phosphotyrosine proteins p62 and p130, and a serine/threonine kinase. The Vav interaction is mediated by the N-terminal phosphotyrosine residues of SLP-76 and involves the Vav SH2 domain. The other three proteins are as yet unidentified but bind to the SLP-76 SH2 domain. To date, the role of SLP-76 during T cell activation events has been paralleled with the one of Vav as SLP-76 similar to Vav can enhance TCR controlled induction of NF-AT and IL-2 promotor activity. This function of SLP-76 is partially dependent on an intact SH2 domain. It is thus possible that SLP-76 recruits effector molecules into the proximity of Vav which contribute to Vav responses. Alternatively, SLP-76 may have independent or synergistic functions as it can also associate with the PLCγ1 SH2/SH2/SH3 domains. The future will tell in which biochemical pathway SLP-76 is acting.
Chapter 4

Studies on the functional link between PI 3-kinase and the TCR. Does the p85 subunit of PI 3-kinase bind to the Grb2 adaptor molecule via proline rich motifs?

4.1 Introduction

Engagement of the TCR, CD28 or the IL-2R activates PI 3-kinase (Remillard et al., 1991; Ward et al., 1993; Ward et al., 1992). The TCR exerts its cellular function via cytoplasmic tyrosine kinases of the src family and the ZAP-70 tyrosine kinase. The form of PI 3-kinase implicated in TCR regulation of D-3 phosphoinositide metabolism comprises a regulatory p85 and a catalytic p110 subunit. Despite intensive studies it is currently not clear how the TCR regulates PI 3-kinase. However, activation of PI 3-kinase requires the translocation of the enzyme to the plasma membrane and at least three principal membrane-recruitment mechanisms have been described: 1. by direct or indirect binding to the cytoplasmic tail of receptor subunits (Cantley et al., 1991); 2. by interaction with src family tyrosine kinases (Cantley et al., 1991; Kapeller and Cantley, 1994); and 3. by association with small GTPases of the ras and rho family (Rodriguez-Viciana et al., 1994; Tolias et al., 1995; Zheng et al., 1994).

In many cells the p85 subunit of PI 3-kinase has been documented to associate via its SH2 domains in a high affinity interaction with specific phosphotyrosines in signalling molecules, such as IRS or the activated PDGF receptor (Backer et al., 1992; Kashishian et al., 1992). From these studies it became evident that the preferred binding motif of PI 3-kinase has the consensus sequence YMXM or YVXM (X for any amino acid). In T cells these high affinity templates exist in the cytoplasmic tail of the CD28 receptor, Y(173)MNM, or the CTLA-4 surface molecule, Y(201)VKM.
(Schneider et al., 1995; Truitt et al., 1994). After IL-2 stimulation the p85 subunit can directly associate with the phosphotyrosine residue 392 in the IL-2R β chain via a non-canonical YCTF motif (Truitt et al., 1994). In marked contrast, the cytoplasmic tails of the TCR/CD3 complex contain no high affinity binding sites for the p85 subunit of PI 3-kinase (Osman et al., 1996). Nevertheless, PI 3-kinase activity could be detected in anti-TCR ζ (Carrera et al., 1994; Prasad et al., 1993) or anti-CD3ε (Thompson et al., 1992) immune complexes and a low affinity interaction of the p85 subunit with the doubly tyrosine-phosphorylated membrane proximal ITAM motif of the TCR ζ chain, LYNELNLGRREEYDVL₆ (Exley et al., 1994) but not the CD3ε chain (Osman et al., 1996) has been reported. However, the association with TCR ζ was only shown in in vitro binding assays with synthetic peptides as affinity matrices (Exley et al., 1994) and was at least 100 fold lower when compared to the binding affinity of the high affinity binding site in the PDGF receptor, Y751, or the association of the ZAP-70 tyrosine kinase with the TCR ζ chain (Osman et al., 1996). This raises the question as to whether a direct association of the p85 subunit with the tyrosine phosphorylated TCR ζ chain via its SH2 domains occurs in intact cells. Moreover, in the experiments where PI 3-kinase activity could be co-precipitated with anti-TCR ζ or anti-CD3ε antibodies (Carrera et al., 1994; Prasad et al., 1993; Thompson et al., 1992) low stringency cell lysis conditions were employed and/or TCR activation was achieved by crosslinking of the TCR/CD3 complex with IgM or with secondary antibodies which is well documented to results in the formation of large protein aggregates.

From the above studies it is clear that any association with the TCR/CD3 complex is of low affinity and only engages a small pool of total cellular PI 3-kinase. It is therefore likely that the recruitment of PI 3-kinase to the plasma membrane in TCR activated cells involves the formation of transient protein complexes. This raises the possibility that PI 3-kinase activity in response to TCR stimulation is not directly associated with the TCR/CD3 complex and hence is recruited into the receptor complex.
via an indirect association with the TCR. In this respect, it is noteworthy that the p85 subunit of PI 3-kinase contains apart of its two SH2 domains at least two more protein binding modules: one SH3 domain and two proline rich motifs which provide putative interaction sites for SH3 domains (Escobedo et al., 1991; Kapeller et al., 1994; Otsu et al., 1991; Skolnik et al., 1991).

Indeed, a second activation/recruitment mechanism in response to TCR engagement is proposed to involve binding of the p85 subunit to the src family tyrosine kinases p59fyn (Prasad et al., 1993) or p56lck (Vogel and Fujita, 1993). In these studies it was demonstrated that the p85 subunit of PI 3-kinase can be isolated with recombinant p59fyn (Prasad et al., 1993) or p56lck (Vogel and Fujita, 1993) SH3 domains from T cell lysates. The level of association of the p85 protein from T cell lines with recombinant p59fyn SH3 domains was not regulated by TCR crosslinking and it was suggested that the p85/p59fyn SH3 domain interaction did not require tyrosine kinase activity (Prasad et al., 1993). Moreover, the p85/p56lck SH3 domain association appeared to be dependent on serine/threonine phosphorylation and independent of tyrosine phosphorylation of PI 3-kinase (Vogel and Fujita, 1993). This is concordant with earlier data from our laboratory (Reif, 1992; Reif et al., 1993; Ward et al., 1992) and from others (Carrera et al., 1994) which demonstrated that PI 3-kinase activity cannot be recovered from anti-phosphotyrosine immune complexes in TCR activated cells. Consistent with this, PI 3-kinase subunits did not associate with tyrosine kinase activity after TCR triggering (Reif, 1992; Ward et al., 1992); in addition, regulation of PI 3-kinase in intact cells correlated with serine/threonine phosphorylation of PI 3-kinase subunits (Reif, 1992; Reif et al., 1993). Thus, in the study showing an interaction between p59fyn and p85 (Prasad et al., 1993), although increased levels of PI 3-kinase lipid activity were observed with recombinant p59fyn proteins after TCR crosslinking, it was not resolved how this raise in activity is regulated. Finally, this study failed to document an increase in PI 3-kinase activity with endogenous p59fyn and other studies could not
detect any PI 3-kinase activity in anti-p59fyn immune complexes (Thompson et al., 1992). In addition, although p56\textit{lck} activity is required for PI 3-kinase activation after TCR triggering (Carrera et al., 1994; Von Willebrand et al., 1994), an in vivo association between p56\textit{lck} and PI 3-kinase activity was not consistently observed (Carrera et al., 1994; Thompson et al., 1992). In this respect, only some studies under low stringency cell lysis conditions found low levels of PI 3-kinase activity in anti-phosphotyrosine immune complexes in TCR activated cells (Thompson et al., 1992; Von Willebrand et al., 1994) but this association was not generally detected (Carrera et al., 1994; Reif, 1992; Reif et al., 1993; Ward et al., 1992).

In conclusion, the precise activation mechanism of PI 3-kinase associated with p59fyn or p56\textit{lck} in response to TCR engagement and the potential biological significance of these interactions has not been resolved: 1. One major criticism is that the data showing an p59fyn-SH3- or an p56\textit{lck}-SH3-p85 interaction stem exclusively from in vitro binding studies with recombinant proteins or from baculovirus co-expression studies. 2. The src family kinases p56\textit{lck} and p59fyn can readily be detected in anti-phosphotyrosine immune complexes in TCR activated cells but PI 3-kinase activity is generally not complexed to phosphotyrosine proteins in TCR stimulated cells. 3. The interaction of p85 with p59fyn was constitutive and not regulated by the TCR; as p59fyn is permanently associated with the plasma membrane, PI 3-kinase is constantly in the proximity of its substrate PI(4,5)P\textsubscript{2} and would constitutively produce PI(3,4,5)P\textsubscript{3}; however, in quiescent T cells PI(3,4,5)P\textsubscript{3} levels are very low (Ward et al., 1992) (hence, a 3'-'inositol phosphatase activity would be required that is negatively regulated by the TCR).

The discrepancies encountered in the association studies of the p85 subunit with src family tyrosine kinases, in particular the lack of membrane-recruitment regulation in response to TCR activation, prompted me to investigate a third possibility of how
translocation of PI 3-kinase into the TCR/CD3 complex and hence to the plasma
membrane after TCR triggering may be achieved. As a low affinity interaction was
required, a SH3-type approximation of signalling molecules remained an attractive
concept. (SH3/proline type interactions exhibit micromolar affinities as compared to the
nanomolar range of phosphotyrosine/SH2 mediated interactions.) To this end, it has
recently become evident that a plethora of adaptor molecules exist which contain SH3
and/or SH2 binding modules and may thus serve as linker proteins (Pawson, 1995;
Pawson and Gish, 1992).

One adaptor molecule that contains SH3 domains and is regulated by the TCR
in an activation dependent manner is Grb2 (Buday et al., 1994; Downward, 1994; Reif
et al., 1994). Originally, identified as the adaptor protein that binds Sos via its SH3
domains, Grb2 SH3 domains have since been shown to interact with a variety of
proteins including dynamin (Gout et al., 1993), C3G (a second Ras guanine nucleotide
exchange factor) (Tanaka et al., 1994), and in T cells a 75-76 kDa protein (SLP-76) (see
chapter 3) (Jackman et al., 1995; Reif et al., 1994) and a 116 kDa molecule (Motto et al.,
1994). Grb2 is a very abundant protein in T cells with more than 500,000 molecules per
cell (my unpublished observation) as compared to the TCR with 30,000 molecules. As
Grb2 appears to have pleiotropic functions it may act in more than one signalling
pathway in T cells. Interestingly, all the proteins described to date that interact with
Grb2 SH3 domains associate with Grb2 SH3 domains constitutively, in quiescent and
stimulated cells, and the regulation of the Grb2/effector complexes is proposed to be
dependent on translocation of these complexes to the activated receptor and/or to the
plasma membrane (Cussac et al., 1994; Downward, 1994). In this respect, the
significance of Grb2 for T cell activation events is emphasised by the notion that
Grb2/Sos rapidly form a complex with a 36 kDa protein that is tyrosine-phosphorylated
after TCR engagement (Buday et al., 1994). Moreover, SLP-76 is a substrate for TCR-
controlled PTKs including ZAP-70 (Wardenburg et al., 1996) and also p116 becomes
tyrosine phosphorylated after TCR triggering (Motto et al., 1994). Finally, the TCR induced 36 kDa tyrosine phosphoprotein can also bind PLCγ1, a Grb2/p36/PLCγ1 complex has been described (Sieh et al., 1994) and tyrosine phosphorylation of Grb2-associated proteins correlates with PLCγ1 activation in T cells (Motto et al., 1996). Accordingly, p36 and Grb2 may be important for TCR regulation of inositol phospholipid metabolism. In the present chapter, I shall investigate the possibility of Grb2 in linking PI 3-kinase to the TCR.

### 4.2 Results

**The p85 subunit of PI 3-kinase binds to Grb2 SH3 domains via proline rich motifs**

First, I tested the hypothesis that p85 subunit of PI 3-kinase binds to the SH3 domains of Grb2. For this purpose I performed a series of binding experiments with GST fusion proteins of Grb2. Grb2 has a single SH2 domain and two SH3 domains. To explore the SH3 domain specificity of p85 binding a panel of mutated and truncated Grb2 fusion proteins were used in the experiments. This panel included GST-Grb2\_iSH3 which has an intact SH2 domain but a mutation in both the C- and N-terminal SH3 domains, GST-Grb2 N-SH3 and GST-Grb2 C-SH3 which are the isolated N- and C-terminal SH3 domains of Grb2, respectively. The p85 subunit of PI 3-kinase can bind to wild type GST-Grb2, GST-Grb2 N-SH3 and GST-Grb2 C-SH3 but not GST-Grb2\_iSH3 as shown by Western blot analysis with p85 mAbs (Fig. 4.1A). The p85 subunit bound preferentially to the Grb2 N-SH3 domain and hence showed a similar binding pattern as Sos. Grb2 complexes isolated from quiescent and TCR activated T cells revealed that the interaction of p85 with Grb2 SH3 domains is not dependent on cell activation via the TCR (Fig. 4.1A). To determine whether the p85 subunit interacts with its catalytic p110 subunit in Grb2 complexes, PI kinase assays were carried out.
Results: p85 associates with Grb2-SH3 domains

A T lymphocytes were untreated (-) or stimulated with 10 μg/ml UCHT-1 mAbs for 2 min (+). Proteins were precipitated from T cell lysates (1.6 x 10^7 cell equivalents per lane) with fusion proteins encoding full length GST-Grb2 (Grb2), a double SH3 mutant GST-Grb2 49L/203R (Grb2mutSH3), the isolated N-terminal GST-huGrb2 SH3 domain (aa 1-58) (N-SH3) and the C-terminal GST-huGrb2 SH3 domain (aa 159-217) (C-SH3). Protein complexes were resolved by 10% SDS-PAGE, analysed by immunoblotting with the p85α mAb U5 (upper panel) or the Sos1 antisera (lower panel) and visualised by ECL detection. The migration of the molecular mass standards is indicated to the left in kilodaltons.

(A) T lymphocytes were untreated (-) or stimulated with 10 μg/ml UCHT-1 mAbs for 2 min (+). Proteins were precipitated from T cell lysates (1.6 x 10^7 cell equivalents per lane) with fusion proteins encoding full length GST-Grb2 (Grb2), a double SH3 mutant GST-Grb2 49L/203R (Grb2mutSH3), the isolated N-terminal GST-huGrb2 SH3 domain (aa 1-58) (N-SH3) and the C-terminal GST-huGrb2 SH3 domain (aa 159-217) (C-SH3). Protein complexes were resolved by 10% SDS-PAGE, analysed by immunoblotting with the p85α mAb U5 (upper panel) or the Sos1 antisera (lower panel) and visualised by ECL detection. The migration of the molecular mass standards is indicated to the left in kilodaltons.

(B) T lymphocytes were left untreated and proteins were precipitated from T cell lysates (2.0 x 10^7 cell equivalents per lane) as described above. The protein complexes were analysed for PI 3-kinase activity using PI as a substrate in the absence (-) or presence (+) of 0.5% Nonidet P-40 (NP40). Extraction and separation of the products was performed as described in Materials and methods. PIP indicates the position of the co-migrated phosphatidylinositolphosphate standard.
The 32P-labelled phosphoinositides were extracted and separated by TLC. The p85 molecules purified with Grb2 fusion proteins are associated with the catalytic domain of the enzyme as evidenced by the PI 3-kinase activity present in the Grb2 complexes (Fig. 4.1B). Thus, intact GST-Grb2 and GST-Grb2 N-SH3 and GST-Grb2 C-SH3 purify PI 3-kinase activity from T cell extracts whereas GST-Grb2μSH3 does not.

The binding of p85 to the SH3 domains of Grb2 fusion proteins could be a direct interaction or involve some intermediate molecule acting as a bridge between p85 and Grb2. To resolve these alternatives a far-Western blot analysis of PI 3-kinase was carried out using intact or the SH3 domain-mutated Grb2 proteins. Endogenous p85
p85, a peptide was synthesised corresponding to one of the p85α proline rich motifs, p85-PRO (aa 302-317, QPAPALPPKPPKPTTV), and tested for its ability to isolate endogenous Grb2 complexes from T cell lysates. Western blot analysis demonstrated that Grb2 could be isolated with the p85-PRO peptide (Fig. 4.3A). In 35S-metabolic labelling experiments a 26 kDa species which co-migrated with Grb2 proteins was detected in p85-PRO peptide complexes (Fig. 4.3B). The level of Grb2 isolated with the p85-PRO peptide was lower than that purified with SOS-PRO peptide. As the p85 subunit contains at least two proline rich motifs a detailed analysis of the precise binding mechanisms would demand to examine the binding affinities of the other proline rich motif of p85 for Grb2.

The p85 subunit of PI 3-kinase associates with endogenous Grb2

To assess whether endogenous Grb2 associates with the p85 subunit of PI 3-kinase via its SH3 domains I adopted the strategy described in chapter 3 that uses a tyrosine phosphorylated peptide from the cytoplasmic domain of the EGFR receptor, EGFR-Y(1068)-P, as an affinity matrix to purify endogenous Grb2 via its SH2 domain from T cell lysates which allows to identify its SH3 domain binding partners. In reciprocal experiments interactions between Grb2 and its SH3 domain associated proteins were competed by a peptide derived from the C-terminal proline rich regions of mSos1, SOS-PRO. This SOS-PRO peptide binds to Grb2 SH3 domains and hence competes any interactions between endogenous proteins and Grb2 SH3 domains thereby permitting purification of endogenous Grb2 and associated SH2 binding proteins. The data in Fig. 4.4A show that the EGFR-Y(1068)-P peptide and SOS-PRO can affinity purify equivalent levels of Grb2 from T cell lysates, however as previously shown in chapter 3 only the EGFR-Y(1068)-P peptide associated Grb2 co-purified Sos and in TCR stimulated cells the p75 phosphotyrosine protein (SLP-76). The data in Fig. 4.4A also show a Western blot analysis with p85 mAbs of Grb2 complexes
proteins were purified with the PDGFR-Y(751)-P peptide which is a high affinity resin for the p85 subunit. The data (Fig. 4.2) show that p85 is reactive with wild type GST-Grb2 but not GST-Grb2μSH3 in far-Western blot analysis. These data support that the association between p85 and Grb2 is direct and mediated by Grb2 SH3 domains.

Figure 4.2. The 85 kDa subunit of PI 3-kinase binds directly to Grb2 SH3 domains in T lymphocytes. Endogenous proteins from unstimulated (-) or 10 μg/ml UCHT-1 stimulated (+) T lymphocytes (2.0 x 10^7 cell equivalents per lane) were purified as indicated with PDGFR-Y(751)-P peptide or with Affi-Gel 10 beads alone (control). Precipitated proteins were analysed by 10% SDS-PAGE and replicate PVDF membranes were subjected to far-Western blot analysis with Grb2myc or Grb2mycpSH3 fusion proteins as indicated. The migration of the molecular mass standards is indicated to the left in kilodaltons.

The p85α subunit contains two proline rich motifs, aa 82-96 (SPPTKPRPPRPLP) and aa 300-314 (ERQPAPALPPKPPKP) (Kapeller et al., 1994; Otsu et al., 1991), which provide potential SH3 domain binding sites (residues potentially involved in SH3 binding of Grb2 are in boldface; one proposed consensus binding site for Grb2 SH3 domains is (Cussac et al., 1994): (PVP)ΨΨPPR; Ψ=P, V, A, L, I, M, F, W). To further assess the interaction between Grb2 SH3 domains and
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Figure 4.3. A peptide corresponding to residues 302-317 of a proline rich region in p85α co-purifies endogenous Grb2 from T cell lysates. (A) Immunoblot analysis of proteins affinity-purified from quiescent (−) or 1.6 μg/ml OKT3F(ab')2 activated (+) T lymphocytes with EGFR-Y(1068)-P, Sos-PRO, and p85-PRO peptides coupled to Affi-Gel 10 beads or beads alone (control). Precipitated proteins (isolated from 2.0 x 10⁶ cells per lane) were analysed by 7-17% SDS-PAGE, immunoblotted with Grb2 mAb and visualised by ECL detection. The migration of the molecular mass standards is indicated to the left in kilodaltons. (B) [³⁵S]Methionine-labelled T lymphocytes were untreated (−) or stimulated with 10 μg/ml UCHT-1 for 3 min (+) and lysed (50 mM NaCl, 50 mM Heps pH7.4, 1% Brij 96, 10 mM NaF, 10 mM Iodoacetamide, 5 mM sodium orthovanadate, 1 mM PMSF, and the small peptide inhibitors leupeptin, pepstatin A, and chymostatin all at 1 μg/ml). Cellular proteins (1.0 x 10⁶ cell equivalents per lane) were precipitated with EGFR-Y(1068)-P, Sos-PRO, p85-PRO peptide coupled beads or beads alone (control). Affinity purified proteins were subjected to SDS-PAGE in a 7-17% polyacrylamide gels and visualised by autoradiography. The migration of the molecular mass standards is indicated to the left in kilodaltons.
isolated from quiescent or TCR activated cells with EGFR-Y(1068)-P, SOS-PRO peptides. These data reveal that p85 is present in the Grb2 complexes isolated with the EGFR-Y(1068)-P peptide but not in those purified with the SOS-PRO peptide. The association between Grb2 and p85 is constitutive and not regulated by the TCR (Fig. 4.4A). Moreover, this association apparently was not regulated by tyrosine phosphorylation as no p85 tyrosine-phosphoproteins were detected in Grb2 complexes purified with the EGFR-Y(1068)-P peptide. To determine the relative level of p85 proteins binding to Grb2 proteins, endogenous p85 proteins were purified with the PDGFR-Y(751)-P peptide. About 2% of total p85 proteins as compared to the amount bound to the PDGFR-Y(751)-P peptide were associated with endogenous Grb2. To determine whether the Grb2 bound p85 is coupled to the catalytic p110 subunit of PI 3-kinase, the Grb2 complexes were assayed for PI 3-kinase activity. PI kinase activity was purified with the Grb2 complexes isolated with EGFR-Y(1068)-P but not with those isolated with SOS-PRO peptide (Fig. 4.4B). The p85 molecules purified with endogenous Grb2 are thus associated with the catalytic domain of the enzyme. To quantitate the Grb2/p85 complexes, the level of p85 and PI 3-kinase activity associated

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Figure 4.4. PI 3-kinase binds to endogenous Grb2 SH3 domains via its p85 subunit in T lymphocytes.

(A) Endogenous proteins from unstimulated (-) or 10 μg/ml UCHT-1 stimulated (+) T lymphocytes (1.6 x 10⁷ cell equivalents per lane) were purified as indicated with 20 μM EGFR-Y(1068)-P, Sos-PRO or PDGFR-Y(751)-P affinity resins or with Affi-Gel 10 beads alone (control). Precipitated proteins were analysed by 10% SDS-PAGE and the PVDF membrane was subjected to repeated Western blot analysis with Grb2 mAbs, Sosi antisera, p85α mAbs or phosphotyrosine mAbs 4G10 as indicated. The migration of the molecular mass standards is indicated to the left in kilodaltons.

(B) Endogenous proteins were purified from T cell lysates with 20 μM EGFR-Y(1068)-P, Sos-PRO or PDGFR-Y(751)-P affinity resins or Affi-Gel 10 beads alone (control) as indicated. Precipitated proteins were divided into three (1.6 x 10⁷ cell equivalents per lane). One third and also total cell lysate corresponding to 4.0 x 10⁷ cell equivalents per lane were analysed by 10% SDS-PAGE followed by immunoblotting with Grb2 mAbs (bottom panel). The migration of the molecular mass standard is indicated to the left in kilodaltons. The affinity complexes were analysed for PI 3-kinase activity using PI as a substrate, one third in the absence (-) and one third in the presence (+) of 0.5% Nonidet P-40 (NP40) (upper panel) (assay time was 14 min). Extraction and separation of the products was performed as described in Materials and methods. PIP indicates the position of the co-migrated phosphatidylinositolphosphate standard.
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Results: p85 associates with Grb2-SH3 domains

A

<table>
<thead>
<tr>
<th>2 min UCHT-1</th>
<th>EGF-P</th>
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<th>PDGF-P</th>
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- Grb2
- Sos
- p85α

Phosphotyrosine
with endogenous Grb2 was compared to the total cellular pool of PI 3-kinase purified with the PDGFR-Y(751)-P affinity resin from T cell lysates. The data in Fig. 4.4B show that a low level, approximately 1-2 % of total PI 3-kinase activity is associated with endogenous Grb2. Collectively, the data in Fig. 4.4 show that PI 3-kinase forms a complex with endogenous Grb2. Attempts to carry out reciprocal experiments and show by Western blot analysis that Grb2 could be co-purified with PI 3-kinase in anti-
p85 immunoprecipitates proved impossible as Grb2 migrates exactly at the position of the light chains of the p85 mAbs.

In chapter 3 two abundant Grb2 SH3 binding proteins could be detected in endogenous Grb2 complexes, Sos and p75/SLP-76 (Fig. 3.3A). Moreover, a molecule of 85 kDa was not present in the large scale purification of Grb2 SH3 domains associated proteins (Fig. 3.6). These data underscore that the Grb2/p85 complex is of low stoichiometry when compared to the Grb2/Sos or Grb2/p75 complex possibly because the interaction of p85 with Grb2 is of very low affinity. Similarly, when the PI 3-kinase subunits were purified with the PDGFR-Y(751)-P peptide via SH2 domain binding of the p85 subunit to this peptide (Fig. 3.6), which should not interfere with associations to the proline rich motifs in p85, no other proteins such as Grb2 or p56lck/p59fyn (see below) co-purified.

A quantitative comparison of Grb2 and p59fyn/p56lck associations with PI 3-kinase

The p85 subunit of PI 3-kinase can form complexes with multiple proteins including an SH3 domain mediated association with src family kinases such as p56lck and p59fyn (Prasad et al., 1993; Vogel and Fujita, 1993). The association between PI 3-kinase and src family kinases in T cells has never been quantitatively assessed. Therefore, I was interested to compare the level of PI 3-kinase association with src family kinases to the Grb2/PI 3-kinase interactions. Initial experiments to affinity purify the T cell src family kinases p56lck and p59fyn were frustrated by a lack of antibodies that would immunoprecipitate these kinases with high efficiency. To overcome this problem I used a tyrosine phosphorylated peptide from the hamster middle T antigen, HaMT-Y(324)-P, that binds the SH2 domains of the src family kinases p56lck and p59fyn with high affinity (Songyang et al., 1993; Songyang et al., 1994). The HaMT-Y(324)-P affinity resin should allow to co-purify these src family kinases and their SH3
Figure 4.5. Quantitative comparison of Grb2 and p59fyn/p56lck associations with PI 3-kinase.

T lymphocytes were lysed and endogenous cellular proteins were purified with 20 μM CD28-Y(173)-P, HaMT-Y(324)-P, EGFR-Y(1068)-P or as control unphosphorylated CD28-Y(173) peptide affinity resins. Supernatants from the HaMT-Y(324)-P and EGFR-Y(1068)-P purified protein complexes were subjected to a second round (2) of precipitation with these affinity resins. Each of the washed protein complexes was divided into four and subjected to Western blot analysis (A) or PI 3-kinase assays (B) (2.0 x 10^7 cell equivalents per lane).

(A) Protein complexes were resolved by 10% SDS-PAGE and analysed by immunoblotting with p59fyn antisera, p56lck antisera, Grb2 mAbs, or p85α mAbs as indicated. The migration of the molecular mass standards is indicated to the left in kilodaltons.

(B) The affinity complexes were analysed for PI 3-kinase activity using PI as a substrate, one fourth in the absence (-) and one fourth in the presence (+) of 0.5% Nonidet P-40 (NP40). Extraction and separation of the products was performed as described in Materials and methods. PIP indicates the position of the co-migrated phosphatidylinositolphosphate standard. Incorporation of ^32P into PIP was quantified using a PhosphorImager. The numbers refer to PI 3-kinase activity, [^32P]PIP (fmol/min).
domain associated proteins. The data in Fig. 4.5A demonstrate that this peptide is an efficient tool to isolate p56lck and p59fyn from cell lysates as judged by the fact that a single round of precipitation with the peptide could preclear the majority of p56lck and p59fyn from T cell lysates (Fig. 4.5A). These Western blot data illustrate moreover that p59fyn and p56lck are not present in the Grb2 complexes isolated with EGFR-Y(1068)-P and vice versa Grb2 is not found in the PTK complexes (Fig. 4.5A). To quantitate the Grb2/p85 and the p59fyn/p56lck/p85 associations, the level of p85 bound to endogenous Grb2 or p59fyn/p56lck was compared to the total cellular pool of p85 purified from T cell lysates. In this experiment a tyrosine phosphorylated peptide corresponding to a high affinity p85 binding site in the cytoplasmic tail of the CD28 T cell co-receptor, CD28-Y(173)-P, was used to isolate endogenous p85 proteins efficiently from T cell lysates. This peptide provides at the same time a low affinity binding site for Grb2 proteins (YXNX motif) (Schneider et al., 1995; Songyang et al., 1993; Songyang et al., 1994). Western blot analysis with p85 mAbs confirmed that p85 could be co-purified with the p59fyn/p56lck complexes as well as with Grb2 (Fig. 4.5A) and lipid kinase assays confirmed the presence of the PI 3-kinase catalytic subunit (Fig. 4.5B).
The data in Fig. 4.5A and 4.5B show that a low level, approximately 1-2% of total PI 3-kinase is associated with endogenous Grb2. Similarly, a comparison of the PI 3-kinase activity present in the PTK complexes with total cellular levels of the enzyme showed that only a small percentage of PI 3-kinase can be co-purified with p56lck and p59fyn. However, in the second round of precipitation of Grb2 complexes with the EGFR-Y(1068)-P peptide a relatively high level of p85 protein compared to Grb2 levels in the peptide complexes was noticed. Hence, it can not be excluded when low levels of Grb2 are present in T cell lysates that the p85 subunit of PI 3-kinase can bind to the EGFR-Y(1068)-P peptide directly via its SH2 domains. The data in Fig. 4.5A show that there is no p59fyn or p56lck in the Grb2 complexes and no Grb2 in the p56lck/p59fyn complexes. Accordingly, the p56lck/p59fyn and Grb2 associated PI 3-kinase are mutually exclusive subpopulations of the enzyme.

It has been reported that binding of the SH3 domain of the src kinases p53/p56lyn or p59fyn to purified PI 3-kinase from B lymphocytes stimulates PI 3-kinase activity five fold (Pleiman et al., 1994). Moreover, association of the p85 subunit with the tyrosine phosphorylated Y(751) peptide corresponding to its cognate binding site in the PDGF receptor has been demonstrated to enhance PI 3-kinase activity (Roche et al., 1994; Rodriguez-Viciana et al., 1996). I therefore was interested to examine whether the interaction of the p85 subunit with Grb2 SH3 domains would influence PI 3-kinase catalytic activity. Hence, the PI 3-kinase activity associated with GST-Grb2 fusion proteins was compared to the lipid kinase activity purified with p85 mAbs or the PDGF-Y(751)-P peptide. The data in Fig. 4.6A show the PI 3-kinase activity and the level of p85 protein present in the different complexes. Interestingly, when the PI 3-kinase activity present in these complexes was normalised to the amount of p85 protein and the specific activity of PI 3-kinase in the Grb2 or the PDGFR-Y(751)-P peptide complexes was compared to the level of PI 3-kinase activity in the anti-p85 immune complexes (Fig. 4.6B, antibody binding to the p85 protein has not been reported to
affect the specific activity of PI 3-kinase), the specific activity of PI 3-kinase associated with Grb2 proteins was markedly reduced by about 75%. Consistent with earlier observations the PI 3-kinase specific activity in PDGFR-Y(751)-P complexes was increased. Due to the lack of recombinant fyn or lck proteins in our laboratory, a direct comparison of the PI 3-kinase specific activities associated with Grb2 SH3 domains versus p56lck/p59fyn SH3 domains in T cells was not possible in this study. The decrease of PI 3-kinase specific activity associated with Grb2 in T cells suggests that this interaction keeps PI 3-kinase in a repressed stage. The significance of this downmodulation of the specific activity of PI 3-kinase is not clear. Finally, although it is generally presumed that the p85 and the p110 subunit exist in a 1:1 complex in cells, a precise study of the specific activity of the PI 3-kinase associated with Grb2 SH3 domains would warrant an analysis of the actual levels of the p110 catalytic subunit associated with the p85 regulatory subunit of PI 3-kinase. Unfortunately, when this study was ongoing no reliable p110 Abs existed which allowed an analysis of p110 protein levels. Thus, it cannot be excluded that the interaction of the p85 subunit with Grb2 affects the binding to its catalytic p110 subunit.

Figure 4.6. Assessment of the specific kinase activity of PI 3-kinase subunits complexed to Grb2 fusion proteins.
(A) The p85 subunits of PI 3-kinase were isolated from cell lysates with GST fusion proteins of Grb2, p85α mAbs (U5), Sos-PRO peptide or beads (control) as indicated. The protein complexes were washed and divided in three (2.5 x 10^7 cell equivalents per lane). The affinity complexes were analysed for PI 3-kinase activity using PI as a substrate, one third in the absence (−) and one third in the presence (+) of 0.5% Nonidet P-40 (NP40) (upper panel). Extraction and separation of the products was performed as described in Materials and methods. PIP indicates the position of the co-migrated phosphatidylinositol phosphophate standard. Incorporation of ^32P into PIP was quantified using a PhosphorImager and is expressed in arbitrary units. One third of the affinity-purified proteins were resolved by 10% SDS-PAGE. Protein complexes were analysed by immunoblotting with p85α mAb followed by ^125I-ProteinA. p85α proteins were visualised by autoradiography (lower panel). The detected protein bands were excised from the PVDF membrane and proteins levels were quantified by measuring the Cerenkov counts of bound ^125I-ProteinA using a Beckman LS6000 series scintillation counter. Protein levels are indicated at the bottom in arbitrary units.
(B) The specific activity (PI kinase activity/p85α protein levels) of the PI 3-kinase present in the affinity complexes was calculated from the values indicated in (A).
Chapter 4

Results: p85 associates with Grb2-SH3 domains

A

<table>
<thead>
<tr>
<th>Grb2</th>
<th>control</th>
<th>p85a</th>
<th>PDGF-Y(751)-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>0.5% NP40</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

- PIP

B

- 143%
- 100%
- 24%

- Grb2
- control
- p85a
- PDGF-P

Experiments with Grb2 fusion proteins can be used for assessing protein interaction. It is important to establish conditions between proteins that are known to interact in vivo and in vitro. Vectors that are derivative of the plasmid pCI-neo are useful for experiments only with Grb2. The vector pCI-neo contains a 3' untranslated region of the HSV TK gene that is expressed in vivo and in vitro.
4.3 Discussion

The present data show that Grb2 SH3 domains can bind to the p85 subunit of PI 3-kinase. This was demonstrated using two approaches: association studies with GST fusion proteins of Grb2 which revealed an in vitro interaction of p85 with Grb2 SH3 domains and co-purification analysis with endogenous Grb2 complexes which proved that this SH3 domains mediated association also occurs in vivo. The p85 subunit complexed to Grb2 in T cells associated with its catalytic PI 3-kinase activity and interacted with Grb2 in both resting and TCR activated T cells. The p85 subunit of PI 3-kinase bound directly to Grb2 SH3 domains as indicated by far-Western blot analysis with recombinant Grb2 proteins. The p85 subunit of PI 3-kinase contains two proline-rich sequences in its N-terminal region, aa 82-96 (SPPTKPKRPPRPLP) and aa 300-314 (ERQPALPPKPPKP) (Kapeller et al., 1994; Otsu et al., 1991), which provide potential SH3 domain binding sites and as shown in the present study at least one of these proline-rich motifs (aa 302-317, QPAPALPPKPKPTTV) can mediate binding to the Grb2 adaptor protein. Interestingly, the PI 3-kinase bound to Grb2 proteins exhibited a 75% lower specific activity when compared to the one present in anti-p85 immune complexes.

Experiments with GST fusion proteins can be valuable for assessing protein-protein interactions but it is important to establish whether the associations between proteins that are identified in such in vitro studies actually occur in vivo. The salient point about the Grb2-p85 binding described herein is that it can be demonstrated both in vitro and in vivo in endogenous Grb2 complexes. It was striking that in the current experiments only a small subpopulation of PI 3-kinase was demonstrably associated with Grb2. This apparent low stoichiometry does not necessarily correlate with the potential of Grb2 to regulate the enzyme. For example, in a similar analysis of the association of Grb2 with the effector molecule Sos, only 5-10% of cellular Sos could be
co-precipitated with Grb2 (Helen Turner, unpublished data). Moreover, previous studies have identified a complex between p85 and the tyrosine kinases p56lck and p59fyn that is mediated by the SH3 domains of these src family kinases. An exploration of the stoichiometry of the p56lck/p59fyn association with PI 3-kinase showed that approximately 1% of cellular PI 3-kinase can be co-precipitated with p56lck and p59fyn which is similar to the level of the enzyme found to associate with Grb2 (Fig. 4.4). The Grb2 and p56lck/p59fyn associated PI 3-kinase were exclusive populations which suggests that there is not a single common pool of PI 3-kinase in T cells but possibly multiple subpopulations of the enzyme that might function in different micro-environments within the cell. It has been shown previously that binding of the SH3 domains of src kinases to PI 3-kinase from B lymphocytes activates the enzyme (Pleiman et al., 1994). It has not been reported that interactions of the src family kinases with PI 3-kinase has an stimulatory effect on the enzyme in T cells. Surprisingly, the association of PI 3-kinase with Grb2 in vitro decreased PI 3-kinase catalytic activity. The significance of this downmodulation of the PI 3-kinase enzymatic activity remains unresolved. The interactions of PI 3-kinase with Grb2 were not regulated by the T cell antigen receptor. However, this does not exclude in vivo regulation of the enzyme by Grb2 and it is likely that by analogy with Sos, Grb2 could control the cell localisation of PI 3-kinase.

Previous studies have shown that Grb2 is regulated by the TCR which induces tyrosine phosphorylation of a 36 kDa protein that binds to the SH2 domains of Grb2. This 36 kDa protein is the functional link between the TCR and Grb2 and is proposed to be central in coupling the TCR to effector molecules that bind to the SH3 domains of Grb2 or PLCγ1 (Buday et al., 1994; Sieh et al., 1994). Similarly, in IL-2 activated T cells tyrosine phosphorylated Shc binds to Grb2 SH2 domains and couples the IL-2R to Grb2 signalling pathways (Osman et al., 1995; Ravichandran and Burakoff, 1994). The original idea was that the TCR regulated p36 molecule and IL-2R
associated Shc are adapters that couple these receptors to Grb2 and the Sos/p21ras signalling cascade. However, the demonstration that PI 3-kinase binds to Grb2 SH3 domains analogous to the Grb2/Sos interaction suggests that the function of both p36 and Shc might be broader than at first anticipated as they could play a role in coupling the TCR and the IL-2R to PI 3-kinase. In addition, the role of Grb2 is not likely to be restricted to the regulation of Sos and PI 3-kinase as the novel TCR induced tyrosine phosphoproteins SLP-76, p116, and the proto-oncogene Cbl are putative Grb2 effectors that have been detected in Grb2 complexes in an SH3 mediated association (Buday et al., 1996; Donovan et al., 1994; Fukazawa et al., 1995; Jackman et al., 1995; Meisner et al., 1995; Motto et al., 1994; Reif et al., 1994). Moreover, Vav, a putative exchange factor for rho family GTPases which is expressed in the haematopoietic lineage, and an inositol polyphosphate 5-phosphatase, SIP-110, have been reported to bind to Grb2 SH3 domains (Kavanaugh et al., 1996; Lahesmaa et al., 1995; Ramos Morales et al., 1994; Ramos Morales et al., 1995; Ye and Baltimore, 1994). Grb2 is thus likely to have pleiotropic functions in T cells and the present data suggest that linking receptors to PI 3-kinase is one potential purpose. However, the function of Grb2 in bridging receptors to PI 3-kinase is likely not to be limited to T cells but may be significant for other receptor/cell system (where p85 subunits do not bind to the receptor directly). For instance, the tyrosine-phosphatase SHP-2 has been proposed to be important for integrating signals from IL-3 and GM-CSF receptors to PI 3-kinase (Welham et al., 1994). As SHP-2 can bind to the Grb2 SH2 domain (Bennett et al., 1994; Li et al., 1994), Grb2 serves as an excellent candidate in connecting SHP-2 and PI 3-kinase. SHP-2 may also be important for the TCR/Grb2/p85 link in T cells as it becomes tyrosine phosphorylated after TCR activation and is found in a complex with Grb2 and p85 (Tailor et al., 1996).

The mechanisms of regulating cellular PI 3-kinase appear to be increasingly complicated. Originally, it was thought that receptor induced tyrosine phosphorylation
of the enzyme was important. This hypothesis was supplanted by the suggestion that the crucial activating event was recruitment of PI 3-kinase to the membrane by interactions of the p85 SH2 domains with selected tyrosine phosphorylated sequences within the cytoplasmic domains of growth factor receptors (Cantley et al., 1991). Such associations might also be mediated via other adaptor proteins, as it has been reported in T cells that the p85 subunits of PI 3-kinase can interact via their SH2 domains directly with the TCR-regulated PTK substrates p36 (Fukazawa et al., 1995) or c-Cbl (Fukazawa et al., 1995; Hartley et al., 1995; Meisner et al., 1995) including a specific association of the p85β subunit with c-Cbl (Hartley et al., 1995). More recently, it has been suggested that the low molecular weight G proteins of the ras and rho family may play a role in PI 3-kinase recruitment to the membrane and hence its activation (Rodriguez-Viciana et al., 1994; Tolias et al., 1995; Zheng et al., 1994). Finally, the associations between PI 3-kinase and the SH3 domains of src family kinases and the herein described Grb2 association are likely to be significant recruitment/activation mechanisms. In this respect, it is noteworthy that PI 3-kinase subunits do not associate with tyrosine kinase activity in TCR stimulated cells and PI 3-kinase activation delivered by the TCR correlates with serine/threonine phosphorylation of PI 3-kinases subunits in vivo (Reif, 1992; Reif et al., 1993; Ward et al., 1992) which prompts the Grb2/PI 3-kinase interaction being an attractive concept. The functional relevance of Grb2/PI 3-kinase complexes to TCR regulation of the enzyme is unknown but there is equally no genetic or functional evidence that any of the other possible routes for PI 3-kinase activation are used in T cells e.g. p21ras/receptor recruitment. Interestingly, in a yeast two-hybrid system screen for p85 binding partners Grb2 was demonstrated to bind to either of the proline rich motifs (see above) in the p85 subunit (Wang et al., 1995). In this study, an association between p85 and Grb2 was also observed in anti-Grb2 or anti-p85 immune complexes isolated from fibroblasts.
The focus of future studies will be to unravel the relative contribution of these different intracellular interactions for regulating PI 3-kinase while recognising that they are not necessarily mutually exclusive. Rather the synergistic interplay of different pathways may be required for optimal PI 3-kinase regulation as suggested by a recent analyses of PI 3-kinase activation in PC12 cells (Ohmichi et al., 1994). In this context, it is known that in T cells a synergy between the TCR and CD28 results in optimal stimulation of PI 3-kinase activity (Ward et al., 1993; Ward et al., 1996). CD28 is tyrosine phosphorylated and PI 3-kinase becomes recruited to the membrane via binding of its p85 SH2 domains (Truitt et al., 1994). Moreover, it has been reported that Grb2 can also bind to phosphotyrosine at position 173 in CD28 (Schneider et al., 1995). The mechanism underlying the TCR contribution to CD28 regulation of PI 3-kinase is unclear but it is likely that PI 3-kinase associations with the SH3 domains of the src kinases and/or Grb2 might be involved.
Chapter 5

The creation of a constitutively active PI 3-kinase, rCD2p110, and other PI 3-kinase chimeras and their biochemical characterisation

5.1 Introduction

Activation of PI 3-kinase is thought to be important in a wide range of biological activities (Carpenter and Cantley, 1996; Fry, 1994), including control of proliferation (Cantley et al., 1991; Cheatham et al., 1994; Roche et al., 1994; Valius and Kazlauskas, 1993), activation of p70S6k (Chung et al., 1994; Monfar et al., 1995), cytoskeletal organisation (Kotani et al., 1994; Wennström et al., 1994), apoptosis (Dudek et al., 1997; Hemmings, 1997; Kauffmann-Zeh et al., 1997; Yao and Cooper, 1995), neurite outgrowth (Kimura et al., 1994), endocytosis and vesicular trafficking (Joly et al., 1995; Joly et al., 1994; Li et al., 1995; Martys et al., 1996; Shpetner et al., 1996), insulin-stimulated glucose transport (Cheatham et al., 1994; Okada et al., 1994) and events leading to T cell activation and growth (Cantrell et al., 1993; Rudd, 1996; Ward et al., 1996). Thus, D-3 phosphoinositide levels increase upon engagement of the TCR, CD28 and cytokine receptors including the IL-2R (Remillard et al., 1991; Ward et al., 1993; Ward et al., 1992); however, at present the knowledge about the function of PI 3-kinase during these processes is limited. In particular, in T cells and in other cell systems the specific components of PI 3-kinase directed pathways are not known.

More is known about upstream regulators of PI 3-kinase and its activation by regulatory receptors. As described in the previous chapter the activation of PI 3-kinase by the TCR may involve the adaptor protein Grb2 or src family tyrosine kinases (see Fig. 5.1). Regulation mechanisms in other cell systems comprise association of PI 3-
kinase with a variety of signalling proteins, including growth factor receptors like the PDGF receptor, non-receptor tyrosine kinases of the src family, abl or crk (Cantley et al., 1991) and GTP-binding proteins such as the GTPase Ras (mediated by p110) (Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1996) or the Rho family proteins Cdc42 and Rac (mediated by the bcr domain of p85) (Tolias et al., 1995; Zheng et al., 1994).

![Figure 5.1. Possible recruitment mechanism implicated in translocating PI 3-kinase to the plasma membrane.](image)

Activation of the enzyme is thought to be achieved by several mechanisms: 1. regulation mediated by direct phosphorylation of the p85 or p110 subunits (Auger et al., 1989; Dhand et al., 1994; Kavanaugh et al., 1994; Reif et al., 1993); 2. stimulation induced by conformational changes brought about by protein/protein interactions, e.g. binding of the SH2 domain of p85 to specific phosphotyrosine residues of signalling proteins (Backer et al., 1992; Carpenter et al., 1993; Panayotou et al., 1992; Shoelson et al., 1993); 3. stimulation due to localisation of PI 3-kinase at the plasma membrane in...
the proximity of its substrate PI(4,5)P$_2$ (Kapeller and Cantley, 1994). This latter translocation event appears to be the common theme underlying all described mechanism and is generally required for D-3 phosphoinositide production (see Fig. 5.1).

Endogenous PI 3-kinase consist of a tight heterodimeric p85 and p110 complex (ratio 1:1). The regulation of enzyme activity by growth factor receptors and the complex protein interactions which may be involved in the function of PI 3-kinase seem to require the presence of both subunits. Indeed, expression studies in insect cells of the p110α subunit either alone or together with its p85α subunit have shown that the p85-p110 complex exerts a higher specific activity than free p110 (Dhand et al., 1994; Woscholski et al., 1994). Analysis of p110 catalytic properties in mammalian cells (Cos cells) has suggested that p110 does not exhibit PI 3-kinase activity unless p85 is co-expressed (Hiles et al., 1992; Klippel et al., 1994). Mapping studies of the p85 and p110 interaction sites have identified the inter-SH2 domain region of p85 (aa 478-513 of p85α and aa 445-485 of p85β) and the N-terminal region of p110 (aa 20-108 of p110α (or aa 31-150 of p110β)) as being required for the binding of the molecules to each other (Dhand et al., 1994; Holt et al., 1994; Hu and Schlessinger, 1994; Klippel et al., 1994; Klippel et al., 1993). Sequence analysis suggests that the inter-SH2 region of p85 forms an anti-parallel coiled-coil of two α-helices (Dhand et al., 1994; Panayotou et al., 1992). Secondary structure prediction of the N-terminus of the p110 subunit indicates that 60% will adopt an α-helical and 20% a β-sheet conformation. Thus, it is thought that the α-helices are engaged in mediating the stable p110-p85 association and may also involve a small hydrophobic pocket on p85. An additional possible regulatory element has been identified in a lipid binding site, presumably for PI(4,5)P$_2$, in the inter-SH2 region of the p85 subunit (End et al., 1993) which may corroborate the importance of the p85-p110 interaction.
The identification of cellular functions for PI 3-kinase stems largely from studies with mutant receptors in which the phosphotyrosine binding sites for PI 3-kinase are replaced with phenylalanine (Fantl et al., 1992; Kazlauskas et al., 1992) or experiments with a p85 mutant that lacks the p110 binding site (Dhand et al., 1994; Hara et al., 1994; Kotani et al., 1994; Wennström et al., 1994). These mutants prevent the recruitment of PI 3-kinases into activated receptor complexes but such dominant interfering approaches cannot distinguish between the requirement for two proteins that bind to the same site (Nishimura et al., 1993). In addition, the analysis of the role of PI 3-kinase during T cell activation and growth has been hampered by the fact that high affinity binding sites for PI 3-kinase in the cytoplasmic tails of the TCR or the IL-2R do not exist which prevents a study of PI 3-kinase immunological functions using specific receptor mutants (see chapter 4). Additional tools for the analysis of PI 3-kinase effector pathways are inhibitors of PI 3-kinase function, such as wortmannin or the structurally unrelated LY294002 compound, which compete for the lipid- or the ATP-binding site on PI 3-kinase, respectively (Arcaro and Wymann, 1993; Ui et al., 1995; Vlahos et al., 1994; Wymann et al., 1996). However, interpretation of these studies is complicated by the finding that these inhibitors are only partially specific for PI 3-kinase (Brunn et al., 1996; Cross et al., 1995; Downing et al., 1996; Nakanishi et al., 1995).

The strategies employed to define the importance for PI 3-kinase have mainly been indirect and no conclusive data has been provided to establish whether PI 3-kinase signals are sufficient to activate downstream effector pathways. I therefore initiated studies to generate a constitutively active form of the enzyme that would allow analysis of the cellular functions of PI 3-kinase and elucidate its relative contribution to a certain effector pathway. Constitutively active versions of signalling molecules are widely used in signal transduction research to assess the potential of a signalling molecule to drive a biological pathway and/or to substitute for a physiological stimulus.
In particular, such activated enzymes are valuable to identify its specific cellular functions and define the components of its downstream effector pathways.

The interaction of PI 3-kinase with signalling proteins appears to have one common element: to promote the relocalisation of PI 3-kinase to the plasma membrane into the proximity of its substrate PI(4,5)P_2. The strategy I chose to generate a constitutively active mutant was therefore to localise the subunits of PI 3-kinase to the plasma membrane. Three principal approaches were possible to target the enzyme to the plasma membrane: 1. by fusing the myristoylation signal of v-Src to the N-terminus (the src myristoylation motif is MGSSKSHPKDSQR) (Buss et al., 1988); 2. by appending the sequence that specifies farnesylation and palmitoylation of c-Ha-Ras to the C-terminus (KLNPPDESFGMCVSCKCVLS), generally referred to as introducing a CAAX motif (C=Cys, A=aliphatic, X: the identity of this residue confers farnesylation or geranylgeranylation; the farnesylation motif for c-Ha-Ras is CVLS) (Hancock et al., 1991; Hancock et al., 1990); 3. by generating chimeras with a transmembrane receptor, normally fused to the enzyme at the N-terminus using the extracellular and transmembrane domain of receptors such as the EGFR or CD16. All three approaches have been successfully used to localise enzymes at the plasma membrane (Aronheim et al., 1994; Desai et al., 1993; Kolanus, 1993; Leevers et al., 1994). The prevailing approach in immune cells has been to generate chimeric transmembrane proteins. This has several advantages: 1. surface expression of the chimeras can easily be detected by immunofluorescent surface staining with specific antibodies to the extracellular domain of the receptor followed by flow cytometric analysis; 2. cells expressing the chimera ectopically can be sorted/identified by flow cytometry; 3. the chimeras can easily be distinguished from the endogenous protein and do not require an additional peptide tag; 4. it is possible to specifically localise the fusion protein into a receptor complex such as the TCR/CD3 complex by generating chimeric antibodies which permit crosslinking of two different surface molecules.
5.2 Results

To localise PI 3-kinase at the membrane, I constructed chimeric molecules in which the cytoplasmic domain of the rat CD2 (rCD2) cell surface antigen was replaced with PI 3-kinase subunits or truncated/mutated versions of the p110 catalytic subunit (Fig. 5.2A). The rCD2 molecule was chosen as it can be distinguished from human or mouse CD2 with a mAb to a xenotypic epitope of the molecule which permits specific detection of the ectopically expressed protein in many T cell lines. The extracellular and transmembrane domains of the rCD2 antigen were linked to the N-terminus of p85α (rCD2p85) or p110α (rCD2p110). As a control, a rCD2p110 construct with an inactivating point mutation (the arginine at position 1130 replaced with a proline; R1130P) in the ATP binding site (Dhand et al., 1994), which abolishes its kinase activity, was generated (rCD2p110-R/P). It had been suggested that the first N-terminal 123 aa residues of p110 containing the p85 interaction site are important for the regulation of the catalytic activity of p110 and deletion of this region may lead to abrogation of enzymatic activity (Klippel et al., 1994). However, it was also possible that such a p110 mutant lacking the N-terminal 123 aa residues, p110-Δ123, has diminished catalytic activity but is not completely inactive. This was concluded from the notion that the p110-Δ123 mutant was expressed in Cos7 cells in which activity of expressed full length wild-type p110 is difficult/impossible to detect whereas when expressed in insect cells activity of wild-type p110 can easily be measured (Hiles et al., 1992; Klippel et al., 1994). To further determine the requirement of this region for p110 enzymatic activity and to possibly create a rCD2-PI 3-kinase constructs with lower catalytic activity, I also generated a rCD2p110-Δ126 chimera with an p110 N-terminal truncation of 126 aa residues and, as a control, its kinase dead counterpart with the inactivating point mutation in the ATP binding site (R1002P), rCD2p110-R/P-Δ126. Additional controls used were full length wild-type rCD2 and a truncated version of rCD2 comprising the extracellular and transmembrane region of rCD2 plus 10 random aa
residues (LSMHLEGPI), rCD2Δ. In addition, for co-expression studies, wild-type p110 and kinase dead p110-R/P (R916P) both containing a Myc-epitope tag at the C-terminus were made. It was anticipated that the Myc-tag would allow an comparison of the specific activities of the different PI 3-kinase constructs.

Figure 5.2A. Schematic of truncated rCD2 constructs, rCD2-PI 3-kinase chimeras and p110 molecules generated in the present study. Numbers correspond to amino acids (aa) in the protein and demarcate the extracellular, transmembrane, and cytoplasmic domains. Amino acid 216 depicts the start of the wild-type p85α or p110α molecules; aa 213 the start of the p110α of which the first 126 aa (p85 binding site) have been truncated. The position of the p85 binding site on p110 (patterned box), the location of the kinase domain (green box) and the inactivating point mutation in the ATP binding site (black dot), R1130P in rCD2p110-R/P, R1002P in rCD2p110-R/P-Δ126, or R916P in p110-R/P, are denoted. All p110 mutants contain a C-terminal Myc-tag (triangle).
Construction of chimeric cDNAs

The cDNAs of bovine p85α, EMBL accession number M61745 (Otsu et al., 1991), in pBluescript I (SK-), and p110α, EMBL accession number M93252 (Hiles et al., 1992), in pBluescript I (SK-) (BamHI clone) were kindly provided by Michael Waterfield, London. The cDNA for rCD2, EMBL accession number X05111 (He et al., 1988; Williams et al., 1987), in pKG5 (BamHI clone) was a gift from Neil Barclay, Oxford. To modify the C-termini and/or N-termini of p85α, p110α, and rCD2, the cDNAs were amplified by PCR with the following oligonucleotide primers (bases coding for the restriction sites are underlined); some of the PCR constructs obtained are depicted in Fig. 5.2B.

*BamHI-*rCD2Δ-NotI (original N-terminus; introduce NotI site and truncate C-terminus):
5′-primer: 5′-GCG CTC GGA TCC AAG ATG AGG TGT AAA TTC C-3′
3′-primer: 5′-GCC ATC GCG GCC GCT GTT CCG TTT TTT CCT CTT GCA GAT ACA G-3′

*Clal-*rCD2Δ-EcoRI (introduce Clal site before start codon at N-terminus; introduce EcoRI site and truncate C-terminus):
5′-primer: 5′-GCG CAT CGA TCC AAG ATG AGG TGT AAA TCC-3′
3′-primer: 5′-CCG CAG GAA TTC GTT CCG TTT TTT CCT CTT GCA G

NotI-p110/myc-SalI (introduce NotI site and omit methionine at N-terminus; introduce glycine linker, Myc-tag and Xhol-SalI sites at C-terminus):
5′-primer: 5′-CGG AAC AGC GGC CGC CCT CCA AGA CCA TCA TCA GGT GAA CTG TGG-3′
3′-primer: 5′-GCC GGA GTC GAC CTC GAG TCA CAA GTC TTC TTC AGA AAT AAG CTT TTG TTC GCC TCC GCC GGT CAA AGC ATG CTG CTT AAT TGT GTG G-3′
**Chapter 5**

**Results: rCD2-PI 3-kinase chimeras**

_Not1-p85-Clal (introduce Not1 site and omit methionine at N-terminus; introduce XbaI-Clal sites at C-terminus):

5'-primer: 5'-CGG AAC AGC GGC CGC AGT GCC GAG GGG TAG CAG TAC CGG GCG C-3'

3'-primer: 5'-GCC CTC ATC GAT TCT AGA TCA TCG CCT CTG CTG CGC GTA CAC TGG G-3'

_BamHI-p110/myc-SalI (original N-terminus; introduce glycine linker, Myc-tag and XhoI-SalI sites at C-terminus):

5'-primer: 5'-AAC TAG TGG ATC CGA ACA ATG CCT CCA AGA CC-3'

3'-primer: 5'-GCC GGA GTC GAC CTC GAG TCA CAA GTC TTC TTC AGA AAT AAG CTT TTG TTC GCC TCC GCC GTT CAA AGC ATG CTG CTT AAT TGT GTG G-3'

**Construction of rCD2Δ, rCD2, rCD2p85, rCD2p110 and rCD2p110-R/P**

The coding regions of bovine p85α and p110α were attached to a truncated rat CD2 molecule (aa 1 to 212) consisting of the extracellular and transmembrane domain plus the intracellular stop transfer motif (aa 207-212; KRKKRN). The rCD2 was truncated at the stop transfer sequence by addition of a Not1 site (BamHI-rCD2Δ-Not1). The p85α subunit (Not1-p85-Clal) and the p110α subunit (Not1-p110/myc-SalI) were adapted with a Not1 site at the 5' end omitting the methionine of the leader sequence which due to the Not1 site introduced the peptide SGR in the coding sequence at the hinge. The Not1-p110 construct was tagged at the C-terminus with the Myc-epitope (EQKLISEEDL) (Evan et al., 1985; Kolodziej and Young, 1991) via a glycine linker (GGG). Each PCR construct obtained was digested with the appropriate restriction enzymes as indicated and ligated into pBluescript I. In the BamHI-rCD2Δ-Not1 PCR product, the AvrII-Hincll fragment (see Fig. 5.2B, restriction sites shaded in grey) was released involving partial digestion of the plasmid and substituted with the
corresponding fragment from the original cDNA. In the NotI-p85-ClaI PCR product, the

BglII-Sall

Figure 5.2B. PCR products generated to construct rCD2-PI 3-kinase chimeras.
All the sites shown are unique restriction sites. However, those which are shaded or framed
have been used in subsequent gene manipulations. The p110 constructs containing the point
mutation arginine to proline in the ATP binding site of p110 also comprised an additional BamHI
site indicated in bold. For further details see text.
Figure 5.2C. Schematic of vectors used to express PI 3-kinase constructs in mammalian cells. The pcDNA3 and the pRK5 direct expression from a cytomegalovirus (CMV) promoter/ enhancing region with the promoter/enhancer in pRK5 being more potent. The pcDNA3 vector contains a neomycin resistance marker, expressed from the SV40 early promoter for the selection of stable transformants in the presence of G418. The pEF-BOS directs expression from the promoter of polypeptide chain elongation factor 1α (EF-1α). EF-1α promotes the GTP-dependent binding of an aminoacyl-tRNA to ribosomes and is one of the most abundant proteins in mammalian cells. All plasmids contain a SV40 origin for transient episomal replication in cells expressing SV40 large T antigen (such as Cos7 or JHTAg). All plasmids yield a high copy number during growth in E. coli due to their ColE1 or pMB1 origin of replication.
fragment was released involving partial digestion of the plasmid and replaced with the corresponding fragment from the original cDNA. In Notl-p110/myc-Sall PCR product, the EcoRI-PstI fragment was released and exchanged with the corresponding fragment from the original cDNA. To construct the inactive rCD2p110-R/P, a 533 bp PstI-Ncol cartridge from the modified Notl-p110/myc-Sall fragment (involving partial digestion of the plasmid) was replaced with the corresponding cartridge from the described p110-BamHI plasmid containing the R916P mutation (Dhand et al., 1994) producing the plasmid Notl-p110-R/P/myc-Sall. In addition to the arginine to proline mutation, this oligonucleotide cartridge also contains a novel BamHI site by means of silent codon changes. The basepairs which originated from the PCR products and the gene fusion sites were verified by nucleotide sequencing, all constructs were checked by intensive restriction enzyme analysis. The rCD2-PI 3-kinase chimeras were assembled by subcloning the modified cDNAs into the pcDNA3 vector (in vitrogen) (see Fig. 5.2C) by first ligating the BamHI-rCD2Δ-Notl fragment into the corresponding sites in pcDNA3. The other fragments were subcloned using the Notl site and the Apal site present in the pbluescript I polylinker to obtain pcDNA3-rCD2p85, pcDNA3-rCD2p110 and pcDNA3-rCD2p110-R/P. The rCD2Δ construct corresponds to the BamHI-rCD2Δ-Notl fragment in pcDNA3 and utilises a stop codon in the vector sequence occurring after about 30 bp. Subsequently, the rCD2p85, rCD2p110 and rCD2p110-R/P chimeras were subcloned in either the pRK5 (Ferrari et al., 1993) or the pEF-BOS (Mizushima and Nagata, 1990) expression vectors (see Fig. 5.2C) using the following restriction sites: pRK5-rCD2p85: BamHI-Sall (partial digest); pEF-rCD2p85: BamHI-ClaI; pRK5-rCD2p110 and pEF-rCD2p110: BamHI-Sall; pRK5-rCD2p110-R/P and pEF-rCD2p110-R/P: BamHI-Sall (partial digest). All obtained plasmids were verified by nucleotide sequencing and/or restriction enzyme analysis. Full length wild-type rCD2 was released from the pKG5 vector (BamHI clone) with a BamHI digest, subcloned into pcDNA3 or pEF expression plasmids, and checked for the correct orientation.
Construction of p110 and p110-R/P

The coding region of bovine p110α was amplified by PCR using an 3'-oligonucleotide primer which encoded a Myc-tag and a Sall restriction site after the stop codon (see above). The obtained PCR fragment was ligated via BamHI-Sall ends into pbluescript I. The BamHI-PstI fragment was then exchanged with the corresponding one from the original cDNA clone. Subsequently, the PstI-NcoI fragment (see above) was released (involving a partial digest). This fragment was replaced with the corresponding PstI-NcoI fragment from the original cDNA to obtain p110/myc or the PstI-NcoI DNA cartridge encoding the inactivating point mutation in the ATP binding site (see above) to generate p110-R/P/myc. The correct sequence of the p110 fragments modified by PCR including gene fusion sites was confirmed by DNA sequence analysis. The respective DNA fragments were subcloned into pcDNA3 via NotI-Apal sites (both from pbluescript I) to generate pcDNA3-p110 and pcDNA3-p110-R/P. These Myc-tagged p110 constructs were subcloned into pRK5 and pEF via BamHI-Sall sites which involved partial digestion of the p110-R/P/myc DNA fragment (additional BamHI site, see above) to generate the expression plasmids pRK5-p110, pRK5-p110-R/P, pEF-p110, and pEF-p110-R/P.

Construction of rCD2p110-Δ126 and rCD2p110-R/P-Δ126

The rCD2 was modified before the start codon to introduce a Clai restriction site and truncated at the stop transfer sequence by addition of a EcoRI site (Clai-rCD2Δ-EcoRI) using PCR (see Fig. 5.2B). The obtained PCR fragment was digested with Clai-EcoRI and subcloned into pbluescript I. In the Clai-rCD2Δ-EcoRI PCR product, the AvrII-HincII fragment (see Fig. 5.2B, restriction sites shaded in grey) was released involving partial digestion of the plasmid and substituted with the corresponding fragment from the original cDNA (generated by Carol Beadling, Lymphocyte Activation Laboratory, ICRF). To obtain p110 or p110-R/P mutants in which the N-terminal 126 aa are truncated, which contain a Myc-tag, and can be adapted with an EcoRI site to the
Chapter 5  Results: rCD2-PI 3-kinase chimeras

Clal-rCD2Δ-EcoRI fragment, an EcoRI-Apal fragment (the Apal site is from pbluescript I) was released both from the pbluescript I plasmids containing the corrected/checked NotI-p110/myc-SalI fragment or the 'kinase dead' NotI-p110-R/P/myc-SalI fragment. There is a convenient unique EcoRI site in the original cDNA of bovine p110 (Otsu et al., 1991) which yields a 126 aa truncation of p110. These EcoRI-Apal fragments were then subcloned into the corresponding sites of pcDNA3. Then, the pbluescript I plasmid containing the corrected Clal-rCD2Δ-EcoRI PCR product was digested with KpnI-EcoRI (the KpnI site is present in the polylinker of pbluescript I) and ligated into the corresponding sites in the pcDNA3 vectors containing the above EcoRI-p110/myc or EcoRI-p110-R/P/myc fragments to produce the plasmids pcDNA3-rCD2p110-Δ126 and pcDNA3-rCD2p110-R/P-Δ126. Subsequently, the rCD2p110-Δ126 and rCD2p110-R/P-Δ126 chimeras were released by a Sall digest and subcloned in the correct orientation in the pRK5 expression vector (see Fig. 5.20) to gain pRK5-rCD2p110-D126 and rCD2p110-R/P-D126. The correct sequence of the obtained plasmids was confirmed by nucleotide sequencing and/or restriction enzyme analysis.

Expression of rCD2-PI 3-kinase chimeras in Cos7 cells and other adherent cell lines

To demonstrate that the newly generated rCD2-PI 3-kinase cDNAs in pcDNA3 vectors encode the correct rCD2-PI 3-kinase fusion proteins, they were transiently transfected into Cos 7 cells by electroporation. Cos7 cells are derived from African monkey kidney cells, are transformed with SV40 large T antigen and widely used for protein expression studies. Forty-eight hours after transfection, Cos7 cells were lysed and Western blot analysis with rCD2 mAb, Ox34, revealed the presence of molecular species which corresponded to the expected masses of rCD2-PI 3-kinase chimeras (Fig. 5.3A, left panel): rCD2Δ = 35 kDa; rCD2p85 = 120 kDa; rCD2p110 or rCD2p110-R/P = 145 kDa; rCD2p110-D126 or rCD2p110-R/P-Δ126 = 130 kDa. The extracellular domain of rCD2 becomes highly glycosylated during its transfer across the
endoplasmatic reticulum and the golgi before its reaches the cell surface, thus, rCD2Δ and the rCD2-PI 3-kinase chimeras exhibit a lower electrophoretic mobility than predicted from their primary amino acid sequence. The rCD2p85 chimera was also detected with the U5 mAb reactive with the bcr domain of p85α (Fig. 5.3A, right panel). The rCD2p85 chimera was highly overexpressed when compared to the protein levels of endogenous p85α which were also detected with p85α mAb. In parallel to the Western blot analysis, cell surface expression of the different rCD2-PI 3-kinase chimeras was

![Figure 5.3A. Expression of rCD2-PI 3-kinase chimeras in Cos7 cells. Cos7 cells were transiently transfected with 10 μg of either rCD2Δ, rCD2p85, rCD2p110, rCD2p110-R/P, rCD2p110-A126, or rCD2p110-R/P-A126 expression vectors (pcDNA3) as indicated and seeded onto two 10 cm dishes. After 48 h cells were lysed, 1/4 of the cell lysates was separated by 8% SDS-PAGE (and Western blot analysis was performed using rCD2 (Ox34) mAb (left panel) or p85α (U5) mAb (right panel). The migration of the molecular mass standards is indicated to the left in kilodaltons.]
Figure 5.3B. Flow cytometry analysis of Cos7 cells expressing rCD2Δ, rCD2p85, rCD2p110, rCD2p110-R/P, rCD2p110-Δ126, and rCD2p110-R/P-Δ126. Cos7 cells from Fig. 5.3A were stained with rCD2 mAb followed by fluorescin-conjugated anti-mouse Ab (black line). As control, each population of transfected cells was incubated with mouse IgG as primary Ab (dotted line).

Figure 5.3C. Time course of rCD2p110 expression in Cos7 cells. Cos7 cells were transfected with the amounts of pcDNA3-rCD2p110 plasmids as indicated, cells were harvested after the times indicated and protein expression was determined by Western blot analysis with rCD2 mAb. The migration of the molecular mass standards is indicated to the left in kilodaltons.
Figure 5.3D. Expression of rCD2-PI 3-kinase chimeras in NIH 3T3 and 293 cells. Western blot analysis with rCD2 mAb of cell lysates from NIH 3T3 cells (left panel) or 293 cells (right panel) transiently transfected with rCD2-PI 3-kinase chimeras as indicated using a Ca\textsubscript{2+}PO\textsubscript{4}\textsuperscript{-} based method. Amounts of DNA used in this experiment were 1 µg per 6 cm dish for the NIH 3T3 cells and 10 µg per 10 cm dish for the 293 cells. NIH 3T3 cells: Cells were harvested after 50 h, and 1/2 of the cell lysates was separated by 10% SDS-PAGE. 293 cells: Cells were harvested after 40 h, and 1/10 of the cell lysates were subjected to SDS-PAGE in a 7.5% polyacrylamide gel. The migration of the molecular mass standards is indicated to the left in kilodaltons.
demonstrated by flow cytometric immunofluorescence (FACS) analysis with rCD2 mAb (Fig. 5.3B). Before the analysis, cells were double-stained with propidium iodide to label dead cells. In the examined cell populations about 90% of the cells were viable and of these cells 5% to 12% expressed rCD2-PI 3-kinase chimeras on the surface. The protein and the surface expression of the 'kinase active' versus the 'kinase dead' rCD2-PI 3-kinase chimeras appeared to be equal; hence, (potential) kinase activity did not seem to change expression levels. Expression of the rCD2p110 protein in Cos7 cells was rapid and maximum protein levels occurred about two to three days after transfection when the cells had reached confluence (Fig. 5.3C). In addition, rCD2-PI 3-kinase chimeras were transfected using a Ca\textsubscript{2+}P\textsubscript{0.4}-based method (from pcDNA3 vectors) in mouse NIH 3T3 (Fig. 5.3D, left panel) and human 293 cells (Fig. 5.3D, right panel); the resulting rCD2-PI 3-kinase fusion proteins were detected by Western blot analysis with rCD2 mAb.

To obtain a first impression of the overall distribution of the rCD2-PI 3-kinase chimeras in cells, Cos7 cells transfected with rCD2-PI 3-kinase chimeras were stained with rCD2 mAb and examined by immunofluorescence microscopy (Fig. 5.4, C-M). In addition, cytoplasmic p85\textalpha (Fig. 5.4A) and a deletion mutant lacking aa 478-513 of p85\textalpha, p85\textDelta, which abrogates p110 binding to p85\textalpha (Fig. 5.4B) (Dhand et al., 1994) were transfected into Cos7 cells and their cellular expression determined by staining with p85\textalpha mAb (U5) (endogenous simian p85\textalpha in untransfected cells could not be detected in these stainings). The p85\textalpha and the p85\textDelta proteins appeared to be mainly expressed in the cytoplasm and at the cell membrane.

Figure 5.4. Distribution of p85\textalpha of PI 3-kinase, p85\textDelta, rCD2\textalpha, or rCD2-PI 3-kinase chimeras in Cos7 cells overexpressing these molecules. Cos7 cells were transiently transfected with (A) p85\textalpha, (B) p85\textDelta, (C) rCD2\textDelta, (D, E) rCD2p85, (F, G, H) rCD2p110, (J) rCD2p110-R/P, (K, L) rCD2p110-\textDelta126 and (M) rCD2p110-R/P-\textDelta126 (from pSR\textalpha (A, B) or pcDNA3 vectors (C-M)) and seeded onto coverslips. Fifty hours after transfection cells were stained as described in Materials and methods with p85\textalpha mAb (A, B) or rCD2 mAb (C-M) followed by FITC-conjugated anti-mouse IgG and analysed by fluorescence microscopy.
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Images showing the translocation of transfected cells. The constructs were transfected into cells and observed under a fluorescent microscope.
in the cytoplasm, whereas the rCD2-PI 3-kinase chimeras could be detected at various stages along their presumptive route of transfer from the endoplasmatic reticulum through the golgi, via transport vesicles to the plasma membrane. It therefore appeared that the rCD2 fusion proteins, similar to wild-type rCD2, were post-translationally modified and transported to the plasma membrane (no specific analysis of the subcellular locations was performed). To investigate by immunofluorescence microscopy whether a protein is expressed at the plasma membrane would require analysis using a confocal microscope; however, such an analysis was not necessary, as it had been shown before by FACS analysis (Fig. 5.3B) that the rCD2-PI 3-kinase chimeras are expressed on the cell surface. (In general it is not possible to assess with ordinary immunofluorescence microscopy analysis whether a protein is expressed at the plasma membrane as fibroblasts spread out their membrane and hence not many 'membrane layers' rest on top of each other. Exceptions are cells with a 'square' topology such as MDCK cells, in which many membrane layers overlay and thus the staining signal becomes strong enough to be visualised by immunofluorescence microscopy.)

As the p110 catalytic subunit of PI 3-kinase exists in a tight complex with its p85 regulatory subunit, the ability of p85 to associate with rCD2 PI 3-kinase chimeras was investigated. Cos7 cells were transfected with rCD2-PI 3-kinase chimeras without or with co-expression of p85α as indicated in Fig. 5.5. Cell lysates were subjected to immunoprecipitation with rCD2 mAb, and proteins present in the immune complexes were visualised by Western blot analysis with rCD2 mAb (upper panel) or p85α mAb (lower panel). The rCD2 mAb precipitated the respective rCD2-PI 3-kinase chimeras (Note: Owing to a not correctly measured DNA concentration, the expression level of rCD2p110-R/P was lower in this experiment). Interestingly, the p85α regulatory subunit was only detected in a complex with rCD2p110 and rCD2p110-R/P when p85α was overexpressed. The rCD2-PI 3-kinase constructs which lack the N-terminal 126 aa, rCD2p110-Δ126 and rCD2p110-R/P-Δ126, did not co-purify p85α which confirms
Figure 5.5. The rCD2p110 and rCD2p110-R/P but not the rCD2p110-Δ126 or rCD2p110-R/P-Δ126 fusion proteins associate with the p85α subunit when overexpressed in Cos7 cells. Western blot analysis with rCD2 mAb (upper panel) or p85α mAb (lower panel) of anti-rCD2 immune complexes isolated from cell lysates of Cos7 cells transiently expressing rCD2-PI 3-kinase chimeras without or together with ectopically expressed bovine p85α as indicated. Cells were transiently transfected with 10 μg of plasmid DNA, except 5 μg of pcDNA3-rCD2p85 or 2.5 μg of pSRα-p85α and lysed 48 h after transfection. Equal amounts of total cellular proteins were subjected to immunoprecipitation with rCD2 mAb and proteins present in immune complexes were separated by 8% SDS-PAGE. The data are from a representative experiment. Similar results were obtained in two further experiments. The migration of the molecular mass standards is indicated to the left in kilodaltons.

Note: Owing to a not correctly measured DNA concentration, the expression level of rCD2p110-R/P was lower in this experiment.

that this regions is required for p85 binding. It has been reported previously that wild-type p110 when expressed in Cos1 cells cannot co-purify endogenous simian p85 in anti-p110 immune complexes (Hiles et al., 1992). Thus, it is possible that ectopically expressed p110, rCD2p110 or rCD2p110-R/P cannot form or do not exist in a complex with endogenous p85 in intact Cos cells. Another possibility is, that there is very little
endogenous p85 present in Cos cells and only a few p110/rCD2p110 molecules find a p85 binding partner. The problem is then one of sensitivity and limited by the affinity of the p85α mAb in a Western blot analysis. Alternatively, endogenous p85/p110 complexes exhibit a higher affinity as ectopically expressed p110/rCD2p110 plus endogenous p85 and hence endogenous associations cannot easily be disrupted. Transfection of pSRα-p85α usually results in a significant elevation of p85α over endogenous protein levels. Thus, rCD2p110/rCD2p110-R/P complex formation with ectopically expressed p85α subunits can occur in cells co-expressing p85 as now free p85 proteins are present, and the rCD2p110/rCD2p110-R/P : p85 ratio in these cells is approximately 1:1. However, it cannot be ruled out that such complex formation only takes place post-lysis. Reciprocal experiments in which pcDNA3-rCD2p85 is transfected into Cos cells without or together with pcDNA3-p110 were not possible at the time, due to the lack of efficient p110 antisera for Western blot analysis. Furthermore, when I attempted to perform (co)-expression studies of myc-tagged wild-type p110 (see Fig. 5.1) 1. alone or 2. together with rCD2p85, I could not detect 1. sufficient expression of Myc-tagged p110 with Myc mAb (9E10) and 2. co-expression of Myc-tagged p110 with rCD2p85 drastically lowered the expression levels of rCD2p85. Point No.1 can partially be explained by the fact that the Myc-tag on my p110/rCD2-p110 fusion proteins could not be detected in Western blot analysis with Myc mAb (9E10-immunoprecipitation followed by lipid kinase assays does work moderately), the main issue, however, appears to be that (Myc-tagged) wild-type p110 cannot be expressed from CMV-based vectors but expresses well when cloned into a pMT2 or pSG5 vector (personnel communication, Pablo Rodriguez Viciana, ICRF). Point No.2 is somewhat dependent on point No.1, nevertheless, it is possible as suggested previously (Kodaki et al., 1994) that high levels of free wild-type p110 is toxic for some cells; additionally, free p110 is a very instable protein. (Toxicity did not seem to be a major problem for rCD2p110 expression).
Expression studies of rCD2-PI 3-kinase chimeras in T cells

As I anticipated to perform most of the functional studies in T cells, I analysed expression of rCD2-PI 3-kinase fusion proteins in a subclone of the human leukaemic cell line Jurkat, JHTAg, which expresses SV40 large T antigen. The first experiments with rCD2-PI 3-kinase constructs in pcDNA3 expression plasmids were frustrated by fairly low expression levels of the fusion proteins in these cells. Therefore, I subcloned the rCD2-PI 3-kinase chimeras in pRK5 or pEF expression plasmids (see above) and transfected them into JHTAg cells by electroporation. The presence of the rCD2-PI 3-kinase fusion proteins (expressed from pRK5 vectors) was assessed by Western blot.

Figure 5.6A. Expression of rCD2-PI 3-kinase chimeras in Jurkat T cells. JHTAg cells were transiently transfected with 30 μg of either rCD2Δ, rCD2p85, rCD2p110, rCD2p110-R/P, rCD2p110-Δ126, or rCD2p110-R/P-Δ126 expression vectors (pRK5). After 14 h cells were lysed, proteins (3/5 of total) were separated by 8% SDS-PAGE and Western blot analysis was performed using rCD2 (Ox34) mAb (left panel) or p85α (US) mAb (right panel). The migration of the molecular mass standards is indicated to the left in kilodaltons.
Figure 5.6B. Flow cytometry analysis of JHTAg cells expressing rCD2Δ, rCD2p85, rCD2p110, rCD2p110-R/P, rCD2p110-Δ126, and rCD2p110-R/P-Δ126 (from pRK5 vectors). JHTAg cells from Fig. 5.6A were stained with rCD2 mAb followed by fluorescin-conjugated anti-mouse Ab (black line). As control, each population of transfected cells was incubated with mouse IgG as primary Ab (dotted line).

Analysis with rCD2 or p85α mAbs (Fig. 5.6A). The rCD2-PI 3-kinase chimeras in JHTAg cells showed similar relative expression levels as in Cos7 cells (Fig. 5.6A versus Fig. 5.3A). In parallel, surface expression of rCD2-PI 3-kinase chimeras was confirmed by flow cytometry analysis with rCD2 mAb (Fig. 5.6B). Before the analysis, cells were double-stained with propidium iodide to label dead cells. About 25% to 30% of the total viable cell population showed surface expression of the rCD2 constructs. In general, about 50% to 70% of the total cell population were dead cells as determined by
double-staining with propidium iodide. A time course of the expression of the rCD2p85 chimera (in pcDNA3) in JHTAg cells showed maximum expression between 8h and 20h as assessed by Western blot analysis in Fig. 5.6C. The first molecules were expressed 2h to 4h after transfection.

![Western blot analysis of rCD2p85 expression](image)

**Figure 5.6C.** Time course of rCD2p85 expression in Jurkat cells. JHTAg cells were transfected with 30 µg per 1.8 x 10^7 cells of pcDNA3-rCD2p85 plasmid, cells were harvested (1.5 x 10^7) after the times indicated and protein expression was determined by Western blot analysis with rCD2 mAb (upper panel) or p85α mAb (lower panel). The migration of the molecular mass standards is indicated to the left in kilodaltons.

Further, I tested the expression of rCD2 (full length), rCD2p85, rCD2p110, and rCD2p110-R/P in JHTAg cells from pEF vectors. The rCD2-PI 3-kinase chimeras encoded in pEF vector showed similar expression levels as the ones in pRK5 vector as demonstrated in the Western blot analyses with rCD2 mAb (Fig. 5.6D versus Fig. 5.6A).

In the parallel flow cytometry analysis (Fig. 5.6E), to examine surface expression of the rCD2 fusion constructs, 20% to 25% of the live cells expressed the rCD2-PI 3-kinase chimeras. In subsequent functional experiments, I also used the Jurkat subclone
Figure 5.6D. Western blot analysis with rCD2 mAb of JHTAg cells expressing rCD2 PI 3-kinase chimeras. JHTAg cells were transiently transfected with 30 µg of pEF vectors encoding full length rCD2, rCD2p85, rCD2p110 or rCD2p110-R/P as indicated.

Figure 5.6E. Flow cytometry analysis of JHTAg cells expressing rCD2, rCD2p85, rCD2p110 and rCD2p110-R/P (from pEF vectors). JHTAg cells from Fig. 5.6D were stained with rCD2 mAb followed by fluorescin-conjugated anti-mouse Ab (black line). As control, each population of transfected cells was incubated with mouse IgG as primary Ab (dotted line).
Figure 5.6F. Surface expression analysis of rCD2-PI 3-kinase chimeras in JKHM1 Jurkat cells using flow cytometry. JKHM1 cells were transiently transfected with 30 μg of plasmid DNA as indicated and stained with rCD2 mAb followed by fluorescin-conjugated anti-mouse Ab (black line). As control, each population of transfected cells was incubated with mouse IgG as primary Ab (dotted line).
JKHM1. Therefore, I analysed expression of the rCD2-PI 3-kinase chimeras in these cells as well. As shown in Fig. 5.6F, all rCD2-PI 3-kinase chimeras expressed from pEF or pRK5 vectors were detected on the cell surface and demonstrated similar levels of expression as seen in JHTAg cells (compare Fig. 5.6F to Fig. 5.6B, E).

The human leukaemic T cell line Kit225 express IL-2R on the surface (but no TCR) and are dependent on IL-2 for growth. Kit225 cells were transfected with rCD2-PI 3-kinase chimeras in pEF or pRK5 plasmids and expression of the resulting rCD2 fusion proteins was detected by Western blot analysis (Fig. 5.7A) or in parallel by FACS.

Figure 5.7A. Expression of rCD2-PI 3-kinase chimeras in Kit225 T cells. Western blot analysis with rCD2 mAb of Kit225 cells expressing rCD2 PI 3-kinase chimeras as indicated. Kit225 cells were transiently transfected with 30 µg of pEF or pRK5 vectors encoding rCD2 molecules of rCD2-PI 3-kinase fusion proteins. The migration of the molecular mass standards is indicated to the left in kilodaltons.
Figure 5.7B. FACS analysis of rCD2-PI 3-kinase chimeras in Kit225 T cells. Kit225 cells from Fig. 5.7A were stained with rCD2 mAb followed by fluorescin-conjugated anti-mouse Ab (black line). As control, each population of transfected cells was incubated with mouse IgG as primary Ab (dotted line).
analysis (Fig. 5.7B) with rCD2 mAb. Expression levels in Kit225 cells were somewhat lower as in Jurkat cells and 5% to 10% (pEF) or 9% to 17% (pRK5) of the live cells exposed rCD2-PI 3-kinase chimeras on the cell surface.

**rCD2p110 and rCD2p85 associate with *in vitro* protein kinase activity**

PI 3-kinase is a dual specificity enzyme which contains both an intrinsic protein-serine/threonine and (phospho)inositide kinase activity (Dhand et al., 1994; Hunter, 1995) which may regulate its biological activity in T cells (Reif, 1992; Reif et al., 1993). This protein kinase activity can be detected in *in vitro* protein kinase assays (Dhand et al., 1994; Reif, 1992): the p85 regulatory subunit serves as a substrate for the p110 catalytic subunit (the major phosphorylation site being serine residue 608 of p85α) and p110 also autophosphorylates (weakly in fibroblasts and more pronounced in Jurkat cells, my unpublished observations). To determine whether rCD2p110 exhibits protein kinase activity, rCD2p110 was expressed in Jurkat cells without or together with 'its substrate' p85. In addition, rCD2p85 was expressed alone or in combination with rCD2p110 to assess whether it associates with and is phosphorylated by endogenous p110, or whether it serves as a substrate for rCD2p110. As shown in Fig. 5.8A (*lane 2* and also *lane 7*), in anti-rCD2 immunoprecipitates, rCD2p85 associates with a protein kinase that can phosphorylate a 120 kDa protein which co-migrates with rCD2p85. The protein kinase present in anti-rCD2 immunocomplexes co-purified with rCD2p85 under medium stringency solubilisation conditions and hence is likely to be endogenous p110 of PI 3-kinase. When rCD2p110 was expressed in cells and anti-rCD2 immune complexes were analysed for the presence of protein kinase activity, a 85 kDa phosphoprotein was detected (Fig. 5.8, *lane 3*) that co-migrates with endogenous p85 of PI 3-kinase in anti-p85α immunoprecipitates (Fig. 5.8A, *lane 9*). Such an 85 kDa band, in much higher levels, was present in anti-rCD2 immune complexes when p85α was overexpressed together with rCD2p110 (Fig. 5.8, *lane 4*). In the latter immunoprecipitate, an additional 145 kDa band can be visualised which co-migrates with
CD2p110, a similar band also being present at a very low level in the CD2p110 containing precipitates in Fig. 5.8A, lane 3. The variance in the level of this 145 kDa phosphoprotein in lane 3 versus lane 4 of Fig. 5.8A can be explained by the much lower expression level of CD2p110 in lane 3 versus lane 4 in this particular experiment as assessed in parallel by Western blot analysis with CD2 mAb (Fig. 5.8B). The 145 kDa phosphoprotein which co-migrates

Figure 5.8. Immune complex protein kinase assays. JHTAg Jurkat cells were transiently transfected with 30 μg of empty vector, CD2Δ, p85α, and/or CD2-PI 3-kinase chimeras in pcDNA3 vectors (except pSRα-p85α) as indicated and harvested 19 h after transfection. Cells were solubilised in 1% NP40 lysis buffer and cell lysates (1.4 x 10^9 live cell equivalents) were subjected to immunoprecipitation with CD2 mAb or p85α mAb as indicated. Before washing, the immune complexes were pelleted by centrifugation. The supernatant were transferred into a separate tube, concentrated as described in Materials and methods and analysed by Western blotting (see 5.8B).

(A) Immune complexes were washed extensively and protein kinase assays were performed as described in Materials and methods except that 10 μM ATP were used. Proteins were separated by 10% SDS-PAGE and visualised by autoradiography.

(B) Western blot analysis of supernatants from anti-CD2/anti-p85α immunoprecipitates (see above) using CD2 mAb (upper panel) or p85α mAb (lower panel). Proteins were separated by 10% SDS-PAGE.

The data are from a representative experiment. Similar results were obtained in one further experiment. The migration of the molecular mass standards is indicated to the left in kilodaltons.
with rCD2p110 can also be detected in anti-rCD2 immune complexes prepared from cells which overexpress rCD2p110 and rCD2p85 (Fig. 5.8, lane 6). Thus, the 145 kDa band ascribes to (auto)-phosphorylated rCD2p110. Anti-rCD2 immunoprecipitates from cell lysates which contained overexpressed rCD2p110 plus rCD2p85 also purified a 120 kDa protein which was phosphorylated in vitro and co-migrates with rCD2p85. When 'kinase dead' rCD2p110-R/P was expressed in Jurkat cells together with p85, the 145 kDa band and also the 85 kDa were not phosphorylated in in vitro protein kinase assays performed on anti-rCD2 immune complexes (Fig. 5.8A, lane 5). This was not a problem of expression as rCD2p110-R/P expressed to a similar level as rCD2p110,
determined in parallel by Western blotting with rCD2 mAb (Fig. 5.8 B). Moreover, rCD2p110-R/P can associate with p85 in anti-rCD2 immune complexes (see Fig. 5.5, data not shown). This suggests that the protein kinase activity which phosphorylates these proteins resides in rCD2p110.

From these data, it appears that endogenous p85, co-expressed p85α of PI 3-kinase, or rCD2p85 associate with rCD2p110 and become phosphorylated by rCD2p110 protein kinase activity. Furthermore, rCD2p85 can associate with and can be phosphorylated by endogenous protein kinase activity, most likely p110 of PI 3-kinase (Fig. 5.8, lane 2 and also lane 7). It had been noted before in the Western blot analysis in Fig. 5.5 that rCD2p110 cannot co-purify endogenous p85 in Cos7 cells whereas in this experiment in Jurkat cells rCD2p110 appeared to phosphorylate endogenous p85 of PI 3-kinase. It is possible that there is more endogenous p85 present in Jurkat cells than in Cos7 cells. Alternatively, and more likely, the co-purification/association of p85 with rCD2p110 can be more easily visualised in in vitro protein kinase assays than by Western blot analysis with p85 mAb as the p85 is a very good in vitro kinase substrate for p110/rCD2p110. Vice versa endogenous protein kinase activity which phosphorylated rCD2p85 in vitro and which may stem from endogenous p110 of PI 3-kinase, did precipitate with anti-rCD2 immune complexes from cell lysates of cells overexpressing rCD2p85. These data suggest that endogenous p85 binds rCD2p110 (and also rCD2p110-R/P) and vice versa endogenous p110 interacts with rCD2p85, however, these associations are of very low level. A more detailed analysis of the association levels of endogenous proteins with rCD2-PI 3-kinase chimeras and/or a comparison of binding properties in Jurkat versus Cos7 cells was not carried out. Nevertheless, from my observations it did not appear that there was any difference in the biochemical properties of rCD2-PI 3-kinase chimeras when expressed in Jurkat cells versus Cos7 cells. In addition, co-expression of p85 did not influence the specific in
vitro protein kinase or PI-lipid kinase (see below) activity of p110, except may be that rCD2p110 generally expressed to a slightly higher level when p85 was co-expressed.

Studies on the in vitro lipid kinase activity of rCD2-PI 3-kinase chimeras

To assess whether rCD2-PI 3-kinase chimeras exhibit lipid kinase activity, Cos7 cells were transfected with rCD2-PI 3-kinase expression vectors and the resulting rCD2-fusion proteins were immunoprecipitated with rCD2 mAb and subjected to PI kinase assays in vitro. rCD2p110 had strong PI 3-kinase activity in vitro, which was dependent on the integrity of the p110 ATP-binding site - there is no detectable lipid kinase activity in comparable immunocomplexes of rCD2p110-R/P (Fig. 5.9A, B). Both proteins, rCD2p110 and rCD2p110-R/P, were present in the anti-rCD2 immune complexes to a similar level as determined by Western blot analysis with rCD2 mAb and p110α antisera (Fig. 5.9C). These results and the ones in Fig. 5.8 show that the fusion of p110 to the rCD2 extracellular and transmembrane domains does not adversely effect the folding of the protein and thus destroy the catalytic activity of the enzyme. There was also low level PI kinase activity associated with the rCD2p85 chimera. Western blot analysis confirmed that this reflected a low level association of rCD2p85 with the endogenous p110α catalytic subunit of PI 3-kinase (Fig. 5.9C, middle panel). Finally, rCD2p110-Δ126 exhibited a very moderate PI 3-kinase activity. As

Figure 5.9. PI kinase activity present in anti-rCD2 immunoprecipitates. Cos7 cells were transiently transfected with 10 μg of either empty vector, rCD2Δ, rCD2p85, rCD2p110, rCD2p110-Δ126, rCD2p110-R/P, or rCD2p110-R/P-Δ126 in pcDNA3 expression vectors as indicated. Cell lysates were subjected to immunoprecipitation with rCD2 mAb, immune complexes were washed and split into two each with the final wash. Immune complexes were subjected to PI kinase assays (A) or Western blot analysis (C). (A) Autoradiograph of the TLC plate from the PI kinase assay. (B) Quantification of [32P]phosphate incorporation into PI from (A) using a PhosphorImager. [32P]phosphate incorporation into PI is expressed as fmol of [32P]P~P per minute. (C) Western blot analysis using rCD2 mAb (top panel), p110 antisera (middle panel) or p85α mAb (bottom panel) of rCD2-PI 3-kinase chimeras present in anti-rCD2 immunoprecipitates assessed in parallel. Proteins were separated by 8% SDS-PAGE. The migration of the molecular mass standards is indicated to the left in kilodaltons. The data are from a representative experiment. Similar results were obtained in two further experiments.
Chapter 5

Results: rCD2-PI 3-kinase chimeras

A

B

\[ \text{PIP} \]

\[ \text{ORI} \]

\[ \text{empty} \quad \text{rCD2}\Delta \quad \text{rCD2p85} \quad \text{rCD2p110} \quad \text{rCD2p110-D126} \quad \text{rCD2p110-R/P} \quad \text{rCD2p110-R/P-D126} \]

\[ \text{PI kinase activity, PIP (nmol/min)} \]

\[ \text{empty} \quad \text{rCD2p85} \quad \text{rCD2p110} \quad \text{rCD2p110-D126} \quad \text{rCD2p110-R/P} \quad \text{rCD2p110-R/P-D126} \]
Results: \( rCD2-PI \) 3-kinase chimeras

To explore the role of \( rCD2p110 \) in D-3 phosphoinositide metabolism and cellular levels of D-3 phosphoinositides, Cos-7 cells were transiently transfected with \( rCD2p110 \) from pSE299, followed by metabolically labelled lipids extracted. The Cos-7 cells were then fractionated using high-pressure liquid chromatography (HPLC) and the data in Fig. 5.1A shows that cells transfected with \( rCD2p110 \) from pSE299 have very high levels of phosphatidylinositol 3,4,5-trisphosphate (IP₃). The results presented in Fig. 5.10 illustrate the ability of \( rCD2p110 \) from pSE299 to induce these membrane changes.
this 126 aa N-terminal region of p110, the p85 binding site, appears to be required for optimal kinase activity (rCD2p110-R/P-Δ126 did not exert any kinase activity). However, as suggested previously (Klippel et al., 1994), PI 3-kinase activity was not completely abolished by deletion of this region.

It had been suggested that co-expression of p85 with p110 raises the specific \textit{in vitro} lipid kinase activity of p110 (PI used as substrate) (Klippel et al., 1994). I did not observe such an increase in PI lipid kinase activity when p85 was co-expressed with rCD2p110 (data not shown, see also above). This is consistent with results from others who only could observe a higher specific activity of p110 after co-expression of p85 when PI(4,5)P$_2$ was used as a substrate but not PI (Woscholski et al., 1994). Attempts to compare the specific activity of cytosolic p110 to the one of rCD2p110 failed as p110 could not be expressed to sufficiently high levels (see above).

\textbf{rCD2p110 is a constitutively active enzyme as it raises intracellular levels of D-3 phosphoinositides}

To explore the effect of membrane recruitment of the PI 3-kinase subunits on cellular levels of D-3 phosphorylated inositol lipids in intact cells, Cos7 cells were transiently transfected with the different rCD2-PI 3-kinase chimeras. Cos7 cells were metabolically labelled with $[^{32}\text{P}]$orthophosphate and phospholipids extracted. The levels of D-3 phosphoinositides were determined by analysing the headgroup structures of $^{32}\text{P}$-labelled inositol phospholipids using High-Pressure Liquid Chromatography (HPLC) in the presence of relevant internal standards (Fig. 5.10). The data in Fig. 5.10A show the elution profile of control Cos7 cells or Cos7 cells transfected with rCD2p110 from three independent experiments. The corresponding PI(4,5)P$_2$, PI(3,4)P$_2$ and PI(3,4,5)P$_3$ peaks from these profiles were quantitated and so were the ones from these experiments of which the elution profile was not depicted in Fig. 5.10A (Fig. 5.10B). Control Cos7 cells, transfected with empty vector or the truncated rCD2Δ
molecule, showed low D-3 phosphoinositides levels. (In some experiments Cos7 cells expressing rCD2Δ exhibited slight fluctuations in D-3 inositol phospholipids which may be due to the very high expression levels of this protein.) Strikingly, in each experiment expression of rCD2p110 caused a potent increase in PI(3,4)P₂ and PI(3,4,5)P₃ levels as compared to basal levels in control cells. When the data were normalised to the PI(4,5)P₂ levels in each separation, the fold inductions for rCD2p110 in the three independent experiments were for PI(3,4)P₂: 4.2-, 4.4-, 14.5-fold and for PI(3,4,5)P₃: 5.8-, 8.6-, 3.4-fold as compared to basal levels in control cells. (When the data was not normalised to the PI(4,5)P₂ levels in each experiment and were calculated directly from the raw data as presented in Fig. 5.10, the fold increases were for rCD2p110 over basal levels: 2.4-, 4.7-, 9.9-fold for PI(3,4)P₂ and 3.3-, 9.3-, 2.3-fold for PI(3,4,5)P₃.) The stimulatory effects of rCD2p110 were due to the catalytic activity of the membrane-bound p110 protein as expression of equivalent levels of the point mutated rCD2p110-R/P, the 'kinase dead' version of rCD2p110, did not significantly alter levels of PI(3,4)P₂ and PI(3,4,5)P₃. Similarly, expression of rCD2p85 did not elevate cellular levels of

**Figure 5.10.** Elevation of PI(3,4)P₂ and PI(3,4,5)P₃ levels in intact Cos7 cells by expression of rCD2p110. Cos7 cells were transfected in three independent experiments with 5 µg plasmid DNA of the indicated constructs (in pcDNA3), labelled with [³²P]orthophosphate and levels of PI(3,4)P₂ and PI(3,4,5)P₃ were determined. 

**(A)** Anion-exchange HPLC separation of the deacylation products derived from lipid extracts of either control cells (empty) or cells transfected with rCD2p110. The left hand graph shows the entire Gro PI(4,5)P₂ peak. The right hand graph is the same profile on a 100-times larger scale; the black arrowhead indicates the deacylated PI(3,4)P₂ peak and the white arrowhead the deacylated PI(3,4,5)P₃ peak. The PI(3,4,5)P₃ peak was identified by comparison with the elution time of the internal ^H-labelled l(1,3,4,5)P₄ peak which elutes 1.5 min after the respective PI(3,4,5)P₃ peak. In some separations deacylated ^H-labelled PI(4,5)P₂ was also included as an internal standard. The identity of the PI(3,4,5)P₃ peak and PI(4,5)P₂ peak was deduced from the elution profile of the respective ^H- or ³²P-labelled standards during a standard separation on the same column.

**(B)** Quantitation of the raw PI(4,5)P₂, PI(3,4)P₂ and PI(3,4,5)P₃ peaks shown in (A) and also of the ones of which the elution profile was not shown in (A) from these experiments. Data were normalised to the ³²P-counts loaded onto the column for each separation. The Western blot analysis using rCD2 mAb was performed in parallel to Experiment 1 to check expression of rCD2-PI 3-kinase chimeras. This analysis only determines qualitatively whether the rCD2-fusion proteins were expressed but does not aim to quantify the exact expression levels. Similar Western blot analyses were carried out for the other experiments (data not shown).
Chapter 5

Results: rCD2-PI 3-kinase chimeras

**Experiment 1**

![Graph showing experiment 1 results](image1)

**Experiment 2**

![Graph showing experiment 2 results](image2)

**Experiment 3**

![Graph showing experiment 3 results](image3)
Results: rCD2-PI 3-kinase chimeras

Experiment 1

[32P]l(4,5)l(6)l (10^3 cpm)

Empty rCD2D rCD2p85 rCD2p110 rCD2p110-D126 rCD2p110-RP

[32P]l(3,4,5)l (10^3 cpm)

Empty rCD2D rCD2p85 rCD2p110 rCD2p110-D126 rCD2p110-RP

[32P]l(0,4,5)l(6)l (10^3 cpm)

Empty rCD2D rCD2p85 rCD2p110 rCD2p110-D126 rCD2p110-RP
Chapter 5 Results: rCD2-PI 3-kinase chimeras

Experiment 2

![Bar chart showing results for Experiment 2]

Experiment 3

![Bar chart showing results for Experiment 3]
PI(3,4)P₂ or PI(3,4,5)P₃ (Fig. 5.10B). The rCD2p110-Δ126 molecule exhibited very
moderate PI 3-kinase activity in in vitro protein kinase assays (see Fig. 5.9A, B).
Consistent with this notion, expression of rCD2p110-Δ126 did not elevate cellular levels
of D-3 inositol phospholipids.

In conclusion, plasma membrane-targeting of p110, the catalytic subunit of PI 3-
kinase, generates a mutant that is constitutively active in vivo, whereas membrane-
targeting of the p85 regulatory subunit has no effect on levels of D-3 phosphorylated
inositol lipids in vivo. Immunocomplexes of rCD2p85 contain PI 3-kinase activity in vitro
as this chimera forms an association with endogenous PI 3-kinase catalytic subunits
(Fig. 5.9). The level of activity in these complexes is extremely low in comparison to the
activity in immunocomplexes containing rCD2p110 explaining the failure of rCD2p85 (or
rCD2p110-Δ126) to regulate endogenous levels of D-3 phosphoinositides.

5.3 Discussion

A chimeric molecule in which the cytoplasmic domain of the rCD2 cell surface
antigen was replaced with the p110α subunit of PI 3-kinase allows plasma membrane
localisation of the catalytic subunit of PI 3-kinase creating rCD2p110. Membrane
placement of rCD2p110 induces accumulation of cellular levels of PI(3,4)P₂ and
PI(3,4,5)P₃: in cells transiently transfected with rCD2p110, there is an approximate 5-
fold increase (and at least an 2.5-fold increase) in cellular levels of PI(3,4,5)P₃. As
shown in Fig. 5.3B about 10% of the total population of transiently transfected Cos7
cells express the rCD2p110 chimeras. Normalised to the transfection efficiency, this
gives an increase in PI(3,4,5)P₃ levels in cells expressing rCD2p110 that is about 25-
to 50-fold which is well within the range described for growth factor induced elevations of
cellular levels of PI(3,4,5)P₃. Therefore, the elevation of PI(3,4)P₂ and PI(3,4,5)P₃ in
cells expressing rCD2p110 is similar to those seen after stimulation with mitogens and rCD2p110 acts as a constitutively active PI 3-kinase. The raise in cellular D-3 phosphoinositide levels in cells expressing rCD2p110 was due to the intrinsic catalytic activity of the rCD2p110 molecule, as expression of a mutated rCD2p110 control construct, which has a point mutation in the catalytic domain that abrogates ATP-binding, 'kinase dead' rCD2p110-R/P, does not lead to an increase in D-3 phosphoinositol lipids in intact cells. Moreover, localising at the plasma membrane a truncated version of p110 which lacks the 126 N-terminal amino acids, rCD2p110-Δ126, does not elevate cellular levels of D-3 phosphoinositides. The data from these analyses of the in vivo levels of PI(3,4)P$_2$ and PI(3,4,5)P$_3$ are consistent with the PI 3-kinase activity of rCD2p110-Δ126 detected in in vitro protein or PI lipid kinase assays: rCD2p110-Δ126 exhibits a very low kinase activity.

Surprisingly, targeting the p85α regulatory subunit of PI 3-kinase to the plasma membrane does not significantly regulate intracellular levels of D-3 inositol phospholipids. This is unexpected, as it was anticipated that rCD2p85 would recruit endogenous cytosolic p110 to the plasma membrane. This idea is based on the fact that p85 and p110 of PI 3-kinase form a tight complex. Indeed, rCD2p85 does associate with endogenous p110 as demonstrated in in vitro protein or PI 3-kinase assays. However, the levels of rCD2p85-associated PI 3-kinase activity/protein are very moderate and do not seem to be reflected by the levels of rCD2p85 protein present in the immune complexes or in cells expressing rCD2p85. Thus, it appears that rCD2p85 does not sufficiently disrupt endogenous p85-p110 complexes and hence the detected low association levels of endogenous p110 with rCD2p85. Such a low stoichiometry in binding to endogenous PI 3-kinase subunits is also observed in reciprocal experiments in which rCD2p110 in co-precipitation studies can only recruit modest levels of endogenous p85. However, the inability of rCD2p85 or rCD2p110 to complex at high levels endogenous p110 or p85, respectively, is not a structural issue:
when free p85 subunits are present after overexpression of the molecule, interactions between rCD2p110 and p85 can readily be observed.

The rCD2-PI 3-kinase chimeras express in every cell line tested to date including epithelial cells and T cells, using a range of different transfection methods. As rCD2p110 is a constitutively active enzyme, it is a useful tool for exploration of the cellular responses triggered by PI 3-kinase.
Chapter 6

Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways

6.1 Introduction

In the previous result chapter, I described the generation of a membrane-localised constitutively active PI 3-kinase, rCD2p110, of which the expression in cells leads to an increase in the \textit{in vivo} levels of D-3 phosphoinositides. I first wished to assess whether this activated form of PI 3-kinase would indeed be a good tool to investigate the contribution of PI 3-kinase to a certain effector pathway. PI 3-kinase has been implicated, by the use of inhibitors, in mediating a variety of cellular functions in response to growth factors such as PDGF or insulin. These include activation of p70S6k (Chung et al., 1994; Monfar et al., 1995) and the regulation of the actin cytoskeleton (Kotani et al., 1994; Nobes et al., 1995; Wennström et al., 1994).

Growth factors trigger membrane ruffling, and the subsequent formation of actin stress fibres, by a mechanism that is dependent on the function of the rho family GTPases Rac and Rho, respectively (Ridley et al., 1992). The expression of constitutively active forms of these proteins, V12Rac and V14Rho, can mimic the responses induced by PDGF: V12Rac induces membrane ruffling and focal complex formation and V14Rho induces actin stress fibres and focal adhesion assembly (Ridley and Hall, 1992; Ridley et al., 1992). The activation of PI 3-kinase is necessary for PDGF activation of Rac- and Rho-mediated rearrangements of the actin cytoskeleton (Nobes et al., 1995; Wennström et al., 1994); indeed, previous studies have suggested that PI 3-kinase may regulate GTP-loading of Rac (Hawkins et al., 1995) and hence,
regulate Rho (Nobes et al., 1995; Ridley et al., 1992). It is unclear, however, whether PI 3-kinase activation is sufficient to induce Rac- and Rho-mediated cytoskeletal rearrangements or whether PI 3-kinase is a single component of more complex activation processes.

Comprehensive studies have recently established an additional role for Rac and Rho in mediating transcriptional regulation of the c-fos serum response element (SRE) (Hill et al., 1995). The data concerning a role of PI 3-kinase in regulating SRE transcription has been controversial: some studies state that PI 3-kinase function is required for SRE induction (Hu et al., 1995; Yamauchi et al., 1993), whereas others could not define a role for PI 3-kinase in this response (Hill et al., 1995). Such discrepancies could be explained by the existence of parallel compensatory pathways. Information concerning the ability of active PI 3-kinase to generate positive signals that regulate Rac/Rho transcription factor responses would thus be of value. A key question is whether PI 3-kinase is a general stimulator of Rac and Rho function in cells.

Experiments with interfering approaches have provided evidence for a role of PI 3-kinase in p70S6k activation (Chung et al., 1994). p70S6k is a serine/threonine kinase, specific for ribosomal protein S6, which is required for cells to enter S phase after mitogen stimulation (Brown and Schreiber, 1996; Proud, 1996). However, studies using PDGF receptor mutants which fail to bind PI 3-kinase came to alternate conclusions whether PI 3-kinase activity is necessary for p70S6k regulation (Chung et al., 1994; Ming et al., 1994). The explanation for these discrepancies remains unclear, but one possibility is that p70S6k can be regulated by different effector pathways (Brown and Schreiber, 1996; Chou and Blenis, 1996; Downward, 1994; Proud, 1996).

In the present chapter I will investigate the ability of a constitutively active PI 3-kinase, rCD2p110 (see chapter 5), to initiate Rac/Rho-mediated cytoskeletal
rearrangements and Rac/Rho-mediated activation of transcription factor pathways. Further, as a functional control, I will assess whether PI 3-kinase signals are sufficient to regulate p70S6k and Erk activity.

6.2 Results

rCD2p110 activates p70S6k but not the Erks in 293 cells

One key regulator of cell proliferation is the serine/threonine kinase p70S6k (Chung et al., 1992; Lane et al., 1993) and PI 3-kinase has been implicated in activation of p70S6k (Chung et al., 1994; Weng et al., 1995). However, p70S6k can be controlled by diverse signalling pathways and it is not clear that recruitment of PI 3-kinase into a receptor complex is necessary for p70S6k activation (Chou and Blenis, 1996; Downward, 1994; Ming et al., 1994). It is thus unclear, whether membrane localisation of PI 3-kinase would be sufficient to induce p70S6k or whether PI 3-kinase derived signals are one component of more complex activation processes. To resolve this point and to assess the effects of active rCD2p110 on p70S6k activity, serum-starved 293 cells were co-transfected with HA-epitope tagged full length p70S6k and rCD2p110. (The regulation of p70S6k is especially well studied in the 293 cell system.) The data in Fig. 6.1A show that the activity of HA-p70S6k in control serum-deprived 293 cells is low but can be induced 10 (to 12) fold by serum. Co-transfection of rCD2p110 induced constitutive activation of p70S6k to the same extent as serum. p70S6k was not constitutively active in cells expressing inactive rCD2p110-R/P confirming that the activation of p70S6k requires the kinase activity of the p110 subunit. rCD2p85 failed to activate p70S6k which is consistent with its inability to induce accumulation of D-3 phosphoinositides. These data show that PI 3-kinase signals can be sufficient to give full activation of p70S6k. Expression of rCD2p110-R/P or rCD2p85 was noted, in some experiments, to suppress serum inducibility of p70S6k, indicating that these chimeras
Figure 6.1. rCD2p110 activates p70S6k but not p44 Erk1. HA-p70S6k (A) or HA-Erk1 (B) activity were analysed from extracts of untreated cells (-) or cells stimulated with either 10% FCS for 60 min (A) or 20% FCS for 5 min (B). Before stimulation, 293 cells were co-transfected in parallel with either HA-p70S6k (A) or HA-Erk1 (B) plasmids in the presence of either vector plasmid (control), or plasmids encoding for rCD2p85, rCD2p110, rCD2p110-R/P or Ha-v-ras, as indicated. The data are from a representative experiment. Similar results were obtained in three (A) or two (B) more experiments. [32P]phosphate incorporation into S6 (see inset) (A) or MBP (B) was quantified using a PhosphorImager and is expressed as arbitrary units. Serum stimulation induced Erk1 activity 5.7-fold over untreated cells in the control population (B).
may be inhibitory mutants of PI 3-kinase pathways. Hence, rCD2p85, similar to as it has been described for p85 or p85Δ, could associate with the activated receptor complex and, when overexpressed to sufficient high levels, could block endogenous p85-p110 binding to the receptor. This would prevent activation of p70S6k by the growth factor receptor due to the fact that most rCD2p85 proteins would not be in a complex with endogenous p110. *Vice versa* rCD2p110-R/P could associate with endogenous p85 (in low stoichiometry) and hence prevent endogenous p110 from binding to its p85 subunit.

Other important regulators of the mitogenic response in cells are the small GTPase Ras and its downstream effectors the Erk group of Map kinases. Previous studies have suggested a role for PI 3-kinase in regulating the small GTPase Ras and the Erks (Hu et al., 1995). To assess whether constitutively active PI 3-kinase could induce Ras/Erk signalling pathways in parallel with p70S6k, the effect of rCD2p110 on the Ras stimulated Map kinase Erk1 was analysed. Serum starved 293 cells were co-transfected with either rCD2p110 or Ha-v-ras expression vectors together with an expression vector encoding HA-epitope tagged p44 Erk1. Co-expression of rCD2p110 did not stimulate Erk1 activity and Erk1 could still be activated by serum in cells expressing active rCD2p110 (Fig. 6.1B).

From these experiments, it was concluded that PI 3-kinase signals are sufficient to activate p70S6k but not the Erks. The constitutively active PI 3-kinase, rCD2p110, appeared to function in a specific manner and therefore is a valuable tool to study PI 3-kinase effector pathways.
Expression of active PI 3-kinase leads to membrane ruffling and stress fibre formation in Swiss 3T3 cells

Cytoskeletal responses triggered by PDGF require PI 3-kinase activation (Nobes et al., 1995; Wennström et al., 1994). In collaboration with Kate Nobes and Alan Hall (CRC Oncogene and Signal Transduction Group, MRC Laboratory for Molecular Cell Biology, University College London, London), membrane-bound rCD2p110 was employed to determine whether PI 3-kinase signals were sufficient to trigger Rac/Rho cytoskeletal signalling pathways. Serum-starved subconfluent Swiss 3T3 cells were microinjected with an expression vector encoding the active PI 3-kinase rCD2p110, and the pattern of filamentous actin distribution was assessed by immunofluorescence in cells expressing rCD2p110 proteins (Fig. 6.2). Four hours after injection polymerised actin could be observed to form localised lamellipodia around the cell margin and, moreover, to form actin stress fibres (Fig. 6.2A). These results were confirmed by time-lapse image recording where sub-confluent cells expressing rCD2p110 showed active membrane ruffling (data not shown). Swiss 3T3 cells expressing rCD2p110 also exhibited a polarised cell shape (Fig. 6.3; 6.2A, 6.2B). To analyse these cytoskeletal rearrangements further, quiescent cells were stained for vinculin distribution, which monitors the formation of Rac-dependent focal complexes and Rho-dependent focal adhesions (Nobes and Hall, 1995). In cells expressing the active PI 3-kinase, vinculin could be found localised in discrete punctuate spots around the cell margin forming focal complexes and focal adhesions in the body of the cell (Fig. 6.2B). Control cells expressing the inactive rCD2p110-R/P chimera did not show these structures (Fig. 6.2C, D). As a comparison, Swiss 3T3 cells were also microinjected with V12Rac or V12Ras expression vectors or left uninjected (Fig. 6.4). The changes in cell morphology triggered by V12Rac (or also V12Ras, Fig. 6.4E) and rCD2p110 are qualitatively the same (Fig. 6.4: C, D versus 6.2A, B), although V12Rac induced the formation of vinculin spots uniformly around the entire periphery of the cell (Fig. 6.4D) whereas the effects of rCD2p110 were localised to discrete areas of the plasma.
Figure 6.2. Distribution of actin and vinculin in Swiss 3T3 cells expressing active and inactive PI 3-kinase chimeras.
Serum-starved sub-confluent Swiss 3T3 cells were microinjected with rCD2p110 (A, B) or rCD2p110-R/P (C, D) encoding plasmids. After 3-4 h cells were fixed. Actin (A, C) or vinculin (B, D) was visualised by immunofluorescence. Note: in (D) both cells shown were microinjected. Scale bar represents 11 μm.

membrane (Fig. 6.2B). These localised areas of vinculin along the cell margin were also observed in cells microinjected with V12Ras (Fig. 6.4F). This observation is in conjunction with previously reported data where microinjection of V12Ras can activate Rac/Rho signalling pathways (Ridley et al., 1992).
Figure 6.3  Swiss 3T3 cells expressing active PI 3-kinase, rCD2p110, exhibit a polarised cell shape. Serum-starved sub-confluent Swiss 3T3 cells were microinjected with rCD2p110. After 3-4h cells were fixed and permeabilised with saponin. rCD2p110 proteins were detected by staining with rCD2 mAb followed by FITC-conjugated goat anti-mouse secondary antibody and visualised by immunofluorescence. Both cells shown express rCD2p110.
Figure 6.4. Distribution of actin and vinculin in Swiss 3T3 cells expressing V12Rac and V12Ras.
Serum-starved sub-confluent Swiss 3T3 cells were microinjected with V12Rac (C, D), or V12Ras (E, F) encoding plasmids or left uninjected (A, B). After 3-4 h cells were fixed. Actin filaments (A, C, E) or vinculin (B, D, F) was visualised by immunofluorescence. Scale bar represents 11 μm.
PI 3-kinase induced cytoskeletal rearrangements are dependent on endogenous Rac and Rho function

Membrane ruffling and focal complex formation induced by rCD2p110 were similar to those described for activators of Rac, whereas the effects of rCD2p110 on actin stress fibres and focal adhesion assembly are identical to those described for activators of Rho. (Nobes and Hall, 1995; Ridley et al., 1992). To assess directly the role of Rac and Rho in PI 3-kinase-mediated cytoskeletal responses we used N17Rac, a dominant inhibitory mutant of Rac that prevents activation of endogenous Rac (Ridley et al., 1992). To inhibit endogenous Rho function, cells were microinjected with Clostridium botulinum C3 transferase which ADP-ribosylates and inactivates Rho (Ridley et al., 1992). The microinjection of N17Rac proteins abolished membrane ruffles and stress fibre formation in cells expressing active rCD2p110 (Fig. 6.5A, compare the right-hand cell with the left hand cell). Hence, cytoskeletal rearrangements triggered by active PI 3-kinase are dependent on the function of Rac proteins. Expression of C. botulinum C3 transferase which inactivates Rho (Ridley et al., 1992) inhibited rCD2p110-induced assembly of actin stress fibres but not the increase in filamentous actin forming membrane ruffles (Fig. 6.5B). Strikingly, in cells where Rho function was blocked, the active PI 3-kinase induced a pattern of vinculin staining indistinguishable from that seen in cells expressing activated V12Rac (compare Fig. 6.4D with 6.5C): focal complexes around the cell margin, but no vinculin aggregation indicative of Rho-mediated focal adhesions, could be detected (compare Fig. 6.4D with 6.2B). Collectively, these data show that PI 3-kinase can stimulate Rac-dependent pathways for formation of focal complexes and membrane ruffling and Rac/Rho pathways that induce stress fibre and focal adhesion assembly.
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Figure 6.5. N17Rac inhibits rCD2p110 expressing cells the accumulation of lamellipodia and stress fibres whereas C3 transferase prevents stress fibre and focal adhesion assembly but not lamellipodia formation. Serum-starved sub-confluent Swiss 3T3 cells were microinjected with rCD2p110 encoding plasmids. rCD2p110 microinjected cells were re-injected after 3h with recombinant N17Rac proteins (A, right hand cell) or C3 transferase (B, C) together with an IgG injection marker. Thirty minutes after the second microinjection cells were fixed. Actin (A, B) or vinculin (C) was visualised by immunofluorescence. Note: in (A) both cells shown were expressing rCD2p110, but only the right hand cell was co-injected with N17Rac proteins. Scale bar represents 11 μm.
rCD2p110 does not induce SRE-, c-fos-, or Elk-1-dependent gene expression

An additional function for the GTPases Rac and Rho has been demonstrated recently: to regulate the transcriptional activity of the c-fos Serum Response Factor (SRF) (Hill et al., 1995). I therefore examined whether PI 3-kinase is a universal regulator of Rac- and Rho-function by assessing the ability of constitutively active PI 3-kinase to induce changes in transcriptional activity of the c-fos serum response element (SRE). In these experiments, we investigated cellular responses to expression of constitutively active mutants of Rac and Rho, and to regulators of endogenous Rho signalling pathways: serum and Dbl (Cerione and Zheng, 1996), a guanine nucleotide exchange protein that regulates Rho family GTPases. SRE activity was monitored with a SRE-CAT (chloramphenicol acetyl transferase) reporter gene, which is known to be the target of a signalling cascade initiated by the GTPases Cdc42, Rac and Rho (Hill et al., 1995). Accordingly, expression of V14Rho or V12Rac stimulated the expression of the SRE-CAT reporter gene to a level equivalent to the response induced by serum (Fig. 6.6A). However, no SRE-CAT induction could be observed in cells expressing the constitutively active PI 3-kinase alone or in conjunction with suboptimal levels of serum. As well, expression of rCD2p110 alone did not stimulate c-fos promoter activity (Fig. 6.6C) nor could it synergise with serum for this response (data not shown). Expression of Dbl induced SRE activity (Fig. 6.6B). Moreover, SRE responses triggered by expression of Dbl or serum are prevented by co-expression of C3 transferase indicating that SRE regulation by these stimuli is mediated by endogenous Rho signalling pathways. Thus, transcriptional activation of the SRE can be regulated by signalling pathways initiated by serum or by the guanine nucleotide exchange protein Dbl and mediated by the GTPase Rho but these responses cannot be induced by a constitutively active PI 3-kinase.

Transcriptional activation from the SRE requires SRF binding. At the c-fos SRE, SRF forms a ternary complex with TCF (ternary complex factor), which cannot
bind the SRE by itself. One member of the TCF is Elk-1, that is a substrate for both Map kinase families, the Erks (Hill et al., 1995; Treisman, 1994) and the stress-activated protein (SAP) kinase (also known as c-jun N-terminal kinases; Sapks or Jnks) (Cavigelli et al., 1995; Gille et al., 1995; Whitmarsh et al., 1995). As Rac has been shown to be involved in regulating the Jnk/Sapk signalling pathway (Coso et al., 1995; Minden et al., 1995), I investigated whether activated rCD2p110 could induce Elk-1 activation. To monitor Elk-1 transcriptional activity a fusion protein comprising the C-terminus of Elk-1 linked to the LexA repressor (Marais et al., 1993) was co-transfected into NIH 3T3 cells with a LexA operator-controlled CAT reporter gene. LexA-Elk-1 transcriptional activity is low in quiescent fibroblasts but can be induced by co-expression of active Ha-v-ras, by stimulation with serum, or by UV irradiation (Fig. 6.6D). However, expression of rCD2p110 did not stimulate Elk-1 activity (Fig. 6.6D) indicating that a constitutively active PI 3-kinase cannot stimulate UV-activated stress kinases Jnk. This was confirmed by direct in vitro kinase assays of Jnk (data not shown, see (Marte et al., 1997)).

Expression of the Fos protein is required for the promoter activity of many genes. Fos is a component of the AP1 complex, and hence increased cellular levels of Fos proteins can be monitored by assessing whether the activity of the transcription factor AP1 becomes upregulated. The AP1 complex also contains the transcription factors c-Jun and ATF2, and is a target for members of both Map kinase families, Jnks

**Figure 6.6.** rCD2p110 does not induce SRE- or c-fos-dependent gene expression. NIH 3T3 cells were transfected with (A, B) the SRE CAT reporter, (C) the c-fos CAT reporter, (D) the LexA operator-controlled CAT reporter plasmid plus the expression plasmid producing the LexA-Elk-1 fusion protein NLexelk or (E) the AP1 CAT reporter plasmid; together with the expression plasmids for rCD2p110, rCD2p110-R/P, V14Rho, V12Rac, Dbl or Ha-v-ras (v-Ras), as described in *Materials and methods*. In (C) 3 μg V14Rho was used. Serum-starved NIH 3T3 cells were treated as indicated with UV-C: 40 Jm² or 20% FCS when not shown otherwise (A-D), or 4% FCS (E). The data are from a representative experiment. Similar results were obtained in four (A, E), two (B, D) or three (C) further experiments. The data are presented as the ratio of CAT activity (percentage conversion) to β-galactosidase optical density (OD) units.
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**A**

SRE.wt

**B**

SRE.wt

**C**

c-fos

**D**

lexOP + Nlex.elk-1

**E**

AP1
and Erks, and their upstream regulators of the ras and rho family of GTPases. I determined the ability of activated Ras and activated PI 3-kinase to induce transcriptional activity of AP1 complexes: Ha-v-ras led to expression of an AP1-CAT reporter gene and synergised with serum for optimal induction of AP1-CAT (Fig. 6.6E); expression of rCD2p110 did not stimulate AP1 transcriptional activity, nor could it synergise with Ras or serum derived signals. The failure of rCD2p110 to activate SRE, c-fos, Elk-1 or AP1 transcription collectively show that PI 3-kinase signals are not sufficient to stimulate Map kinase effector pathways activated by the GTPases of the ras and rho family.

6.3 Discussion

In the present chapter, I demonstrate that the membrane localised catalytic subunit of PI 3-kinase, rCD2p110, is sufficient to induce signalling responses characteristic of growth factor induction: rCD2p110 is able to fully replace serum for activation of p70S6k in fibroblasts and to stimulate cytoskeletal rearrangements similar as described for PDGF-triggered changes of the actin cytoskeleton. However, constitutively active PI 3-kinase does not uniformly substitute for growth factor signals. Firstly, distinct serine/threonine kinase pathways such as the Erk Map kinase pathway cannot be induced by PI 3-kinase signals alone. Secondly, PDGF-triggered changes in the architecture of the actin cytoskeleton are mediated by the GTPases Rac and Rho, but importantly, PI 3-kinase cannot activate other Rac/Rho-dependent effector pathways such as regulation of gene transcription (see below).

Membrane-targeted PI 3-kinase, rCD2p110, activates p70S6k but not the Erk Map kinase to a similar level as growth factors

Here, I show that localising the catalytic p110 subunit of PI 3-kinase at the plasma membrane is sufficient to stimulate p70S6k in fibroblasts. My data is in
accordance with recent studies in which membrane-bound catalytic subunits of PI 3-kinase, using the Src-myristoylation or the H-Ras farnesylation-palmitoylation signals to anchor p110 in the plasma membrane, activate p70S6k (Klippel et al., 1996). The serine/threonine p70S6k plays a critical role in the regulation of protein translation and cell cycle progression (Brown and Schreiber, 1996; Proud, 1996). p70S6k activation is dependent on phosphorylation of the kinase at specific serine/threonine residues but the kinases that phosphorylate these sites are not known. Nevertheless, it is hypothesised that more than one kinase cascade is involved in regulating the activity of p70S6k including another member of the PI 3-kinase family, Frap (for FKBP12-rapamycin associated protein), also termed Raft1 (rapamycin and FKBP12 target 1), Rapt1 (rapamycin protein target) or mTor (mammalian target of rapamycin) (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994; Sabers et al., 1995), see chapter 7. Therefore, the ability of constitutively active PI 3-kinase to regulate p70S6k is important, as it is possible that PI 3-kinase may orchestrate diverse kinase cascades which contribute to the activation of p70S6k. One kinase which may deliver PI 3-kinase activation signals to p70S6k has recently been characterised, the serine/threonine kinase PKB also termed Akt or Rac-kinase. Interestingly, an activated form of PKB, when overexpressed, can stimulate p70S6k in fibroblasts (Burgering and Coffer, 1995; Franke et al., 1995). Moreover, it has been suggested that PKB can bind D-3 phosphoinositides to its N-terminal pleckstrin homology (PH) domain which may regulate its activity and/or subcellular localisation (Franke et al., 1995; Franke et al., 1997; James et al., 1996; Klippel et al., 1997). Therefore, it had been thought that PKB is a direct target of PI 3-kinase. Indeed, recent reports demonstrate that constitutively active PI 3-kinase is sufficient to stimulate PKB in fibroblasts (Didichenko et al., 1996; Klippel et al., 1996; Marte et al., 1997). Whether PKB is the sole mediator of PI 3-kinase action leading to p70S6k stimulation or whether p70S6k activation pathways bifurcate/branch prior to stimulation of PKB awaits analysis. As well, it will be interesting to assess whether PI
3-kinase regulation of p70S6k confers, at least in part, the ascribed role of PI 3-kinase in mediating cell growth and transformation.

In contrast to the pivotal role of PI 3-kinase in mediating growth factor activation of p70S6k, PI 3-kinase plays a backstage role in Erk activation pathways: expression of constitutively active PI 3-kinase, rCD2p110, is not sufficient to stimulate Erk1 activity in fibroblasts. Moreover, PI 3-kinase did not significantly synergise with serum (or phorbol ester, data not shown) to activate Erk. These data are in agreement with recent studies in which membrane-bound forms of the catalytic subunit of PI 3-kinase (see above) did not raise Erk activity (Didichenko et al., 1996; Klippel et al., 1996; Marte et al., 1997). However, in some cell systems PI 3-kinase may contribute to signalling pathways controlling Erk. Thus, in Xenopus oocytes PI 3-kinase signals can stimulate the Ras/Raf/Erk cascade (Hu et al., 1995); in the monoblastic cell line GM-1 (clone of U937 cells) stably transfected with p110-CAAX, the presence of constitutively active PI 3-kinase may have an inhibitory effect on Erk activity (Didichenko et al., 1996); and in IL-2 dependent T lymphocytes PI 3-kinase is required for IL-2 activation of Mek and Erk (Karnitz et al., 1995), see chapter 7. PI 3-kinase signals thus may participate in Erk stimulation in some cells, but are not sufficient for Erk activation in mammalian cells.

**PI 3-kinase is a selective regulator of Rac/Rho-signalling pathways**

In the present chapter, in collaboration with Kate Nobes and Alan Hall, it was established that PI 3-kinase signals are sufficient to induce cytoskeletal changes including actin polymerisation resulting in Rac-mediated formation of membrane lamellipodia and focal adhesions and Rho-induced formation of actin stress fibres and focal complexes. Moreover, endogenous Rho- and Rac-function are required for these processes, respectively. It is now recognised that rho family GTPases play an important role in the activation of transcription factors and hence are contributing to the regulation of cell growth. The ability of PI 3-kinase signals to initiate the Rac/Rho-
mediated responses in the context of the actin cytoskeleton raises the issue of whether PI 3-kinase is a universal activator of Rac/Rho signal transduction pathways. The present data show that expression of constitutively active PI 3-kinase cannot induce Ras/Rac/Rho signalling pathways that regulate gene transcription from SRE, c-fos, Elk-1 or AP1 reporters. PI 3-kinase signals also could not synergise with serum to induce gene expression from these CAT reporters. In contrast, activation of endogenous GTPases with serum or by expression of Dbl, a guanine nucleotide exchange protein for rho family GTPases, could activate Rho-mediated stress fibre formation (Khosravi-Far et al., 1994; Olson et al., 1996) and Rho-mediated SRE transcriptional activation (Fig. 6.6B). Similarly, studies have shown that Dbl can induce Rac-mediated actin cytoskeleton responses (Olson et al., 1996) and Rac-mediated Jnk/Sapk activation (Coso et al., 1995; Minden et al., 1995). Our results demonstrate that PI 3-kinase induces a selective subset of cellular responses but is not sufficient to stimulate the full range of Rac- or Rho-coordinated pathways. PI 3-kinase is therefore not a universal activator of Rac/Rho-mediated signalling cascades. To explain this phenomenon, it is necessary to evoke discrete subpopulations of these GTPases that are linked to different upstream regulatory proteins and diverse effector pathways. The existence of diverse effector molecules for Rac and Rho has been established, mainly by studies aimed to identify Rac and Rho interacting proteins; and recently specific sites required for the association of different Rac effector molecules with Rac have been mapped (Lamarche et al., 1996): the p21-associated kinase (Pak1) binds to and is activated by GTP-bound Rac or Cdc42 (Martin et al., 1995). Pak binding to active RacL61/Cdc42L61 is abrogated when RacL61/Cdc42L61 contain a Y40C effector site substitution and these mutants are unable to activate the Jnk Map kinase pathway (Lamarche et al., 1996). Two different novel targets of Rac, POR1 and putatively p160ROCK, may link Rac to cellular responses controlling membrane ruffling and G1 cell cycle progression (Lamarche et al., 1996; Van Aelst et al., 1996); the residue being required on Rac for this Rac function involves the phenylalanine at position 37.
(demonstrated with a RacL61-F37A effector mutant). Similarly, two different protein kinases, PKN (Amano et al., 1996; Watanabe et al., 1996) and p164 Rho-kinase (ROK) (Leung et al., 1995; Matsui et al., 1996) associate with active Rho. ROK may act as the Rho effector controlling stress fibre formation by inhibiting myosin light-chain phosphatase (Amano et al., 1997; Bussey, 1996; Kimura et al., 1996), but its role in coupling Rho to the regulation of transcription factors is unclear.

The present study suggests that different Rac and Rho effectors are not necessarily coordinately activated and may be triggered by distinct intracellular signalling pathways. Hence, PI 3-kinase signals can induce Rac/Rho-mediated cytoskeletal changes without activating Rac/Rho-mediated transcription factor pathways. Expression of Dbl activates a wider repertoire of Rac/Rho cellular responses than the membrane-targeted PI 3-kinase. These observations could be explained by several models, described below, which imply spatial restrictions of Rac/effector complexes.

In a simplistic model, the failure of PI 3-kinase-activated Rac to interact with the full range of Rac effector molecules could result from subcellular compartmentalisation of different pools of these proteins. For example, pool 1 of Rac/Rho effectors involved in cytoskeletal rearrangements could be constitutively membrane-localised: receptor activation of PI 3-kinase will generate D-3 phosphoinositides in the plasma membrane, which would spatially restrict the regulatory capacity of these lipids to only membrane-localised pools of Rac/Rho effectors. The expression of oncogenic Dbl is not confined to the plasma membrane and Dbl would hence come into contact with both pools of Rac/effector complexes, including pool 2, that has a more cytoplasmic distribution and transduces transcriptional activation. The discrepancy between the functional effects of Dbl and active PI 3-kinase could then be explained by their different cellular localisation. Evidence from a recent study supports this type of model: the PH domain of Dbl targets
the protein to specific cytoskeletal locations, and a plasma membrane-targeted Dbl with a truncated PH domain is not fully active (Zheng et al., 1996).

A second spatial model, in which pool 2 of effector proteins are selectively recruited, allows for the potential of additional signals derived from growth factors (spatial model 2; Fig. 6.4.A). Such signals would recruit different pools of Rac effector molecules into the proximity of Rac activated by PI 3-kinase. Hence, PI 3-kinase signals may be able to cooperate with other growth factor generated signals to allow full induction of Rac/Rho-mediated signalling pathways, in particular the activation of transcription factor responses. In this context, recent reports describe that the adapter protein Nck binds via its SH3 domains to the Rac effector kinase Pak1 (Galisteo et al., 1996; Lu et al., 1997). This affords a mechanism to recruit Pak1 into activated growth factor receptor complexes and hence to sites in the membrane containing Rac activated by PI 3-kinase. Whether this is the case remains to be elucidated, yet PI 3-kinase signals did not concur with serum-derived signals to give activation of gene expression. Therefore, an

Figure 6.7. Models for the contribution of PI 3-kinase signals to the activation of Rac and Rho GTPase effector pathways.

(A) Spatial model 2: recruitment of pool 2 of effector proteins.
PI 3-kinase signals activate Rac via the stimulation of guanine nucleotide exchange factors (GEF) or possibly via the inhibition of GTPase activating proteins (GAPs). The activated GTP-bound Rac is subsequently able to interact with the pool of effector molecules that link Rac activation to the actin cytoskeleton. Further induction of Rac/Rho-mediated pathways for activation of gene transcription requires a second growth factor-derived signal that will recruit the Rac effector molecules involved in Map kinase activation into the proximity of PI 3-kinase activated Rac. Y indicates a phosphotyrosine residue and PH indicates a pleckstrin homology domain.

(B) Spatial model 3: autonomous regulation of pool 2 of effector proteins.
PI 3-kinase activation triggers membrane ruffles and stress fibre formation that entangles pool 1 of Rac/effector complexes situated in the proximity of the plasma membrane. To trigger GDP/GTP exchange on Rac GTPases that couple to pool 2 of effector proteins, an independent mechanism is evoked by the activated growth factor receptor. This may involve an uncharacterised upstream signalling molecule (X) that activates Rac at a different subcellular location where Rac proteins are networked to pool 2 of effector proteins.

Note: In these models, only guanine nucleotide exchange factors (GEFs) or GTPase activating proteins (GAPs) are depicted as regulators of rho family GTPase activity. Equally possible is the regulation of rho family GTPases is via guanine nucleotide dissociation inhibitors (GDIs). Which regulatory mechanism is engaged is currently not clear, and may involve a combination of all three sets of rho family regulators.
A. Spatial model 2: recruitment of pool 2 of effector proteins

- Growth factor receptor
- PI(4,5)P_2
- PI(3,4,5)P_3
- GDP/Rac
- GTP/Rac
- PI 3-kinase
- GEF
- GAP
- Activation of MAP kinase cascades
- Regulation of gene transcription
- Membrane ruffling
- Rho activation
- Stress fibre formation

B. Spatial model 3: autonomous regulation of pool 2 of effector proteins

- Growth factor receptor
- PI(4,5)P_2
- PI(3,4,5)P_3
- GDP/Rac
- GTP/Rac
- PI 3-kinase
- GEF
- GAP
- Activation of MAP kinase cascades
- Regulation of gene transcription
- Membrane ruffling
- Rho activation
- Stress fibre formation
alternative model to activate Rac/Rho-mediated gene transcription may involve an independent, as yet uncharacterised, mechanism that will not involve PI 3-kinase - autonomous regulation of pool 2 of effector proteins (spatial model 3; Fig. 6.4B).

The identity of the direct targets for D-3 phosphoinositides that regulate the activation of Rac and Rho is currently unknown. The activity of rho family GTPases is controlled by the concerted action of guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation inhibitors (GDIs) and GTPase activating proteins (GAPs). Which of these will be targeted by D-3 phosphoinositides is not clear but it is tempting to speculate that pleckstrin homology (PH) domains, which have been shown to bind phosphoinositol-lipids and are present in rho family GEFs, could provide the link between lipid kinases and Rac/Rho. However, any model for regulation of rho family GTPases must include a mechanism to explain how growth factors apparently have the ability to activate Rac and Rho by mechanisms autonomous of PI 3-kinase. For example, the lysosphosphatic acid (LPA) receptor can bypass PI 3-kinase and Rac to stimulate Rho-dependent responses by a different route. Finally, as demonstrated by the present report, models for activation of Rac and Rho must be superimposed by models that allow for selective activation of different Rac/Rho effectors by diverse regulatory proteins with the subsequent stimulation of divergent cellular responses.

Summary on the contribution of PI 3-kinase to the activation of Rac-/Rho-mediated signalling pathways

Rho family GTPases have been demonstrated to be important for regulation of different cellular responses including regulation of gene transcription, activation of certain members of the Map kinase family and changes in the actin cytoskeleton. Although it is becoming evident that these diverse cellular responses are mediated by different effector proteins that associate with the activated GTPase complexes, little is known of how growth factor receptors orchestrate these distinct effector pathways, in particular
whether triggering of the receptor will uniformly activate all cellular pathways mediated by a certain GTPase.

PI 3-kinase is activated by many growth factor receptors, leading to an increase in D-3 phosphoinositides in the plasma membrane. PI 3-kinase derived signals are sufficient to induce growth factor-like changes of the actin cytoskeleton mediated by the GTPases Rac and Rho, but are not sufficient to stimulate Rac/Rho-mediated gene transcription. Hence, PI 3-kinase activates a distinct subset of Rac/Rho effector pathways but does not give the full repertoire of GTPase responses. The data suggest, therefore, that it is possible to selectively activate different GTPase effector pathways. I propose that specific subcellular compartmentalisation mechanisms exist that localise different exchange factor/GTPase complexes to divergent downstream effector pathways.
Contributions of phosphatidylinositol 3-kinase to the regulation of serine/threonine kinase pathways in lymphoid cells:

† PI 3-kinase links the IL-2R to PKB and p70S6k.
† PI 3-kinase acts to potentiate Erk activity in IL-2-dependent T lymphocytes.
† PI 3-kinase signals are not sufficient to stimulate Jnk, a Map kinase activated by the TCR and CD28.

7.1 Introduction

T cell activation is initiated by a combination of signals evoked by engagement of the TCR and costimulatory molecules. This engagement results in G0 to G1 transition, the production of the cytokine IL-2 and expression of the high affinity IL-2R. The high affinity IL-2R, which comprises α, β, and γ subunits controls G1 to S progression, T cell clonal expansion and functional differentiation (Nakamura et al., 1994; Nelson et al., 1994; Smith, 1988). The IL-2R orchestrates downstream effector pathways by protein tyrosine kinase-dependent activation mechanisms engaging the src family tyrosine kinases Lck and Fyn (Taniguchi, 1995) and the Janus kinases 1 and 3 (Beadling et al., 1994; Miyazaki et al., 1994; Russell et al., 1994). Signalling cascades integrated by the action of these tyrosine kinases include activation of the Ras/Raf/Erk pathway (Fairhurst et al., 1993; Izquierdo et al., 1992; Karnitz et al., 1995; Perkins et al., 1993; Turner et al., 1991; Zmuidzinas et al., 1991), activation of the transcription factors STAT3 and STAT5 (Schindler and Darnell, 1995), and the regulation of PI 3-kinase (Augustine et al., 1991; Merida et al., 1991; Remillard et al., 1991; Williamson et al., 1993). Following IL-2R stimulation PI 3-kinase is recruited to the plasma membrane by at least two mechanisms:
engagement of the IL-2R leads to binding of the p85 regulatory subunit of PI 3-kinase to tyrosine 392 in the IL-2R β chain (Truitt et al., 1994); and, IL-2 stimulation results in the interaction of PI 3-kinase with the src family kinases Fyn (Karnitz et al., 1994) and Lck (Taichman et al., 1993).

The activation of PI 3-kinase is a response that IL-2 shares with other cytokines that control lymphoid cell growth and development such as IL-4 and IL-7 (Gold et al., 1994; Venkitaraman and Cowling, 1994). It is clear that PI 3-kinase activation is necessary for the growth and differentiation inducing properties of these cytokines (Corcoran et al., 1996; Mills et al., 1993; Monfar et al., 1995; Myers et al., 1994). However, despite the pivotal role of PI 3-kinase in lymphoid cells, there is only a preliminary and incomplete understanding of the targets for this enzyme in the mitogenic signalling pathways regulated by the haematopoietin family of cytokines. To date, the identification of biochemical targets for PI 3-kinase in T cells stems mainly from studies employing the PI 3-kinase inhibitors wortmannin or the LY294002 compound (Karnitz et al., 1995; Monfar et al., 1995): IL-2 activation of the Erk Map kinase is sensitive to wortmannin (Karnitz et al., 1995). Similarly, IL-2 activation of the serine/threonine kinase p70S6k is prevented by these PI 3-kinase inhibitors (Monfar et al., 1995). The activation of p70S6k by IL-2 is also impeded by the immunosuppressant rapamycin which targets another member of the PI 3-kinase family of enzymes, Frap/mTor/Raft1/Rapt1 (Abraham, 1996; Brown and Schreiber, 1996; Carpenter and Cantley, 1996; Proud, 1996). The observation that wortmannin and rapamycin have identical inhibitory effects on IL-2-mediated p70S6k activity, generated a model for the regulation of p70S6k in which PI 3-kinase acts as an upstream regulator of Frap (Brown and Schreiber, 1996; Proud, 1996). However, this model has been challenged by a recent study showing that the action of Frap is directly inhibited by wortmannin and LY294002 (Brunn et al., 1996). These results raise the issue of whether PI 3-kinase itself has any upstream regulatory role in p70S6k activation in T lymphocytes. Similar
caution must be applied to interpretations of data involving PI 3-kinase in Erk activation in T cells. As eluded to in chapter 6, Erk regulation may differ in respect to the involvement of PI 3-kinase and is dependent on the cell system: expression of an active PI 3-kinase is sufficient for Erk activation in Xenopus oocytes (Hu et al., 1995); whereas PI 3-kinase signals do not stimulate Erk activity in a variety of fibroblasts and in a monoblast cell line (Didichenko et al., 1996; Klippel et al., 1996; Reif et al., 1996). Whether PI 3-kinase signals are sufficient to stimulate p70S6k or Erk activation in T cells awaits analysis.

A distinct Map kinase pathway, the Jnk/SapK pathways, integrates signals from the TCR and the co-stimulatory molecule CD28. The TCR and CD28 are central to G0 to G1 progression in T cells and, therefore, Jnk is thought to be important in T cells (Su et al., 1994). It is currently not known, which molecules deliver the signals from the TCR and CD28 to activate Jnk. In fibroblasts, however, activated Rac and to a lesser extent activated Ras can stimulate Jnk activity (Coso et al., 1995; Minden et al., 1995). As demonstrated in the previous chapter, PI 3-kinase can stimulate Rac/Rho effector pathways controlling rearrangements of the actin cytoskeleton, but does not induce Rac/Rho-mediated effector pathways leading to gene transcription on the SRE or Ras/Rac-induced activation of Jnks (Marte et al., 1997; Reif et al., 1996). It is possible that the Map kinase pathway of the Jnks, similar to the Erk pathway, can be regulated by divergent mechanisms in different cells. In this respect, the Jnk pathways in fibroblasts is engaged in response to environmental stress whereas in T lymphocytes growth regulatory receptors control Jnk activity. The TCR and the costimulatory receptor CD28, both stimulate PI 3-kinase and can synergise to increase D-3 phosphoinositides in T lymphocytes. It will be interesting to assess whether PI 3-kinase is sufficient to stimulate Jnk activity in T cells.

Constitutively active PI 3-kinase finally allows to assess the relative contribution of PI 3-kinase-derived signals to a certain effector pathway, in particular
whether PI 3-kinase activation is sufficient to promote a specific cellular response. In the present chapter, I have used membrane-localised rCD2p110 as a tool to explore the regulation of serine/threonine kinase pathways by PI 3-kinase in T lymphocytes. In particular, I have analysed the importance of PI 3-kinase in controlling Erk, Jnk and p70S6k activity. p70S6k regulation by PI 3-kinase may involve the serine/threonine protein kinases B (PKB) also known as c-Akt or Rac protein kinase (Burgering and Coffer, 1995; Franke et al., 1995; Kohn et al., 1995). Although treatment of cells with growth factors such as insulin and PDGF activates PKB through a mechanism that requires PI 3-kinase and an intact PH domain, at present, it is not known whether cytokine receptors such as the IL-2R activate PKB in lymphoid cells. PKB was originally identified as the cellular homologue of the directly transforming oncogene of the murine retrovirus AKT8 which causes thymic lymphomas (Staal and Hartley, 1988). In this respect, the putative role of PKB during normal growth in T lymphocytes will be important to analyse.

7.2 Results

IL-2 and PI 3-kinase signals activate p70S6k in Kit225 cells

The IL-2-dependent T cell line Kit225 is a valuable model cell system for analysing IL-2 receptor signal transduction mechanisms in human T cells. The data in Fig. 7.1A show that p70S6k activity is low in quiescent rIL-2-deprived Kit225 cells but can be rapidly stimulated by rIL-2. The activity of p70S6k is increased in response to phorbol esters that stimulate protein kinase C (PKC) (Fig. 7.1A). I asked whether PI 3-kinase signals could substitute for IL-2 in inducing p70S6k activity. To address this question I used membrane-bound constitutively active PI 3-kinase, rCD2p110 and as control 'kinase dead' rCD2p110-R/P, described in chapter 5. To assess the effects of PI 3-
kinase signals on p70S6k activity, rIL-2-starved Kit225 cells were co-transfected with HA-epitope tagged p70S6k and rCD2p110. The HA-tagged p70S6k was

**Figure 7.1.** Interleukin-2 activates p70S6k which can be mimicked by co-expression of membrane-localised constitutively active PI 3-kinase, rCD2p110.  
(A) Kit 225 cells were deprived of rIL-2 for 68h, treated with 20 ng/ml rIL-2 or 50 ng/ml PdBu for the indicated times and p70S6k activation/phosphorylation was assessed by electromobility shift assays (upper panel). p70S6k was precipitated from lysates with M5 Abs and p70S6k activity was analysed in immune complex kinase assays using S6 as a substrate. [32P]phosphate incorporation into S6 (middle panel) was quantified (graph) using a Phospholmager and is expressed as arbitrary units. Protein levels of p70S6k present in immune complexes were assessed in parallel by Western blotting with M1 Abs (lower panel).  
(B, C) HA-p70S6k activity was analysed from extracts of untreated cells (−) or cells stimulated with either 20 ng/ml rIL-2 or 50 ng/ml PdBu for 15 min. (B) Before stimulation, Kit225 cells were co-transfected with HA-p70S6k plasmids and vector plasmid (empty), or plasmids encoding for rCD2p110 as indicated. (C) Kit225 cells were co-transfected with HA-p70S6k plasmids and 15 μg each of vector plasmid (empty), or plasmids encoding for rCD2p110 or rCD2p110-R/P. (B) S6 substrate phosphorylation from S6 kinase assays (upper panel) were analysed by autoradiography. Levels of p70S6k in immune complexes (lower panel) were analysed by immunoblotting using M1 antibodies followed by 125I-conjugated Protein A and autoradiography. (C) The data were analysed as in (B), quantified using a Phospholmager and are presented as the ratio of [32P]phosphate incorporated into S6 to 125I-conjugated Protein A bound to p70S6k (expressed as arbitrary units). (A-C) The data are from a representative experiment. Similar results were obtained in two (A, B) or five (C) more experiments.
immunoprecipitated from transiently transfected cells and assayed for its ability to phosphorylate S6 ribosomal subunits (Fig. 7.1B). Expression of the active PI 3-kinase, rCD2p110, resulted in constitutive IL-2-independent p70S6k activation (Fig. 7.1B). p70S6k was not constitutively activated in cells expressing 'kinase dead' rCD2p110-R/P confirming that the p70S6k activation requires the kinase activity of the p110 subunit (Fig. 7.1C). Therefore, PI 3-kinase signals are sufficient to activate p70S6k in IL-2-dependent cells and can substitute for IL-2 to stimulate p70S6k. These data are similar to the one in fibroblasts described in chapter 6, where PI 3-kinase signals could replace serum to activate p70S6k.
IL-2 and membrane-localised PI 3-kinase activate PKB

PKB can be activated by receptor tyrosine kinases such as the PDGF receptor and has been identified as a target of PI 3-kinase in fibroblasts (Burgering and Coffer, 1995; Franke et al., 1995; Klippel et al., 1996). However, whether this pathway is conserved in the haematopoietic system has not been explored. In particular, although PKB can become oncogenic and initiate thymic tumours, its regulation and significance for normal T cell growth processes is not known. As cytokine receptors have essential functions in the development and maintenance of the haematopoietic system, I was interested to assess whether members of the haematopoietin receptor family, such as the prototypical IL-2R, regulate PKB. To examine whether IL-2 activates PKB, immunoprecipitates of this kinase were prepared from rIL-2-deprived and rIL-2-activated Kit225 cells and subjected to \textit{in vitro} kinase assays using histone 2B (H2B) as a substrate: IL-2 induced a rapid activation of PKB (Fig. 7.2A). A two to three fold increase inhibited PI 3-kinase activity with an IC50 of about 5 nM (Fig. 7.2B). Pre-treatment of cells with LY294002 and wortmannin prevented stimulation of PKB by IL-2 (Fig. 7.2C), which suggests that PI 3-kinase delivers signals from the IL-2R to PKB. It is becoming increasingly evident that experiments using these PI 3-kinase inhibitors must be interpreted with caution as other enzymes also fail to work in the presence of LY294002 and wortmannin. In particular, LY294002 and to a lesser extent wortmannin prevent the autokinase activity of Frap/mTor (Brunn et al., 1996), a member of the PI 3-kinase family (Abraham, 1996; Carpenter and Cantley, 1996). Frap is the cellular target for the drug rapamycin which prevents IL-2 coordinated cell cycle progression and proliferation of T lymphocytes (Brown and Schreiber, 1996; Proud, 1996). Frap activity is absolutely required for p70S6k action in T cells (Brown and Schreiber, 1996; Calvo et al., 1992; Kuo et al., 1992; Proud, 1996; Sawami et al., 1992). I therefore assessed whether Frap function is necessary for IL-2-induced stimulation of PKB using rapamycin. Rapamycin had no effect on IL-2-triggered activation of PKB (Fig. 7.2C), although rapamycin completely abolished IL-2- or PI 3-kinase-controlled induction of p70S6k (Fig. 7.2C,
bottom: lower panel). The inhibition of PKB by wortmannin and LY294002, therefore, cannot be caused by prevention of Frap activity and indicate that IL-2 regulation of PKB employs PI 3-kinase.

**Figure 7.2.** Interleukin-2 activates PKB: PI 3-kinase activity is necessary for IL-2 mediated activation of PKB and PI 3-kinase signals are sufficient to stimulate PKB activity in Kit225 T cells.

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**Fig. 7.2 (A)** Interleukin-2 activates PKB. Kit 225 cells were deprived of rIL-2 for 68h, treated with 20 ng/ml rIL-2 or 50 ng/ml PdBu for the indicated times and PKB activation/phosphorylation was assessed by electromobility shift assays (upper panel). PKB was precipitated from lysates with Rac-PK-CT Abs and PKB activity was analysed in immune complex kinase assays using histone 2B (H2B) as a substrate. $[^{32}P] \text{phosphate incorporation into H2B (middle panel)}$ was quantified (graph) using a Phospholmager and is expressed as arbitrary units. Protein levels of PKB present in immune complexes were assessed in parallel by Western blotting with Rac-PK-CT Abs (lower panel).
To investigate directly whether PI 3-kinase signals are sufficient to activate PKB, rIL-20-deprived Kit225 cells were co-transfected with either rCD2p110 or rCD2p110-R/P expression vectors together with an expression vector encoding HA-epitope tagged PKB. In addition, the ability of activated forms of the small GTPases Ha-v-ras and V12Rac to activate PKB was assessed. Immunoprecipitates of HA-tagged PKB were assayed for kinase activity using H2B as a substrate. The constitutively active PI 3-kinase, rCD2p110, induced a robust activation of PKB (Fig. 7.2D). This stimulatory effect of rCD2p110 was dependent on the kinase activity of the enzyme as co-expression of kinase inactive rCD2p110-R/P did not stimulate PKB activity. As observed previously in other cell systems (Chou and Blenis, 1996; Franke et al., 1995; Klippel et al., 1996) co-expression of activated Ha-v-ras but not of active V12Rac led to a moderate rise in PKB activity in Kit225 T cells.
Fig. 7.2 (C) The PI 3-kinase inhibitors wortmannin and LY294002 but not rapamycin inhibit IL-2 dependent activation of PKB. Kit225 cells starved of rIL-2 for 72h were pretreated for 30 min with the vehicle DMSO, 20 ng/ml rapamycin, 5 µM LY294002 or 100 nM wortmannin and then stimulated with rIL-2 for the times indicated before lysis. PKB activity was measured in immune complex kinase assays using H2B as a substrate. [³²P]phosphate incorporation into H2B (top: upper panel) was quantified (graph) using a Phospholmager and is expressed as arbitrary units. Protein levels of PKB present in immune complexes were assessed in parallel by Western blotting with Rac-PK-CT Abs (top: lower panel). In parallel, postnuclear total cell lysates were subjected to SDS-PAGE analysis followed by Western blotting with Rac-PK-CT or M1 antisera to assess PKB (bottom: upper panel) and p70S6k (bottom: lower panel) activation/phosphorylation, respectively.
Fig. 7.2 (D) PI 3-kinase signals trigger a potent stimulation of PKB activity. Kit225 cells were co-transfected with HA-PKB plasmids and vector plasmid (empty), or plasmids encoding for rCD2p110, rCD2p110-R/P, Ha-v-ras or V12Rac as indicated. HA-PKB activity was analysed in anti-HA tag immune complex kinase assays using H2B as a substrate. [³²P]phosphate incorporation into H2B (upper panel) was quantified (graph) using a PhosphorImager and is expressed as arbitrary units. Protein levels of HA-PKB present in immune complexes were assessed in parallel by Western blotting with Rac-PK-CT Abs (lower panel).

Co-expression of an activated form of PKB stimulates p70S6k in Kit225 T cells

p70S6k is activated by multiple serine/threonine phosphorylation in response to mitogenic stimuli. The retroviral oncogene v-Akt is a chimeric molecule, consisting of the retroviral gag protein fused to the N-terminus of c-Akt. The gag protein is myristoylated and therefore v-Akt is predominantly found at the plasma membrane which may give rise to its oncogenicity (Ahmed et al., 1993). Expression of a constitutively active PKB, gagPKB, has been described to activate p70S6k in Rat-1 cells (Burgering and Coffer, 1995) and Cos1 cells (Franke et al., 1995). Nevertheless, the ability of phorbol esters to stimulate p70S6k without any discernible activation of PKB indicated that PKB-independent pathways for activation of p70S6k must exist in T cells. To determine the role of PKB in p70S6k activation in T cells, rIL-2-deprived Kit225 cells were co-transfected with a gagPKB expression vector together with an expression vector
Figure 7.6. rCD2p110 does not activate the Map kinase Jnk in Jurkat T cells. HA-p54β Sapk (here termed Jnk) activity was analysed from extracts of untreated cells (-) or cells stimulated with 50 ng/ml PdBu, 500 ng/ml ionomycin or with PdBu and ionomycin in combination for 15 min. Before stimulation, Jurkat T cells were co-transfected with HA-p54β Sapk plasmids and vector plasmid (empty), or plasmids encoding for rCD2p110, Ha-v-ras, V12Rac, or Dbl as indicated. Jnk assays were performed as described in Materials and methods. Gels labelled Jun are autorads of the GST-Jun substrate after immune complex assay of Jnk phosphotransferase activities. Blots labelled tag-Jnk2 show the immunoblot analysis performed on the p54β Sapk proteins present in the anti-HA-tag immune complex kinase assays using the anti-Jnk2 antisera, JNK2 (FL). The data are from a representative experiment. Similar results were obtained in two more experiments.
Figure 7.3. Co-expression of constitutively active forms of PKB and PI 3-kinase but not of the GTPases Rac and Cdc42 stimulate p70S6k activity in Kit225 cells. HA-p70S6k activity was analysed from extracts of untreated cells (-) or cells stimulated with 20 ng/ml rIL-2 for 15 min. Before stimulation, Kit225 cells were co-transfected with HA-p70S6k plasmids and vector plasmid (empty), or plasmids encoding for rCD2p110, gagPKB, V12Rac, V12Cdc42 or rCD2p85 as indicated. p70S6k assays were performed as described in Materials and methods. The data were quantified using a Phospholmager and are presented as the ratio of [32P]phosphate incorporated into S6 to [25]l-conjugated Protein A bound to p70S6k (expressed as arbitrary units). The data are from a representative experiment. Similar results were obtained in one further experiment.

encoding HA-epitope tagged p70S6k. p70S6k activity was analysed in anti-HA tag immune complexes with S6 ribosomal subunits as a substrate. Co-expression of constitutively active PKB induced a strong activation of p70S6k that was comparable to increases in p70S6k activity seen by co-expression of rCD2p110 (Fig. 7.3). rCD2p85 which does not regulate cellular levels of D-3 phosphoinositides (see chapter 5, (Reif et al., 1996)) did not stimulate p70S6k. In contrast to data described in fibroblasts (Chou and Blenis, 1996), co-expression of V12Rac and V12Cdc42 had no effect on p70S6k activity in Kit225 cells (Fig. 7.3).
IL-2 regulates the transcription factor Elk-1 in Kit225 cells in a PI 3-kinase dependent fashion

In IL-2 dependent T cells, a PI 3-kinase sensitive pathway for regulating the activity of Erk kinase (Mek) and the Erks has been reported to co-exist alongside the PI 3-kinase/p70S6k pathway (Karnitz et al., 1995). Whilst p70S6k is thought to exert its mitogenic function by controlling translation initiation and protein synthesis, the Map kinase Erk is implicated in regulating the phosphorylation and activity of certain transcription factors. One well characterised cellular substrate for Erks in fibroblasts and T cells is the transcription factor Elk-1 (Genot et al., 1996; Hill et al., 1995; Treisman, 1994). I therefore tested the ability of IL-2 to regulate Elk-1 transcriptional activity and hence Erk in Kit225 cells. To monitor Elk-1 transcriptional activity a fusion protein comprising the C-terminus of Elk-1 linked to the LexA repressor (Marais et al., 1993) was co-transfected into Kit225 cells with a LexA operator-controlled CAT reporter gene. The data in Fig. 7.4A demonstrate that IL-2 can regulate Elk-1 transcriptional activity in Kit225 cells. To confirm that Elk-1 transactivation is induced by a Mek/Erk sensitive pathway, I investigated the ability of the well characterised inhibitor of Mek action, PD098059 (Alessi et al., 1995), to prevent IL-2-mediated activation of Elk-1: treatment of Kit225 cells with the PD098059 component inhibited stimulation of Elk-1 transcriptional activity triggered by rIL-2 (Fig. 7.4B). Moreover, rIL-2-induction of Elk-1 activity was prevented by the PI 3-kinase inhibitor wortmannin in a dose dependent manner (Fig. 7.4C) which corroborates

Figure 7.4. Interleukin-2 but not rCD2p110 induces Elk-1-dependent gene expression. Kit225 cells were transfected with the LexA operator-controlled CAT reporter plasmid (lexOP-CAT) plus the expression plasmid producing the LexA-Elk-1 fusion protein Nlex.elk. In (E) the Nlex.elk/lexOP-CAT reporter plasmids were co-transfected with vector plasmid (empty), or the expression plasmids for rCD2p110, rCD2p110-R/P, Ha-v-ras, gagPKB or V12Rac as indicated. Kit225 cells were treated over night with (A) various concentrations of rIL-2, (B) 20 ng/ml rIL-2 plus various concentrations of PD098059, (C) 20 ng/ml rIL-2 plus various concentrations of wortmannin, (D) 20 ng/ml rIL-2 plus 20 ng/ml of rapamycin, or (E) 50 ng/ml phorbol ester (PdBu) or left untreated as indicated. CAT activity was analysed as described in Materials and methods. The data are from a representative experiment. Similar results were obtained in one (A-D) or three (E) further experiments. The CAT activity is presented as percentage conversion.
Chapter 7

Results: PI 3-kinase targets in lymphoid cells

A

B

C

D

E

Table of values:

- **rIL-2 (ng/ml)**: 0, 0.02, 0.2, 2, 20, 100
- **PD098059 (µM)**: 0, 0.5, 1, 5, 10, 25
- **WMN (nM)**: 0, 0.8, 100
- **Rapamycin (ng/ml)**: 0, 0.2, 0.6

Graphs and data points:

- **Graph A**: CAT activity vs. rIL-2 (ng/ml)
- **Graph B**: CAT activity vs. PD098059 (µM)
- **Graph C**: CAT activity vs. WMN (nM)
- **Graph D**: CAT activity vs. Rapamycin (ng/ml)
- **Graph E**: CAT activity vs. Treatments (empty, rCD2p10, rCD2p10-RP, Hex4-m, gagPKB, Y12Phos, PdBu)

Legend:

- **Empty**
- **rCD2p10**
- **rCD2p10-RP**
- **Hex4-m**
- **gagPKB**
- **Y12Phos**
- **PdBu**
earlier studies that Erk activation by IL-2 requires PI 3-kinase function (Karnitz et al., 1995). Treatment of Kit225 cells with rapamycin did not affect Elk-1 transactivation in Kit225 cells (Fig. 7.4D). To assess whether constitutively active PI 3-kinase and the in vivo production of D-3 phosphoinositides could induce Map kinase signalling pathways in T cells, the ability of rCD2p110 to induce transcriptional activation of Elk-1 was analysed. LexA-Elk-1 transcriptional activity was low in quiescent Kit225 cells but can be instigated by co-expression of active Ha-v-ras and by stimulation with phorbol esters, whereas expression of rCD2p110 did not stimulate Elk-1 transactivation (Fig. 7.4E). However, rCD2p110 signals could potentiate phorbol ester induction of the transcriptional activity of Elk-1. This potentiating effect was not observed in cells expressing the 'kinase dead' rCD2p110-R/P and was therefore dependent on the integrity of the lipid kinase and the cellular production of D-3 phosphoinositides.

Moreover, gagPKB cannot mimic the effects of PI 3-kinase on the Erk/Elk-1 pathway (Fig. 7.4E). Interestingly, activated Rac, V12Rac, could induce moderately Elk-1 transcriptional activity. As discussed in chapter 6, Elk-1 is also a substrate for the Sapk or Jnk subgroup of Map kinases (Cavigelli et al., 1995; Gille et al., 1995; Whitmarsh et al., 1995) and these Jnks can be activated by Rac (Coso et al., 1995; Minden et al., 1995). It is possible that Rac can regulate Jnk activity in Kit225 cells and therefore can increase Elk-1 transcriptional activity. Whether Jnks are stimulated by the IL-2R (or by Rac in IL-2 dependent cells) awaits analysis. Finally, V12Rac signals could also synergise with phorbol ester to trigger Elk-1 transactivation. This effect could be mediated by both the Jnk or Erk pathway as activated forms of Rac have recently be reported to cross-talk with the Erk Map kinase pathway (Frost et al., 1996). This data is supported by a recent study where an effector of Rac, the p21-activated kinase-1 (Pak1), in an activated form, can stimulate Erk1 in epithelial 293T cells (Lu et al., 1997). Nevertheless, the failure of rCD2p110 to induce Elk-1 activity indicates that a constitutively active PI 3-kinase is not sufficient to stimulate the Sapk/Jnk pathway in Kit225 cells.
PI 3-kinase signals synergise with phorbol esters to induce Erk activity in Kit225 cells

To assess the effect of membrane-localised PI 3-kinase (and of active Rac) on Erk activity directly rIL-2 deprived Kit225 cells were co-transfected with expression vectors encoding rCD2p110 and HA-epitope tagged p42 Erk2 and cells were stimulated with phorbol esters or left untreated. Co-expression of rCD2p110 did not stimulate Erk2 activity although Erk2 could be activated by co-expressing the oncogenic Ras, Ha-v-ras (Fig. 7.5). These results confirm the data in Fig. 7.4E that PI 3-kinase signals are not sufficient to activate the Erk/Elk-1 pathway. The data in Fig. 7.5 demonstrate that active PI 3-kinase markedly potentiated the level of Erk2 activation triggered by phorbol esters, an effect that was not observed in cells expressing the 'kinase dead' rCD2p110-R/P. PI 3-kinase signals did not enhance IL-2 activation responses on Erk (data not shown).

Figure 7.5. PI 3-kinase signals are not sufficient to stimulate Erk2 activity but can synergise with phorbol esters to give an increase in Erk2 activity in Kit225 cells. HA-Erk2 activity was analysed from extracts of untreated cells (-) or cells stimulated with 50 ng/ml PdBu for 5 min. Before stimulation, Kit225 cells were co-transfected with HA-Erk2 plasmids and vector plasmid (empty), or plasmids encoding for rCD2p110, rCD2p110-R/P, Ha-v-ras or V12Rac as indicated. Erk assays were performed as described in Materials and methods. The data were quantified using a Phospholmager and are presented as the ratio of $^{32}\text{P}$ phosphate incorporated into S6 to $^{125}\text{I}$-conjugated Protein A bound to Erk (expressed as arbitrary units). The data are from a representative experiment. Similar results were obtained in two more experiments.
Similarly, active Rac also increased Erk2 activity in combination with phorbol ester but did not stimulate Erk2 activity in unstimulated cells. It is possible that Rac may contribute to Erk regulation in Kit225 cells. Taken together the results in Fig. 7.4E and Fig. 7.5 clearly demonstrate that although PI 3-kinase signals are not sufficient for Erk/Elk-1 activation they can synergise with phorbol esters to induce a maximal response. These results are concordant with a model where PI 3-kinase signals bifurcate to activate the PKB/rapamycin-sensitive/p70S6k pathway and independently contribute to the Mek/Erk/Elk-1 pathway via an as yet undefined mechanism (see Fig. 7.8).

**PI 3-kinase signals are not sufficient to stimulate Jnk and Jnk effector pathways in Jurkat T cells**

At present, it is not known whether the Sapk/Jnk subgroup of Map kinases is regulated by cytokine receptors such as the IL-2R. Therefore, in initial experiments, I did not pursue an analysis of PI 3-kinase effects on Jnk activity in IL-2 dependent Kit225 cells. Jnks are thought to play an important role in integrating signals from the TCR and the costimulatory molecule CD28 (Su et al., 1994). Therefore, I examined the effects of the active PI 3-kinase on Jnk activation in the Jurkat T leukaemic cell line, JKHM1. Cells were co-transfected with HA-epitope tagged Jnk (p54β Sapk) and rCD2p110 or Ha-v-ras, V12Rac, or Dbl (a guanine nucleotide exchange protein for Rac/Rho family GTPases). Jnk activation in T cells can be stimulated by the combined stimulation of calcium ionophore and phorbol ester; neither stimuli alone is sufficient (Su et al., 1994). Co-expression of activated Ha-v-ras or V12Rac led to a potent increase in Jnk activity, as did activation of endogenous Rac/Cdc42 signalling pathways by expression of an activated version of Dbl (Fig. 7.6). However, expression of the constitutively active PI 3-kinase, rCD2p110, did not stimulate Jnk activity, either alone or in combination with phorbol esters or calcium ionophore (Fig. 7.6). Membrane-targeted PI 3-kinase was constitutively active in Jurkat T cells as rCD2p110 could sufficiently regulate PKB.
activity in these cells: co-expression of rCD2p110 stimulated the activity of HA-epitope tagged PKB in immune complex kinase assays using H2B as a substrate. The level of activation was comparable with that triggered by stimulation of the TCR/CD3 complex with anti-CD3 (UCHT-1) mAbs (see Fig. 8.3A, chapter 8). The data in Fig. 7.6 hence demonstrate that PI 3-kinase signals are not sufficient to activate the Rac/Jnk pathway in T cells nor can they cooperate with phorbol esters or calcium signals to stimulate Jnk activity. The failure of the active PI 3-kinase to stimulate Jnk activity in Jurkat T cells was corroborated by experiments which analysed the inducibility of well the characterised transcription factor targets of Jnk, Jun, Elk-1, or AP1: rCD2p110 failed to regulate CAT reporter genes that monitor the activity of these transcription factors (Fig. 7.7). In these experiments co-transfection of activated forms of the GTPases Ras, Rac, Cdc42 or the exchange factor Dbl were potent activators of Jun, Elk-1 or AP1 transcriptional activity (Fig. 7.7A-C). The failure of rCD2p110 to activate Jnk, Jun, Elk-1 or AP-1 collectively demonstrate that PI 3-kinase signals are not sufficient to stimulate these Map kinase effector pathways in Jurkat T cells.
Figure 7.7. rCD2p110 does not induce Jun-, Elk-1- or AP1-dependent gene expression in Jurkat T cells. Jurkat T cells were transfected with (A) the LexA operator-controlled CAT reporter plasmid plus the expression plasmid producing the LexA-Jun fusion protein Nlex.jun, (B) the LexA operator-controlled CAT reporter plasmid plus the expression plasmid producing the LexA-Elk-1 fusion protein Nlex.elk or (C) the AP1-CAT reporter; together with the expression plasmids for rCD2p110, rCD2p110-R/P, Ha-v-ras, V12Rac, Dbl or V12Cdc42. Jurkat T cells were treated with 50 ng/ml PdBu plus 500 ng/ml ionomycin as indicated. The CAT activity is presented as percentage conversion. The data are from a representative experiment. Similar results were obtained in three (A), one (B) or four (C) more experiments.
7.3 Discussion

In the present chapter, I have used membrane-targeted constitutively active PI 3-kinase, rCD2p110, as a tool to identify direct targets of PI 3-kinase action in IL-2 signal transduction pathways: the serine/threonine kinase PKB/Akt is activated by the cytokine IL-2 via a PI 3-kinase-dependent pathway. Importantly, PI 3-kinase signals alone are sufficient to activate PKB in T cells demonstrating that PI 3-kinase acts as an upstream regulator of this serine/threonine kinase in lymphoid cells. PKB contains an N-terminal pleckstrin homology (PH) domain that can directly bind D-3 phosphoinositides (Franke et al., 1995; Franke et al., 1997; James et al., 1996; Klippel et al., 1997) which may contribute to the regulation of the enzyme. As PI 3-kinase signals are sufficient to substitute for IL-2 in PKB activation, PKB could be a direct target for PI 3-kinase signals during IL-2 signal transduction. PKB/c-Akt is highly expressed in the thymus (Bellacosa et al., 1991) and the oncogenic form of this kinase causes thymic malignancies. Therefore, PKB has a pivotal role in controlling T cell proliferation/differentiation. The present data identify one function for PKB in T cells - PKB action is sufficient to stimulate p70S6k. PI 3-kinase signals are also sufficient for activation of p70S6k which stresses the close link between PI 3-kinase and PKB in regulating p70S6k activity in T cells. Questions regarding the selectivity of the inhibitors which were first used to define a role for PI 3-kinase in T cell biology, have challenged the involvement of this enzyme in the regulation of p70S6k in T cells (Brunn et al., 1996). The present data resolve this controversy and provide unequivocal evidence that PI 3-kinase can function as an upstream regulator of p70S6k in T cells.

Results obtained recently with p110 constructs that are membrane-targeted by myristoylation or farnesylation-palmitoylation signals show that PI 3-kinase is sufficient to activate PKB (Klippel et al., 1996; Marte et al., 1997) and p70S6k (Klippel et al., 1996) in Cos cells. In addition, the GTPases Rac and Cdc42 induce p70S6k activation in
fibroblasts (Chou and Blenis, 1996). I find no evidence for Rac/Cdc42 activation of p70S6k in T cells illustrating that cells of different lineages can differ markedly in their cellular mechanisms for kinase activation. Nevertheless, the present data show a striking conservation of the PI 3-kinase/PKB/p70S6k link in human T cells and simian fibroblasts. The conservation of the PI 3-kinase/PKB/p70S6k signalling cascade in T cells implies a physiological importance of this pathway which has guaranteed its evolutionary conservation.

The role of PI 3-kinase as an upstream regulator of the Erk pathway seems to vary to a greater extent with the cell system: expression of an active form of PI 3-kinase is sufficient for Erk activation in Xenopus oocytes (Hu et al., 1995), but not in fibroblasts or monoblasts (Didichenko et al., 1996; Klippel et al., 1996; Marte et al., 1997; Reif et al., 1996). The present data show directly that PI 3-kinase can have a positive regulatory role in Erk activation in IL-2 dependent T cells (see Fig. 7.8): PI 3-kinase signals markedly potentiate Erk responses in combination with phorbol esters, however, PI 3-kinase signals alone fail to stimulate Erk signalling pathways. Erk regulation of downstream nuclear targets is hereby analysed using the transactivation capacity of the ternary complex factor Elk-1, a well characterised substrate for Erks in fibroblasts and Jurkat T cells (Genot et al., 1996; Hill et al., 1995). I established that Elk-1 is regulated by IL-2 via Mek and PI 3-kinase sensitive pathways. Furthermore, as observed in direct Erk activation assays, PI 3-kinase signals potently enhanced phorbol ester induction of Elk-1 transcriptional activity. PI 3-kinase signals may therefore be required for activation of Map kinase pathways in IL-2-dependent T cells, but they are not sufficient, and hence are one component of a more complex signalling network. I have not yet explored the PI 3-kinase effector pathways involved in Erk activation, although previous data (and the data in Fig. 7.4D) exclude the involvement of the Frap/p70S6k pathway, as activation of Erk is not sensitive to rapamycin inhibition. Moreover, PKB which is a potent activator of p70S6k cannot mimic the effects of activated PI 3-kinase on the Erk/Elk-1 pathway.
These data best fit a model in which PI 3-kinase regulation of Map kinases and PKB/p70S6k bifurcate prior to activation of PKB (Fig. 7.8). Members of the Rho family of GTPases can potentiate Erk activation pathways in fibroblasts (Frost et al., 1996). Interestingly, an activated form of Rac potentiates Erk activity in conjunction with phorbol ester in Kit225 cells, but is not sufficient to induce Erk. Activation of PI 3-kinase is sufficient to trigger cytoskeletal rearrangements mediated by the GTPase Rac and Rho in Swiss 3T3 cells (see chapter 6, (Reif et al., 1996)), and thus has the potential to regulate Rac/Rho signalling pathways in T cells. Although PI 3-kinase did not elicit Rac/Rho effector pathways leading to gene transcription in fibroblasts (see chapter 6, (Reif et al., 1996)), it cannot be excluded that Rac/Rho family GTPases could mediate PI 3-kinase regulation of Erk. In this respect, PI 3-kinase signals can potentiate phorbol ester induction of Erk in IL-2 dependent T cells but not serum (or phorbol ester, data not shown) induction of Erk in fibroblasts (compare Fig. 6.1B with Fig. 7.5). However, several other candidate in vivo targets for D-3 phosphoinositides have been proposed including members of the novel PKC family (Moriya et al., 1996; Toker et al., 1994), and the atypical PKC family, PKC-λ (Akimoto et al., 1996) and PKC-ζ (Nakanishi et al., 1993) which has recently been implicated as a regulator of Mek and Erk activity in Cos cells (Berra et al., 1995).

Unlike Erk regulation, PI 3-kinase signals do not contribute to Jnk regulation. This was examined in Jurkat T cells, where Jnk integrates signals from the TCR and CD28 and can be stimulated by a combination of phorbol ester and calcium ionophore (Su et al., 1994). Accordingly, membrane-targeted PI 3-kinase signals are not sufficient to activate the Jnk pathway in T cells nor do they cooperate with phorbol esters or calcium signals either alone or in combination to evoke Jnk activity. Similar to previous reports in fibroblasts (Coso et al., 1995; Minden et al., 1995), in T cells Jnk activity can be induced by Rac or Dbl (Fig. 7.6). Therefore, PI 3-kinase apparently cannot deliver stimulatory signals to Rac effector pathways that lead to Jnk activation in Jurkat T cells.
This is in accordance with my previous data where PI 3-kinase signals failed to evoke Rac/Jnk effector pathways: PI 3-kinase did not induce Elk-1 or AP1 transcriptional activity in NIH 3T3 cells; PI 3-kinase does not trigger Elk-1, AP1 or Jun transactivation in Jurkat T cells; and PI 3-kinase is not sufficient to stimulate Elk-1-mediated transcription in Kit225 T cells. These data are supported by experiments by Marte et al.: constitutively active PI 3-kinase failed to elicit Jnk activity in Cos cells monitored in direct kinase assays of Jnk activity (Marte et al., 1997). Unlike, Klippel et al. who show an increase in Jnk activity in Cos cells co-expressing constitutively active PI 3-kinase (Klippel et al., 1996), the study by Marte et al. and my data provide no evidence that PI 3-kinase is sufficient to stimulate Jnk. The basis of the discrepancy between the study by Klippel et al. versus the one by Marte et al. and my data, is not clear. In support of my data and that from Marte et al., however, UV induction of Jnk is not prevented by wortmannin, on the contrary, wortmannin enhances UV-mediated activation of Jnk (Kharbanda et al., 1995); in addition, growth factors which are potent regulators of PI 3-kinase are moderate activators of Rac/Jnk or Cdc42/Jnk pathways. Whether the TCR and CD28 regulate Jnk activity via Rac-dependent pathways awaits analysis.

The failure of active PI 3-kinase to initiate Rac/Cdc42 mediated pathways that stimulate the Jnk/Sapk pathway in T cells might seem to exclude the possibility that Rac family GTPases play a role in PI 3-kinase regulation of Erk. However, my data suggest that PI 3-kinase signals function to selectively activate certain subsets of Rac/Rho-mediated responses (see chapter 6, (Reif et al., 1996)). Furthermore, growth factors such as PDGF can stimulate Rac- or Cdc42-dependent pathways for activation of p70S6k but do not induce Rac/Jnk or Cdc42/Jnk responses (Chou and Blenis, 1996; Coso et al., 1995). Accordingly, it should not be excluded that Rac or Cdc42 transmit PI 3-kinase signals to certain effector pathways in T cells (Fig. 7.8).
p70S6k plays a key role in cellular growth control mechanisms by coordinating protein biosynthesis via phosphorylation of the S6 subunit of 40S ribosomes or via regulating the activity of the eukaryotic initiation factor 4E binding protein, 4E-BP1 (Brown
and Schreiber, 1996; Proud, 1996; Von Manteuffel et al., 1996). Expression of an activated PKB can stimulate p70S6k activity in T cells indicating that PKB substrates are part of the p70S6k activation pathways. Moreover, given the ability of PI 3-kinase signals to stimulate PKB and p70S6k, it seems probable that PKB mediates PI 3-kinase activation of p70S6k in T cells. The immunosuppressive drug rapamycin inhibits the cell cycle progression and proliferation of T lymphocytes and has been shown previously to block IL-2 activation of p70S6k (Kuo et al., 1992). Rapamycin forms a complex with the intracellular protein FKBP12 which subsequently provides a high affinity inhibitor of Frap. The Frap kinase is a member of the PI 3-kinase family of enzymes (Abraham, 1996; Carpenter and Cantley, 1996) and plays an established, but poorly defined, role as an upstream regulator of p70S6k (Brown and Schreiber, 1996; Proud, 1996). Rapamycin prevents the activation of p70S6k induced in T cells by the constitutively active PI 3-kinase, rCD2p110, (data not shown) or by active PKB (Burgering and Coffer, 1995; Franke et al., 1995), thus indicating that PI 3-kinase or PKB activation signals cannot bypass the role of Frap in p70S6k activation pathways. A simplistic interpretation of these data is that PI 3-kinase and PKB activation of p70S6k is mediated by Frap, although, it is equally possible that Frap regulates p70S6k by an essential signalling pathway operating in parallel with PI 3-kinase/PKB signals (Fig. 7.8). Frap controls p70S6k activation by regulating the phosphorylation of key residues in the enzyme (Dennis et al., 1996; Pearson et al., 1995). Nevertheless, p70S6k is not a direct substrate for Frap and some intermediate p70S6k kinase(s), as yet uncharacterised, must be invoked to explain the role of Frap in p70S6k activation. Although the evidence that PKB mediates PI 3-kinase effects on p70S6k are compelling, these data do not exclude that there are PKB-independent mechanisms for p70S6k activation of T cells. In this context, the present data show that activation of PKC by phorbol esters stimulates p70S6k without any discernible stimulatory effect on PKB.
Recent studies showing that cytokine activation of serine kinases is important for the regulation of apoptosis (Gajewski and Thompson, 1996; Zha et al., 1996) have focused attention on cytokine induced serine kinase cascades. PI 3-kinase action has been implicated in the prevention of apoptosis in other cell systems (Yao and Cooper, 1995). The present study demonstrate that PI 3-kinase can couple the IL-2R to a selective subset of serine/threonine kinase pathways in T cells, and in this respect, the PI 3-kinase/PKB link is intriguing as PKB mediates activation of the Frap/p70S6k pathway, but may also induce other kinase cascades that bifurcate from the PKB/p70S6k pathway including glycogen synthase kinase-3 (GSK3) signalling pathways (Cross et al., 1995). Therefore, PI 3-kinase and/or PKB have the potential for pleiotropic functions in T cells and their downstream effectors may include additional serine/threonine kinases evoked by IL-2R engagement.

Finally, PI 3-kinase is activated by members of the cytokine receptor family such as the IL-2R, the IL-4R, the IL-7R and the IL-13R. Signalling pathways regulated by PI 3-kinase can hence have an impact on lymphocyte biology at multiple points. Accordingly, it is important to establish the function of this enzyme in lymphoid cells. The IL-2R is a prototypical member of this haematopoietin receptor family. The present results directly define PI 3-kinase function in T cells and position PKB for the first time in a physiologically relevant cytokine induced signal transduction pathway in lymphoid cells. The model described herein may also be applicable to serine/threonine kinase pathways regulated by other receptors that activate PI 3-kinase in T cells.
Chapter 8

A negative role for phosphatidylinositol 3-kinase in regulating T cell antigen receptor function

8.1 Introduction

During T cell activation both positive and negative regulatory signalling cascades are engaged that are vital for a balanced immune response and immune homeostasis. T cell activation is orchestrated by the T cell antigen receptor (TCR) and a plethora of accessory molecules such as integrins, CD45, CD4, CD8, CD28 (Cantrell, 1996; Lenshow et al., 1996; Rudd, 1996; Trowbridge and Thomas, 1994; Wange and Samelson, 1996; Weiss and Littman, 1994). It are these regulatory receptors that will determine the outcome of TCR engagement: As these surface molecules employ intracellular effector cascades, they can modulate TCR function and influence the thresholds of TCR occupancy required to initiate a T cell immune reaction.

One effector molecule regulated by the TCR and CD28 whose function in T cells is as yet poorly understood is PI 3-kinase (Cantrell et al., 1993; Ward et al., 1996). Elevation of cellular levels of PI(3,4,5)P$_3$ correlates with PI 3-kinase action. Studies of PI 3-kinase activation in T cells established that the TCR and the co-stimulatory receptor CD28 can act in concert to give a substantial rise in PI(3,4,5)P$_3$ levels (Ward et al., 1993). Initially, the regulatory effect of CD28 on PI 3-kinase raised the possibility that this enzyme mediated some of the co-stimulatory effects of CD28 (Rudd, 1996; Ward et al., 1996). However, numerous alternative CD28-induced signalling pathways have since been described that are candidates for positively modulating TCR thresholds for activation (August et al., 1994; Boucher et al., 1995; Nunes et al., 1996; Schneider et al., 1995; Su et al., 1994); and a role for PI 3-kinase in CD28-delivered co-stimulation of
cytokine production has not been validated (Crooks et al., 1995; Truitt et al., 1995; Ward et al., 1996). As CD28 has diverse biological functions (Lenshow et al., 1996; Rudd, 1996), the significance of PI 3-kinase in these other CD28 responses e.g. cell survival (Boise et al., 1995) remains to be assessed. Intriguingly, PI 3-kinase is also associated with inhibitory receptors such as CTLA-4 (Hutchcroft and Bierer, 1996; Schneider et al., 1995) or CD5 (Dennehy et al., 1997; Tarakhovsky et al., 1995). Moreover, in mice lacking expression of the cytokine interleukin-2 (IL-2) which regulates PI 3-kinase activity, a key phenotype is immune hyperactivity (Sadlack et al., 1993). It is thus clear that receptors which activate PI 3-kinase have major functions as negative regulators of the immune system.

TCR signal transduction events are subject to important auto-regulatory and para-regulatory negative feedback control mechanisms: it is increasingly recognised that receptors on T cells that generate terminating signals such as CTLA-4 or inhibitory MHC class I molecules are extremely important in immune homeostasis (Lanier and Phillips, 1996; Leach et al., 1996; Walunas et al., 1994; Waterhouse et al., 1995). Recent studies indicate that protein tyrosine phosphatases (PTPases) including SHP-1 and SHP-2 may be vital for these processes (DeFranco and Law, 1995; Imboden and Koretsky, 1995). For example, the PTPase SHP-1 has been shown to negatively regulate B cell antigen receptor signalling (Cyster and Goodnow, 1995; D’Ambrosio et al., 1995). The PTPase SHP-2 can associate with CTLA-4 and possibly plays a role in CTLA-4 action (Marengere et al., 1996). SHP-2 may be a valid theoretical candidate for mediating some aspects of negative signalling pathways in T cells, but to date, there is no functional evidence that it is actually responsible for the CTLA-4 inhibitory events. In reality, there is only a primitive understanding of the effector molecules that downmodulate TCR signal transduction, particularly in the context of negative feedback mechanisms generated by the TCR itself. As PI 3-kinase has the potential for
pleiotropic function, this enzyme could be critical in defining and revising the biological reactions of the TCR by eliciting negative signals.

In the present chapter, I employed the activated PI 3-kinase, rCD2p110, and a inhibitory mutant of PI 3-kinase, p85Δ (which lacks the p110 binding site) to analyse its cellular functions in T cell activation governed by the TCR. The transcription factors coordinate and implement various activating or inhibitory input messages and are ultimately responsible for fine-tuning of the genetic program. I therefore examined the importance of PI 3-kinase signals for TCR-induced transcription factor responses. I specifically investigated the function of transcription factors which are thought to be important for early gene activation orchestrated by the TCR. In particular, I concentrate on the effect of PI 3-kinase on the TCR-controlled transcription from the NF-AT (‘nuclear factor of activated T cells’) response element. Moreover, I attempted to define the effectors which PI 3-kinase may employ to impinge on the genetic program evoked by TCR engagement. Like in the previous chapters, I will focus on the role of the serine/threonine protein kinase B (PKB) (also termed c-Akt) and the GTPase Rac.

8.2 Results

The results presented in this chapter were performed with the help of Susan Lucas, Lymphocyte Activation Laboratory, ICRF.

PI 3-kinase signals block TCR-induced transcription from NF-AT response elements

Immediate responses to TCR triggering include the activation of transcription factors such as AP1, SRF and NF-AT (Ullman et al., 1990) which are ultimately important for cytokine gene expression. In the present report, I analysed the consequences of PI
3-kinase activation on transcription factor regulation controlled by the TCR. The T cell line Jurkat was transfected with reporter genes for different transcription factors together with constitutively active PI 3-kinase, rCD2p110 (see chapter 5, (Reif et al., 1996)). As control, cells were transfected with 'kinase dead' rCD2p110-R/P, rCD2p85 and wild-type rCD2. The transcription factor NF-AT can be induced by triggering of the TCR or by the combined action of calcium ionophore and phorbol ester (PdBu). TCR induction of NF-AT was completely abrogated by expression of activated rCD2p110 (Fig. 8.1). The repressory effect of the active PI 3-kinase on NF-AT induction was selective in that only TCR, but not calcium ionophore and phorbol ester responses were prevented (Fig. 8.1). TCR induction of NF-AT still occurred in the presence of wild-type rCD2, 'kinase dead' rCD2p110-R/P or rCD2p85(Fig. 8.1), demonstrating that the inhibitory effect of rCD2p110 on TCR/NF-AT

![Figure 8.1](image-url)

**Figure 8.1.** PI 3-kinase functions as a negative regulator of TCR-mediated NF-AT induction. Jurkat cells were transfected with NF-AT.CAT reporter plasmid together with empty vector or plasmids encoding rCD2p110, rCD2p110-R/P, rCD2p85, or rCD2 as indicated. Cells were left untreated or stimulated with the CD3 mAb UCHT-1 (*left panel*), or with PdBu and ionomycin (Iono) (*right panel*) for 13 h and then assayed for CAT activity. The CAT activity is presented as percentage conversion. The data are from a representative experiment. Similar results were obtained in seven more experiments.
responses requires the lipid kinase activity of the enzyme (Reif et al., 1996). It was noted that expression of rCDp85 could to some extent induce NF-AT activity. I have observed previously that rCD2p85 acts as a moderate inhibitory mutant of PI 3-kinase signalling pathways which could explain this potentiating effect of rCD2p85 on NF-AT transcriptional activity (see Fig. 8.5).

Figure 8.2. Expression of active PI 3-kinase does not interfere with TCR induced transcriptional activity from the SRE or of AP1. Jurkat cells were transfected with (A) SRE.CAT or (B) AP1.CAT reporter plasmids together with empty vector or plasmids encoding rCD2p110, or rCD2p110-R/P as indicated. Cells were left untreated or stimulated overnight with the CD3 mAb UCHT-1, PdBu, and PdBu plus ionomycin (Iono) as indicated and subsequently assayed for CAT activity. The CAT activity is presented as percentage conversion. The data are from a representative experiment. Similar results were obtained in four (A) or three (B) more experiments.
PI 3-kinase signals do not impair TCR-controlled signalling pathways leading to AP1- or SRF-mediated gene transcription

I next explored the role of PI 3-kinase signals on TCR induction of the transcriptional activity of the c-fos serum response element (SRE) or AP1-driven enhancer elements central to regulation of immediate early genes. Activation of SRE is mediated by p21ras/Raf/Erk pathways that can be elicited in T cells by TCR ligation or by phorbol ester (Fig. 8.2A). Expression of rCD2p110 had no stimulatory effect on SRE activity, but more importantly, it did not compromise TCR or phorbol ester induction of SRE. Transcriptional activity of AP1 complexes could be induced by TCR triggering or treatment with phorbol ester plus ionomycin (Fig. 8.2B). Co-expression of rCD2p110 did not alter these AP1 cellular responses. Therefore, expression of a constitutively active PI 3-kinase has a selective modulatory effect on the repertoire of transcription factor pathways orchestrated by the TCR: PI 3-kinase signals negatively regulate the TCR signalling pathways that lead to NF-AT-mediated transactivation, whilst leaving unaffected TCR induction of SRE and AP1-responsive elements.

The serine/threonine kinase PKB is stimulated by PI 3-kinase but active PKB cannot mimic the negative regulatory effect of active PI 3-kinase on NF-AT transactivation

PI 3-kinase signals are able to activate the serine/threonine kinase PKB (also termed c-Akt) in IL-2 dependent T cells (see chapter 7) and in fibroblasts (Klippel et al., 1996; Marte et al., 1997). This PI 3-kinase pathway is important in regulating cell survival responses in fibroblasts and neuronal cells (Dudek et al., 1997; Hemmings, 1997; Kauffmann-Zeh et al., 1997). The negative effects of active PI 3-kinase on TCR/NF-AT responses and its neutral role in TCR/SRE/AP1 activation prompted us to examine the effects of rCD2p110 on PKB activity in Jurkat cells. TCR triggering lead to an increase in PKB activity (Fig. 8.3A). Moreover, expression of membrane-localised PI 3-kinase is sufficient to stimulate PKB activity to a level comparable to that initiated
Chapter 8  Results: Negative role for PI 3-kinase in TCR events

Figure 8.3. PKB is activated by PI 3-kinase but PKB does not mediate PI 3-kinase regulatory effects on TCR triggered NF-AT induction.

(A) Jurkat cells were co-transfected with HA-PKB plasmids and vector plasmid (empty) or plasmid encoding rCD2p110 as indicated. Cells were left untreated or stimulated with the CD3 mAb UCHT-1 mAb for 5 min prior to lysis. HA-PKB activity was analysed in anti-HA-tag immune complex kinase assays using H2B as a substrate. [32P]phosphate incorporation into H2B (upper panel) was visualised by autoradiography. Protein levels of HA-PKB present in immune complexes were assessed in parallel by Western blotting with Rac-PK-CT Abs (lower panel).

(B) Jurkat cells were transfected with NF-AT.CAT reporter plasmid together with empty vector or plasmids encoding rCD2p110, or gagPKB as indicated. Cells were left untreated or stimulated for 14h with the CD3 mAb UCHT-1 and assayed for CAT activity. The CAT activity is presented as percentage conversion. - The data are from a representative experiment. Similar results were obtained in two (A, B) more experiments.

by triggering of the TCR/CD3 complex. PI 3-kinase action is thus not generally antagonistic and can positively regulate proximal TCR signal transduction events. I next investigated whether PKB is involved in transmitting the inhibitory effects of PI 3-
kinase on TCR signalling. I therefore examined the role of PKB in the modulation of TCR/NF-AT responses by PI 3-kinase: expression of gagPKB, a constitutively active PKB, did not mimic the effect of active PI 3-kinase to abrogate TCR/NF-AT induction (Fig. 8.3B). PKB is thus not a candidate to mediate the inhibitory effect of PI 3-kinase on TCR signalling.

Expression of an activated form of the GTPase Rac, V12Rac, does not abrogate NF-AT transactivation and induces transcription from AP1- and SRE-responsive elements

As demonstrated in chapter 6, active PI 3-kinase, rCD2p110, can initiate signalling pathways mediated by the GTPase Rac that lead to actin cytoskeleton rearrangements in fibroblasts (Reif et al., 1996). Rac can deliver signals for transcriptional activation including stimulation of stress activated protein kinases (see chapter 7, and (Coso et al., 1995; Hill et al., 1995; Minden et al., 1995)). I therefore tested the possibility that PI 3-kinase may exert its function via Rac. Expression of constitutively active Rac, V12Rac, does not substitute for PI 3-kinase and inhibit TCR/NF-AT responses (Fig. 8.4A). Moreover, V12Rac could stimulate SRE- (Fig. 8.4B) and AP1-controlled gene transcription (Fig. 8.4C) as could expression of Dbl (Fig. 8.4B, C), a guanine nucleotide exchange factor for Rho family GTPases, that activates endogenous Rac and Rho (Olson et al., 1996). Expression of rCD2p110 failed to elicit transcriptional activity from the SRE or by AP1 indicating that PI 3-kinase signals do not apparently initiate Rac-mediated transcription factor pathways in T cells. In addition, expression of inhibitory Rac, N17Rac, suppresses rather than potentiates TCR/NF-AT signal transduction cascades (Genot et al., 1996). These data demonstrate that effector pathways distinct from PKB- or Rac-governed signalling cascades must mediate PI 3-kinase effects on TCR function.
Figure 8.4. Expression of active V12Rac or Dbl does not mimic PI 3-kinase signals on gene transcription. Jurkat cells were transfected with (A) NF-AT.CAT, (B) SRE.CAT or (C) AP1.CAT reporter plasmids together with empty vector or plasmids encoding rCD2p110, V12Rac, or Dbl as indicated. Cells were left untreated or stimulated overnight with the CD3 mAb UCHT-1, PdBu, or PdBu plus ionomycin (lono) as indicated and subsequently assayed for CAT activity. The CAT activity is presented as percentage conversion. The data are from a representative experiment. Similar results were obtained in two (A), three (B) or four (C) more experiments.
Expression of inhibitory alleles or treatment with chemical inhibitors of PI 3-kinase enhance TCR/NF-AT responses

It has been known for some time that TCR activation of NF-AT is transient and subject to as yet uncharacterised negative feedback regulation (Hivroz-Burgaud et al., 1991; Loh et al., 1996; Rothenberg and Ward, 1996). The inhibitory effects of active PI 3-kinase on TCR/NF-AT-initiated transactivation coupled with the fact that the TCR activates PI 3-kinase raises the possibility that PI 3-kinase may elicit de-activation of TCR-provoked NF-AT responses. To test this hypothesis, I examined the effect of p85Δ, an inhibitory mutant of PI 3-kinase, on TCR induction of NF-AT. p85Δ is a p85 regulatory subunit that lacks the binding site for the p110 catalytic subunit, and therefore prevents regulation and recruitment of the active enzyme complex to the plasma membrane (Dhand et al., 1994). Expression of inhibitory p85Δ led to a small but reproducible basal induction of NF-AT, and strikingly synergised with TCR stimulation to induce NF-AT transcriptional activity (Fig. 8.5A and Fig. 8.5B). In contrast, in parallel experiments expression of p85Δ had no effect on TCR induction of SRE (Fig. 8.5C). Additional tools to study PI 3-kinase effector pathways are chemical inhibitors of PI 3-kinase function such as wortmannin or LY294002. The data in Fig. 8.5D show that wortmannin significantly potentiated NF-AT transcriptional activity triggered by the TCR. Similar potentiation of NF-AT responses were observed in cells treated with LY294002 (data not shown, performed by Elisabeth Genot, Lymphocyte Activation Laboratory, ICRF). This is consistent with the notion that a basal PI 3-kinase activity exists in quiescent T cells that prevents NF-AT-mediated gene transcription: upon TCR ligation D-3 phosphoinositide levels increase and antagonise the TCR signals that initiate NF-AT transcriptional activity.
Figure 8.5. Expression of inhibitory alleles or treatment with chemical inhibitors of PI 3-kinase potentiate TCR/NF-AT responses. Jurkat cells were transfected with NF-AT.CAT (A, B, D) or SRE.CAT (C). (A-C) Cells were co-transfected with empty vector or plasmids encoding rCD2p110, rCD2p110-R/P or p85Δ as indicated. Cells were left untreated or stimulated overnight with the CD3 mAb UCHT-1, or PdBu plus ionomycin (lono) as indicated. The experiments shown in (B) and (C) were performed in parallel. (D) Jurkat cells (JHTAg) were left unstimulated or were stimulated overnight with 1 μg/ml UCHT-1 in the presence of 50 nM wortmannin or vehicle DMSO as indicated. The CAT activity is presented as percentage conversion. The data are from a representative experiment. Similar results were obtained in four (A), two (B), or three (C, D) more experiments.
8.3 Discussion

A delicate balance between positive and negative signals is obligatory for an immune response. Therefore, it is important to characterise the full spectrum of intracellular molecules that integrate these input signals and their cellular targets. Although substantial progress has been made to delineate positive/proliferative events, much less is known about effectors that negatively modulate or terminate T cell activation. The present data reveal a previously unrecognised function for PI 3-kinase as a selective negative regulator of TCR responses. The importance of this negative signalling role for PI 3-kinase, defined herein, is underscored by its selectivity: PI 3-kinase signals thus are sufficient to stimulate PKB, allow TCR induction of AP1 or SRE, but abolish TCR-controlled NF-AT transactivation. Interestingly, an active allele of PKB did not abrogate NF-AT activity triggered by the TCR, nor did expression of an activated form of the GTPase Rac, a second well characterised effector of PI 3-kinase action. This implicates a novel as yet uncharacterised effector pathway as mediator of PI 3-kinase regulation of the TCR/NF-AT response. The failure of Rac to mediate PI 3-kinase signals on NF-AT or the SRE in T cells, is consistent with my earlier observations where PI 3-kinase could not substitute for Rac in inducing transcription from the SRE in fibroblasts or Elk-1 controlled transactivation in fibroblasts, Jurkat cells and Kit225 cells (see chapters 6, 7).

The transcriptional activation of NF-AT is a complex process that involves translocation of the cytosolic NF-AT component (NF-ATc) from the cytoplasm to the nucleus where it associates with its nuclear component (NF-ATn) which contains Fos/Jun family members that facilitate NF-AT DNA binding and transcriptional activity (Rao, 1994). The cytosolic component is encoded by an extensive gene family including NF-ATp/NF-AT1, NF-ATc, NF-AT3 and NF-AT4/NF-ATx. In Jurkat cells NF-ATp is expressed and can induce NF-ATc expression. Both can drive transcription from
NF-AT regulatory elements in Jurkat T cells (the role of the 'other' NF-ATs in this process is currently not known). The precise mechanism for NF-AT inhibition by PI 3-kinase awaits analysis. However, NF-AT induction by the TCR requires the coordinate action of signalling pathways mediated by the phosphatase calcineurin, the GTPase p21ras, and may involve the adapter protein SLP-76 and Vav (Cantrell, 1996; Wange and Samelson, 1996) (see chapters 1, 3). PI 3-kinase could thus exert its regulatory effects on NF-AT induction at multiple sites.

The first studies to document cytokine gene expression in T lymphocytes noted that this was a transient phenomenon subject to powerful negative feedback control. NF-AT family members govern the transcription of a number of genes including the cytokines IL-2, IL-3, and IL-4, and CD40 ligand and Fas ligand (Hodge et al., 1996; Rao, 1994). In this respect, it has been recognised for several years that negative feedback signalling mechanisms for the TCR-mediated induction of NF-AT exist (Hivroz-Burgaud et al., 1991; Loh et al., 1996; Rothenberg and Ward, 1996). These downregulating mechanisms are likely to be responsible for the transience of cytokine gene expression during T cell activation. The present data showing the reciprocal effects of activating and inhibitory mutants of PI 3-kinase on TCR-delivered NF-AT responses are consistent with a model in which PI 3-kinase has a negative feedback or modulatory role in TCR-controlled induction of NF-AT activity (Fig. 8.6). TCR-triggering itself activates PI 3-kinase and the physiological relevance of the inhibitory PI 3-kinase signals, described herein, may be a homeostatic control mechanism in TCR function that would temporally restrict cytokine production to a limited period of T cell activation. Moreover, TCR induced cytokines such as IL-2 and IL-4 act in autocrine loops to drive T cell cycle progression (Cantrell et al., 1988; Fernandez Botran et al., 1986). These growth factors are powerful activators of PI 3-kinase (Gold et al., 1994; Remillard et al., 1991; Wang et al., 1992), and a PI 3-kinase-mediated NF-AT inhibitory pathway would ensure that cytokine production is restricted to cells that had not yet received a growth signal. This
may be salient, as it seems of little value for a cell that is committed to proliferate to continue to produce growth regulatory cytokines (Fig. 8.6). In this context, it has been reported that NF-ATp/NF-AT1 deficient mice are hyperproliferative or show enhanced immune responses (Hodge et al., 1996; Xanthoudakis et al., 1996), which suggests that NF-AT family proteins are involved in downregulating certain biological responses.

Figure 8.6. Schematic representation of the selective negative role of PI 3-kinase in T cell antigen receptor (TCR) signalling leading to gene transcription. During T cell activation PI 3-kinase is engaged by the TCR. Other receptors which regulate PI 3-kinase activity include CD28, CTLA-4, the IL-2R and the IL-4R. TCR engagement delivers positive signals to transcription factors such as AP1, TCFs, SRF, and NF-AT which ultimately coordinate early gene transcription. This induction of transcription factors is subject to negative feedback mechanisms generated by the TCR itself and activation-dependent receptors such as CTLA-4, the IL-2R and the IL-4R.

PI 3-kinase mediates positive aspects of TCR signal transduction as it stimulates protein kinase B (PKB), an effector of PI 3-kinase which can regulate cell survival and protein synthesis. PI 3-kinase has the potential to activate the GTPase Rac in order to control cytoskeletal responses as demonstrated in fibroblasts. PI 3-kinase also delivers potent inhibitory signals and negatively regulates NF-AT activity induced by the TCR via an unknown effector protein. This negative effect of PI 3-kinase is selective and does not affect TCR-controlled transcription from AP1- or TCFs/ SRF-responsive elements. (For further details see text.)
There could also be broader implications for the terminating effects of PI 3-kinase on TCR function described herein: molecules such as CTLA-4 that negatively regulate T cell activation (Leach et al., 1996; Walunas et al., 1994; Waterhouse et al., 1995) associate with PI 3-kinase (Schneider et al., 1995). Previous studies have ascribed the negative effects of CTLA-4 signalling to the association of this receptor with the tyrosine phosphatase SHP-2 (Marengere et al., 1996). However, there is no direct evidence that SHP-2 acts to negatively regulate T cell functions and it is noteworthy that the p85 subunit of PI 3-kinase and SHP-2 interact via their SH2 domains with the same phosphotyrosine motif, YVKM, in the cytoplasmic domain of CTLA-4 (Marengere et al., 1996; Schneider et al., 1995). PI 3-kinase could thus be a candidate to mediate the negative effects of CTLA-4 on T cell biology. In addition, PI 3-kinase could play a role in the negative signalling pathways initiated by cytokines such as IL-2 and IL-4. Finally, cytokines such as IL-4 control the differentiation of Th1/Th2 T cell subsets. The TCR is well documented to elicit different genetic programs in the presence of IL-4. Thus, IL-4 can inhibit IL-12R β2 expression - a component of the IL-12R which is selectively expressed on Th1 subsets - leading to the loss of IL-12 signalling and the promotion of Th2-dependent responses (Rogge et al., 1997; Szabo et al., 1997). Consistent with this notion, PI 3-kinase can alter the transcription factor responses induced by the TCR which makes PI 3-kinase a plausible candidate to deliver modulatory effects of cytokines on TCR immune reactions. These are obviously complicated issues as PI 3-kinase signals will undoubtedly be influenced by other signalling pathways concurrently evoked in a cell. PI 3-kinase may thus have multiple roles and the precise outcome of PI 3-kinase activation may be determined by the number of different receptors that simultaneously engage PI 3-kinase (quantitative modulation) (Fig. 8.6), and/or the disparate effector pathways that are induced by diverse receptors and can cross-talk to PI 3-kinase effector cascades (qualitative modulation).
As shown in the present chapter, PI 3-kinase signals will modulate patterns of transcription factor activity and hence alter gene expression orchestrated by the TCR in a specific manner, rather than generally disrupt TCR responses and operate as a simple 'off signal' for the TCR. Modulation of TCR function occurs at repeated points during T cell development and activation e.g. during the processes of thymocyte positive versus negative selection or T cell activation versus anergy. The present data identify PI 3-kinase as a determinant of TCR signalling thresholds for activation.
Conclusions and perspectives

Picking up the loose ends

When PI 3-kinase first moved into molecular view a few years ago, we learnt a great deal about its lipid products, the identity of cell surface receptors activating PI 3-kinase and their regulatory mechanisms resulting in PI 3-kinase activation. We now know that quite a few receptors stimulate the production of D-3 phosphoinositides. In T cells D-3 phosphoinositiode generation has been measured in response to engagement of the TCR, CD28 and the IL-2R. Many other receptors such as the IL-4R, the IL-7R or CTLA-4 are thought to regulate PI 3-kinase activity based on the observation that these receptors associate with PI 3-kinase activity upon stimulation.

TCR regulation of PI 3-kinase

One well studied model system which revealed some of the principles of PI 3-kinase regulation is the PDGF receptor. Upon PDGF treatment the p85 subunit of PI 3-kinase associates via its SH2 domains with the tyrosine phosphorylated cytoplasmic receptor tail. The first attempts to show a similar mechanism in T cells after TCR triggering failed, as PI 3-kinase activity could not be recovered from anti-phosphotyrosine immunoprecipitates and high affinity associations with the TCR were not observed (Osman et al., 1996; Ward et al., 1992). I was able to identify a possible mechanism how the TCR could regulate PI 3-kinase. The p85 subunit of PI 3-kinase can associate with the adaptor molecule Grb2 in intact T cells - an association which is constitutive and not regulated by the TCR. The interaction is mediated by proline-rich motifs in p85 and the SH3 domains of Grb2. This was shown by affinity purifying Grb2 and its associated partners from cells with synthetic phosphotyrosine- or proline-
peptides encoding the cognate binding sites for Grb2 SH2 or Grb2 SH3 domains, respectively. In parallel studies, other investigators established that a similar constitutive association with the SH3 domain of src family tyrosine kinases can occur (Prasad et al., 1993). Complexes of both Grb2/p85 or Src-PTK/p85 are alike regarding their affinity and stoichiometry. Which complex is prevalent in TCR function is currently not clear, however, the regulation via an interaction with Grb2 is attractive as Grb2 is likely to shuttle from the cytosol to the plasma membrane upon TCR stimulation. This is based on the notion that Grb2 can associate with a 36 kDa protein that becomes tyrosine phosphorylated in TCR-activated cells and is found predominantly in the membrane fraction in stimulated cells (Buday et al., 1994). Although the precise mechanism of PI 3-kinase regulation during TCR signal transmission remains unresolved, I would favour a model relaying on several adaptor-type proteins as this has the advantage to position different pools of PI 3-kinase into the proximity of multiple effector molecules. Here the magic word may be 'caveolae' and their composition. Caveolae are rosettes of fused vesicles or glycolipid islands in the plasma membrane enriched in tyrosine kinases such as the src family PTKs Fyn and Lck (Lisanti et al., 1995; Parton, 1996). Other molecules including Ras, Grb2, or Raf are also concentrated in the caveolae. It is possible that the highly organised features of caveolae allow contact to certain subsets of effector molecules in a timely and spatial restriction.

**Grb2 associates via its SH3 domains with a novel adaptor protein**

The p85 subunit of PI 3-kinase is not the only signalling molecule bound to Grb2 SH3 domains in T lymphocytes. On the contrary, the major Grb2 SH3 domains associated proteins in T cells are Sos and a novel protein that I was able to identify, p75. All the above protein complexes are mutually exclusive. p75 is constitutively bound to Grb2 SH3 domains and is tyrosine phosphorylated in response to TCR stimulation. Another feature sparking the interest of immunologists concerned with signal transduction is the fact that it is exclusively expressed in haematopoietic cells. p75 is
identical with SLP-76, a protein containing several tyrosines, proline-rich motifs and an C-terminal SH2 domain that was cloned 1995 by Jackman and colleagues (Jackman et al., 1995). SLP-76 is now well established to be an interesting adaptor molecule and is likely to have a role in signalling pathways leading to NF-AT induction and IL-2 transcription (Motto et al., 1996; Wu et al., 1996).

**Cellular effectors of PI 3-kinase No. 1: Rac delivers PI 3-kinase signals to the actin cytoskeleton**

After a year or so analysing the regulation of protein complexes by the TCR I came to realise that these interactions are complex and it may be time to move on and find out about possible biological functions of PI 3-kinase. One approach to tackle this problem, is to create a constitutively active enzyme which allows to assess the relative contribution of a signalling pathway and facilitates the identification of possible downstream effector molecules. I generated a constitutively active PI 3-kinase by targeting the p110 catalytic subunit of PI 3-kinase to the plasma membrane. p110 was fused to a truncated rat CD2 cell surface receptor giving rise to rCD2p110. rCD2p110 is a true constitutively active PI 3-kinase as it raises the levels of D-3 phosphoinositides in intact cells.

In collaboration with Kate Nobes and Alan Hall, we used active PI 3-kinase to demonstrate that PI 3-kinase signals are sufficient to activate Rac- and Rho-dependent effector pathways leading to changes in the architecture of the cytoskeleton. Expression of rCD2p110 induced actin reorganisation in the form of Rac-mediated lamellipodia and focal complexes, and Rho-mediated stress fibres and focal adhesions. How PI 3-kinase puts the GTP on Rac to initiate this cytoskeletal Rac/Rho-GTPase cascade is not clear, however, it could involve binding of D-3 phosphoinositides to the PH domain of putative Rac GEFs or it could involve any other regulatory protein such as GAPs or GDIs. Active PI 3-kinase was born into a time when we started to realise that
Rac/Rho GTPases like to keep their options open and are attached to quite a few downstream effector pathways (Lamarche et al., 1996; Tapon and Hall, 1997; Westwick et al., 1997). Thus, Rac and Rho do not only trigger cytoskeletal changes but activate signalling pathways leading to gene transcription (Coso et al., 1995; Hill et al., 1995; Minden et al., 1995). In marked contrast, PI 3-kinase can only sufficiently activate the Rac/Rho effector cascade leading to actin rearrangements but not the one resulting in gene transcription. This was initially assessed in fibroblasts, but similar results were obtained in Jurkat T cells where PI 3-kinase signals also failed to activate Rac/Rho-dependent gene transcription pathways. Moreover, receptors such as the PDGF receptor that are potent activators of PI 3-kinase and a PI 3-kinase-dependent Rac/Rho cascade resulting in cytoskeletal changes, are poor activators of Rac/Rho-induced transcription factor responses. Finally, expression of Dbl, a GEF for Rac/Rho proteins, can stimulate the full repertoire of Rac/Rho-mediated responses. To cut a long discussion about possible mechanisms to explain these data short (see chapter 6), the conclusion is again: specific localisation - this time of regulator / GTPase / effector complexes to different cellular compartments. How this is achieved and what is the nature of these compartments is difficult to guess, but may involve different protein lipidation signals (Casey, 1995) or may be just simply plasma membrane, cytoplasmic or nuclear compartmentalisation per se.

**Cellular effectors of PI 3-kinase No. 2: PKB controls the suppression of apoptosis**

PI 3-kinase may be finally moved from the margin into the spot light when it turned out to regulate cell survival (Yao and Cooper, 1995). Recent reports establish that this function of PI 3-kinase is mediated by the serine/threonine kinase PKB/Akt. Expression of activated forms of PI 3-kinase or PKB protects Cos7 cells from apoptosis by UV irradiation (Kulik et al., 1997) or neuronal cells (PC12) from cell death induced by the withdrawal of the survival factor IGF-1 (Dudek et al., 1997). The protooncogene
Myc induces apoptosis under conditions where serum survival factors including cytokines are not present (Harrington et al., 1994) and active PI 3-kinase or active PKB can protect from this process (Kauffmann-Zeh et al., 1997). In this respect, I demonstrate that IL-2 regulates PKB activity, PI 3-kinase activity is required for PKB stimulation by IL-2 and active PI 3-kinase signals are sufficient to fully activate PKB. The cytokines IL-2, IL-4, and IL-7 promote the survival of resting T cells that do not proliferate in response to these signals. These findings may provide an inroad of how IL-2 regulates cell survival. IL-2 has been reported to induce in BAF-B03 derived cells bcl-2, bcl-xL and bax gene expression (Miyazaki et al., 1995), members of a burgeoning family of genes that participate in the control of apoptosis (referred to as the bcl-2 family) (Gajewski and Thompson, 1996). The bcl-2 family contains both anti-apoptotic and pro-apoptotic members. It will be interesting to assess whether any of the gene products of the bcl-2 family mediate the effects of PI 3-kinase/PKB on cell survival. One hint may derive from the notion that bcl-2 expression by IL-2 in BAF-B03 cells is sensitive to rapamycin. In the cell systems mentioned above, the PI 3-kinase/PKB signals leading to suppression of apoptosis are insensitive to rapamycin. Thus, it is unlikely that bcl-2 is a direct target of PI 3-kinase/PKB in the IL-2 system. These data also exclude p70S6k as an mediator of PI 3-kinase/PKB anti-apoptotic signals. Nevertheless, I and others have demonstrated that active PKB and active PI 3-kinase are sufficient to stimulate p70S6k in T cells or fibroblasts (see chapters 6, 7). This warrants additional roles for PI 3-kinase and PKB in regulating distinct biological functions including the regulation of protein biosynthesis and cell cycle control (see below). Finally, the ability of PKB to promote cell survival in the absence of normal protective means may be critical to its function as an oncogene in the AKT8 virus. PKB can synergise with the Raf/Map kinase pathway to cause transformation in NIH 3T3 cells (Marte et al., 1997). Moreover, PI 3-kinase/PKB signals may contribute to cell cycle progression through induction of E2F (Paul Brennan and Doreen Cantrell, personal communications).
Cellular effectors of PI 3-kinase No. 3: unknown

One question which will be standing for some time, I guess, is concerning the identity of 'The Third Effector' that mediates the negative signals of PI 3-kinase on TCR regulation of NF-AT. Expression of PI 3-kinase prevents the TCR to induce NF-AT transcriptional activity. This negative regulatory effect is selective: PI 3-kinase signals do not abolish (1) phorbol ester plus ionomycin induction of NF-AT, (2) TCR-regulation of AP1 transcriptional activity and (3) SRE induction triggered by the TCR. Moreover, expression of p85A, a dominant negative mutant of PI 3-kinase actions, potentiates NF-AT induction by the TCR. PI 3-kinase can stimulate PKB activity to a similar levels as the TCR. However, the negative effect of PI 3-kinase on NF-AT transcriptional activity is not mediated by PKB, nor by the GTPase Rac. Therefore, it is likely that a novel as yet unknown effector molecule is responsible for these effects. The many roads that lead to NF-AT induction have to be analysed in detail to gain a clue at which level this effector may act. The most feasible approach to begin with is probably to assess whether the cytosolic form of NF-AT, NF-ATc, can still translocate to the nucleus. This could be achieved by co-expressing rCD2p110 together with a tagged version of NF-ATc - using e.g. GFP (green fluorescence protein) as a tag - and an (immunofluorescence) analysis of its capacity to translocate to the nucleus in the presence of PI 3-kinase. On the speculative side, it could also be worth to examine whether Cbl phosphorylation or association patterns are altered when PI 3-kinase is co-expressed. However, such an analysis most likely would require the generation of an inducible PI 3-kinase. This would allow to obtain stable cell lines in which PI 3-kinase expression can be induced for functional analyses. It would thus be possible to grow large numbers of cells for biochemical studies.
PI 3-kinase in T lymphocyte biology and immune function

What are my predictions from these studies for the role of PI 3-kinases in T cell immune function? Three major functions come to mind along the lines of the three PI 3-kinase effectors. One implications is certainly that PI 3-kinase may be important during responses that require the T cell to rearrange its cytoskeleton. There are two major examples where the T cell gets into shape by polarising its actin cytoskeleton. During antigen presentation and contact formation between a T cell and an APC, the T cell becomes highly polarised forming a bulge in the contact zone towards the APC. Indeed, experiments using wortmannin suggest that PI 3-kinase is required for cytoskeletal polarisation in T cells (Stowers et al., 1995). Polarisation of the cytoplasm can also be observed when cytotoxic T cells attack cancer cells or other cells bearing foreign invaders (Fig. 9.1). In general, PI 3-kinase may play a role in coordination of cell-cell contact, polarised cytokine release or T cell motility including migration into tissues and chemotaxis.

Figure 9.1. T lymphocyte fighting cancer cell.
Part of a cancer cell with several cytotoxic T lymphocytes attacking. The typical polarisation of the activated T lymphocyte towards the cell-cell contact zone is well demonstrated by the two cells in the centre of the picture. The T lymphocyte in the foreground is not yet in contact with the cancer cell and inactive.

Reproduced from Boehringer Ingelheim International GmbH; T lymphocytes fighting cancer 1; photo: Dr. h.c. Lennart Nilsson. © 1985

As mentioned above, PI 3-kinase may have a role in regulating cell survival by cytokines such as IL-2, IL-4 and IL-7, or also CD28 (Boise et al., 1995), under
circumstances where the cells do not want to proliferate. This might be directly connected to the role of PI 3-kinase in preventing NF-AT induction. NF-AT family members are essential for the activation-dependent transcription of cytokine genes. Therefore, PI 3-kinase signals may prevent induction of cytokine genes under conditions where proliferation is put on halt. In this respect, it is worth stressing that different subpopulations of T cells can vary dramatically in their cellular responsiveness to signalling pathways. Thus, the susceptibility of cells to induction of apoptosis by the 'death-inducing' receptor Fas can vary markedly (Nagata, 1997). PI 3-kinase may have a role in protection from Fas-induced apoptosis. The differences in susceptibility to Fas may be explained by quantitative or qualitative differences in the activation levels of PI 3-kinase. Together with the possible consequences of PI 3-kinase-mediated inhibitory effects on NF-AT regulatory pathways mentioned in chapter 8, this identifies PI 3-kinase as a major candidate in immunomodulatory signal transduction pathways and as a determinant of lymphocyte homeostasis.

Final remarks

A good model system for studying the role of PI 3-kinase in cell cycle control and/or cell size control may exist in the PI 3-kinase homologue in *Drosophila melanogaster*, Dp110 (Leevers et al., 1996). Overexpression of an activated membrane-targeted Dp110 mutant in wing or eye imaginal discs results in adult flies with enlarged wings or eyes. These size differences are mediated both by changes in cell size (in the wing and eye) and cell number (in the wing). PI 3-kinase is an upstream regulator of p70S6k. This regulation requires the activity of Frap the cellular target of rapamycin. Rapamycin prevents the IL-2 induced degradation of p27\textsuperscript{Kip1} - a cell-cycle dependent kinase inhibitor of Cdc2 - and hence prevents cell cycle progression from G1 to S (Nourse et al., 1994). Targeted disruption of the murine p27\textsuperscript{Kip1} gene enhances growth in mice and leads to increased numbers of cells in all of the organs examined (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). In oligodendrocytes
the progressive accumulation of p27Kip1 appears to determine the timing of differentiation - differentiation being here analogous to a stop in cell division (Durand et al., 1997). There must be a link between PI 3-kinase, growth and the control of p27Kip1 levels in T cells!

Key work ahead lies in the discovery of the cellular acceptor molecules of D-3 phosphoinositides and their cellular substrates linking PI 3-kinase action to cytoskeletal changes, cell survival, protein biosynthesis, growth control and immunomodulatory functions. After all PI 3-kinase likes to keep its options open!
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Chapter 10 References


Phosphatidylinositol 3-Kinase Links the Interleukin-2 Receptor to Protein Kinase B and p70 S6 Kinase

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Phosphatidylinositol 3-kinase (PI 3-kinase) is activated by the cytokine interleukin-2 (IL-2). We have used a constitutively active PI 3-kinase to identify IL-2-mediated signal transduction pathways directly regulated by PI 3-kinase in lymphoid cells. The serine/threonine protein kinase B (PKB/Akt) can act as a powerful oncogene in T cells, but its positioning in normal T cell responses has not been explored. Herein, we demonstrate that PKB is activated by IL-2 in a PI 3-kinase-dependent fashion. Importantly, PI 3-kinase signals are sufficient for PKB activation in IL-2-dependent T cells, and PKB is a target for PI 3-kinase signals in IL-2 activation pathways. The present study establishes also that PI 3-kinase signals or PKB signals are sufficient for activation of p70 S6 kinase in T cells. PI 3-kinase can contribute to, but is not sufficient for, activation of extracellular signal-regulated kinases (Erks) and Erk effector pathways. Therefore, PI 3-kinase is a selective regulator of serine/threonine kinase signal transduction pathways in T lymphocytes, and this enzyme provides a crucial link between the interleukin-2 receptor, the protooncogene PKB, and p70 S6 kinase.

The high affinity interleukin-2 receptor (IL-2R), which comprises α-, β-, and γ-subunits controls Q_{10} S progression, T cell clonal expansion, and functional differentiation (1–3). The IL-2R orchestrates downstream effector pathways by protein tyrosine kinase-dependent activation mechanisms engaging the Src family tyrosine kinases Lck and Fyn (4) and the Janus kinases 1 and 3 (5–7). Signaling cascades integrated by the action of these tyrosine kinases include activation of the Ras/Raf/extracellular-signal-regulated kinase (Erk) pathway (8–10), activation of the transcription factors STAT3 and STAT5 (11), and the regulation of phosphatidylinositol 3-kinase (PI 3-kinase) (12).

PI 3-kinase is a ubiquitously expressed enzyme that catalyzes the phosphorylation of phosphoinositides at the D-3 hydroxyl of the myo-inositol ring generating PI 3-phosphate, PI 3,4-bisphosphate, and PI 3,4,5-trisphosphate (13,14). The form of PI 3-kinase involved in protein-tyrosine kinase-dependent receptor signal transduction comprises a regulatory subunit of PI 3-kinase to tyrosine 392 in the IL-2R β-chain (15); in addition, interleukin-2 (IL-2) stimulation results in the interaction of PI 3-kinase with the Src family kinases Fyn (16) and Lck (17).

The activation of PI 3-kinase is a response that IL-2 shares with other cytokines that control lymphoid cell growth and development such as IL-4 and IL-7 (18,19). It is also clear that PI 3-kinase activation is necessary for the growth- and differentiation-inducing properties of these cytokines (20–23). However, despite the pivotal role of PI 3-kinase in lymphoid cells, there is only a preliminary and incomplete understanding of the targets for this enzyme in the mitogenic signaling pathways regulated by the hematopoietin family of cytokines. To date, the identification of biochemical targets for PI 3-kinase in T cells stems mainly from studies employing the PI 3-kinase inhibitor wortmannin or the LY294002 compound (10, 20). Hence, IL-2 activation of the mitogen-activated protein (MAP) kinase Erk is sensitive to wortmannin (10). Similarly, IL-2 activation of the serine/threonine kinase p70 S6 kinase (p70S6k) is prevented by these PI 3-kinase inhibitors (20). In addition, IL-2 activation of p70S6k is impeded by the immunosuppressant rapamycin, which targets another member of the PI 3-kinase family of enzymes, Frap (FKBP12-rapamycin-associated protein) also termed "mammalian target of rapamycin" (mTor) (24, 25).

Observations that wortmannin and rapamycin have identical inhibitory effects on IL-2 activation of p70S6k generated a model for the p70S6k signaling pathway in which PI 3-kinase acts as an upstream regulator of Frap (24, 25). However, this model has been challenged by a recent study showing that the action of Frap is directly inhibited by wortmannin and LY294002 (26). These results raise the issue of whether PI 3-kinase itself has any upstream regulatory role in p70S6k activation in T lymphocytes. Similar caution must be applied to interpretations of data involving PI 3-kinase in Erk activation in T cells. In this context, expression of an active PI 3-kinase is sufficient for Erk activation in Xenopus oocytes (27), but it would be fallacious to extrapolate data obtained in Xenopus cells to T cells, since the role of PI 3-kinase as an upstream regulator of kinase pathways can vary depending on the cell system; to this end, PI 3-kinase signals did not stimulate Erk activity in a variety of fibroblasts and in a monoblast.
cell line (28–31). Whether PI 3-kinase signals are sufficient to stimulate p70S6k or Erk activation in T cells awaits analysis.

We and others have recently reported that targeting the catalytic p110 subunit of PI 3-kinase to the plasma membrane generates a constitutively active enzyme that induces cellular accumulation of D-3 phosphoinositides (28–31). A constitutively active PI 3-kinase finally allows assessment of the relative contribution of PI 3-kinase-derived signals to a certain effector pathway, in particular whether PI 3-kinase activation is sufficient to promote a specific cellular response. In the present study, we have used a membrane-localized p110 construct, rCD2p110, that induces accumulation of cellular levels of PI 3,4-bisphosphate and PI 3,4,5-trisphosphate (29) as a tool to explore the regulation of serine/threonine kinase pathways by PI 3-kinase in T lymphocytes. We show that activation of PI 3-kinase is sufficient to stimulate p70S6k activity, although PI 3-kinase signals were not sufficient to induce activation of the MAP kinase Erk in T cells. The present study also characterizes a previously unrecognized IL-2-mediated signal transduction pathway in T cells that involves the serine/threonine protein kinase B (PKB) also known as AKT or Rac protein kinase (32–34). PKB was originally identified as the cellular homologue of the directly transforming oncoprotein of the murine retrovirus AKT8, which causes thymic lymphomas (35). Herein, we demonstrate that PKB is rapidly activated by IL-2 via a wortmannin- and LY294002-sensitive but rapamycin-insensitive pathway. PI 3-kinase signals alone were sufficient to activate PKB in T cells, and expression of a constitutively active PKB could stimulate the activity of p70S6k. Therefore, PI 3-kinase is a selective regulator of serine/threonine kinase signal transduction pathways in T lymphocytes, and this enzyme is an upstream regulator of the IL-2-activated kinases PKB and p70S6k.

EXPERIMENTAL PROCEDURES

Reagents—Phorbol 12,13-dibutyrate (PdBu) and wortmannin were from Calbiochem. LY294002 was a gift from Zeneca. PD098059 was from Sigma Corp. 

Antibodies—Ox34 monoclonal antibody (mAb) is raised against rat CD2 (rCD2) (29); 12CA5 mAb is reactive with hemagglutinin (HA), and 2E10 mAb is reactive with the Myc epitope (36); anti-human S6 kinase M5 antiserum (37) was from Santa Cruz Biotechnology; M1 antiserum (37) was reactive with p70S6k (37) was a gift from G. Thomas; Rac-PK-CT Ab (Upstate Biotechnology, Inc.) is reactive with PKB.

Plasmids and Reporter Constructs—HA-p70S6k (pBJ5) (38); HA-PKB (pSBG) and gagPKB (pSOG) (32); HA-Erk2 (pCEP4) (39); Myc-V12Rac (pEF), Myc-V12Cdc42 (pEF), and Myc-V14Rho (pEF) (40); and HA-vas (pEF) (39) vector constructs have been described. The described rCD2p110, rCD2p110-RP, and rCD2p95 chimeras (29) were subcloned into the pEF-BOS expression vector. The reporter plasmids Neps-Elk-1 (pEF) and 2lexoph.CAT (41) as well as Nels.C2 (pMLV) (42) have been described.

Cell Culture and Transient Transfections—The Kit225 T leukaemic cell line (43) was maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum supplemented with 20 ng/ml of recombinant IL-2 (rIL-2) (Euroclonex) under normal growth conditions. For IL-2 activation assays of endogenous proteins, Kit225 cells were washed three times with PBSA to remove the IL-2 and cultured further in RPMI supplemented with 5% fetal calf serum in the absence of rIL-2 for 48–72 h prior to IL-2 activation assays. When Kit225 cells were transfected, cells were treated as above but only deprived of rIL-2 for 24 h prior to transfection.

Kit225 cells were transfected by electroporation with 20–40 µg of plasmid DNA. The amounts of plasmid DNA were kept constant per cuvette by adding vector plasmid. Kit225 cells (1.5 × 10⁶ cells/0.625 ml) were pulsed at 320 V and 960 microfarads using a Gene Pulsor (Bio-Rad). The amounts of plasmid vector were as follows (unless indicated otherwise): 7.5 µg of HA-p70S6k, 12.5 µg of HA-PKB, 10 µg of HA-Erk2, 20 µg of the plasmid pEF-empty, rCD2p110, rCD2p110-RP, Ha-vas, rCD2p110-CAT, and 7.5 µg of p2lexoph.CAT; 15 µg of pEF-Ne-Eek1-1 or pMLVNe-Eek2. For gene reporter assays, cells were stimulated as indicated 2–4 h after transfection. Cells were collected 14–18 h after transfection.

Immunoprecipitation, p70S6k Assays and Western Blot Analysis—After stimulations as indicated, Kit225 cells were lysed in lysis buffer 1 (120 mM NaCl, 50 mM Tris pH 8.0, 20 mM NaF, 1 mM benzamidine, 1 mM EDTA, 6 mM EGTA, 7.5 mM PFP, 15 mM p-nitrophenyl phosphate, 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM

**Fig. 1.** Interleukin-2 activates p70S6k, which can be mimicked by co-expression of membrane-localized constitutively active PI 3-kinase, rCD2p110. A, Kit225 cells were deprived of rIL-2 for 68 h and treated with 20 ng/ml rIL-2 or 50 ng/ml PdBu for the indicated times, and p70S6k activity/phosphorylation was assessed by electrophorography (top) and autoradiography. C, the data were analysed by densitometry and quantified using a Phosphorlmager. Data are presented as the ratio of [32P]phosphate incorporated into S6 to [35S]conjugated protein A bound to p70S6k (expressed in arbitrary units).
A, ability shift assays; PK-CT Abs and PKB activity was analyzed in immune complex kinase assays using H2B as a substrate. Phosphate incorporation into H2B complexes were assessed in parallel by Western blotting with Rac-LY294002, but not rapamycin, inhibit IL-2-dependent activation of PKB. Kit225 cells starved of rIL-2 for 72 h were pretreated for 30 min with the vehicle dimethyl sulfoxide (DMSO), 20 ng/ml rapamycin, 5 μM wortmannin or 100 nM wortmannin and then stimulated with rIL-2 for 60 min. Gene expression assays were carried out as described (45). The data are presented as percentage of conversion.

**PKB Assays**—Cells were treated as for p70S6K assays except that lysis buffer 2 (120 mM NaCl, 50 mM Hepes, pH 7.4, 10 mM NaF, 1 mM EDTA, 40 mM β-glycerophosphate, pH 7.5, 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na3VO4) was used to lyse cells. To immunoprecipitate endogenous PKB, 2 μg of Rac-PK-CT Abs were used. The immunoprecipitates were washed twice in lysis buffer 2, twice in high salt wash buffer (500 mM LiCl, 100 mM Tris, pH 7.5, 1 mM EDTA, pH 7.5), and once in PKB assay buffer (50 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol). The reaction was initiated by the addition of 15 μl of PKB reaction buffer containing 3 μCi of [γ-32P]ATP, 50 μM ATP, 7.3 μM MgCl2, 7.3 μM dithiothreitol, 500 μM protein kinase inhibitor (Sigma), 40 mM Tris, pH 7.5, and 2.5 μg of histone 2B (H2B) (Boehringer Mannheim). After 30 min at 37 °C, the reaction was terminated by adding reducing SDS-PAGE sample buffer and boiling. Proteins were resolved by SDS-PAGE, and the gel was treated as for p70S6K assays. To detect PKB proteins, Western blot analysis was performed with Rac-PK-CT Abs.

**Erk Assays**—Cells and cell extracts were processed as for p70S6K assays. HA-tagged Erk2 was immunoprecipitated with 12CA5 mAbs. Immunoprecipitated immune complexes were washed three times with lysis buffer 1 and once with Erk wash buffer (30 mM Tris, pH 8.0, 20 mM MgCl2, 2 mM MnCl2). The reaction was initiated by the addition of 10 μl of Erk reaction buffer containing 4 μCi of [γ-32P]ATP, 20 μM ATP, 20 mM MgCl2, 2 mM MnCl2, 5 mM p-nitrophenyl phosphate, 500 nM protein kinase inhibitor (Sigma), 30 nM Tris, pH 8.0, and 15 μg of myelin basic protein (Sigma). After 30 min at 37 °C, the reaction was terminated by adding reducing SDS-PAGE sample buffer and boiling. Proteins were resolved by SDS-PAGE, and the gel was treated as for p70S6K assays. To detect Erk proteins, Western blot analysis was performed with 12CA5 mAbs as primary Ab, rabbit anti-mouse IgG as secondary Ab, and 32P-conjugated protein A.

**Gene Expression Analysis**—Fourteen to 16 h after inductions, as indicated, Kit225 T cells were harvested and cells were lysed in 200 μl of lysis buffer (0.65% Nonidet P-40, 10 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl). Gene expression assays were carried out as described (45). The data are presented as percentage of conversion.
expression of the rCD2p110 chimera was confirmed by flow cytometric immunofluorescence analysis with rCD2 mAbs (data not shown). The HA-tagged p70S6k was immunoprecipitated from transiently transfected cells and assayed for its ability to phosphorylate S6 ribosomal subunits (Fig. 1B). Expression of the active PI 3-kinase, rCD2p110, resulted in constitutive IL-2-independent p70S6k activation (Fig. 1B). p70S6k was not constitutively activated in cells expressing "kinase-dead" rCD2p110-R/P, confirming that the p70S6k activation requires the kinase activity of the p110 subunit (Fig. 1C). The expression of rCD2p110-R/P was noted in some experiments to suppress rIL-2 inducibility of p70S6k, indicating that this chimera may be an inhibitory mutant of PI 3-kinase pathways.

IL-2 and Membrane-localized PI 3-Kinase Activate PKB—PKB can be activated by receptor tyrosine kinases such as the platelet-derived growth factor receptor and has been identified as a target of PI 3-kinase in fibroblasts (28, 32, 33). However, whether this pathway is conserved in the hematopoietic system has not been explored. In particular, although PKB can become oncogenic and initiate thymic tumors, its regulation and significance for normal T cell growth processes is not known. Since cytokine receptors have essential functions in the development and maintenance of the hematopoietic system, we were interested to assess whether members of the hematopoietin receptor family, such as the prototypical IL-2R, regulate PKB. To examine whether IL-2 activates PKB, immunoprecipitates of this kinase were prepared from rIL-2-deprived and rIL-2-activated Kit225 cells and subjected to in vitro kinase assays using H2B as a substrate. The data in Fig. 2A show that IL-2 induced a rapid activation of PKB. A 2–3-fold increase in enzyme activity over basal levels was sustained for more than 60 min in response to rIL-2. PKB activity is regulated by phosphorylation as indicated by the reduced electrophoretic mobility of PKB isolated from rIL-2-activated cells (Fig. 2A). PKB activity was not induced by exposure of Kit225 cells to phorbol esters that activate PKC (Fig. 2A). The data in Fig. 2B show the failure of rIL-2 to stimulate PKB in cells pretreated with LY294002 or wortmannin, two well-characterized PI 3-kinase inhibitors that bind to the ATP or lipid binding sites on the p110 catalytic subunit, respectively. These inhibitors also prevent the autokine activity of Frap/mtor (26), a member of the PI 3-kinase family (47), which is the cellular target for the drug rapamycin and which prevents IL-2-coordinated cell cycle progression and proliferation of T lymphocytes (24, 25). Frap activity is absolutely required for p70S6k action in T cells (24, 25). We therefore assessed whether Frap function was necessary for IL-2-induced stimulation of PKB. Rapamycin had no effect on IL-2-triggered activation of PKB (Fig. 2B), although rapamycin completely abolished IL-2- or PI 3-kinase-controlled induction of p70S6k (data not shown). Thus, the inhibition of PKB by wortmannin and LY294002 cannot be caused by prevention of Frap activity and indicate that IL-2 regulation of PKB employs PI 3-kinase.

To investigate directly whether PI 3-kinase signals are sufficient to activate PKB, rIL-2-deprived Kit225 cells were co-transfected with either rCD2p110 or rCD2p110-R/P expression vectors together with an expression vector encoding HA epitope-tagged PKB. In addition, the ability of activated forms of the small GTPases Ha-v-ras and V12Rac to activate PKB was assessed. Immunoprecipitates of HA-tagged PKB were assayed for kinase activity using H2B as a substrate. The constitutively active PI 3-kinase rCD2p110 induced a robust activation of PKB (Fig. 2C). This stimulatory effect of rCD2p110 was dependent on the kinase activity of the chimera, since co-expression of kinase-inactive rCD2p110-R/P did not stimulate PKB activity. As observed previously in other cell
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systems (28, 33, 48), co-expression of activated Ha-v-ras but not of active V12Rac led to a moderate rise in PKB activity in Kit225 T cells.

Co-expression of an Activated Form of PKB Stimulates p70S6k in Kit225 T Cells—p70S6k is activated by multiple serine/threonine phosphorylation in response to mitogenic stimuli. The retroviral oncogene v-Akt is a chimeric molecule, consisting of the retroviral Gag protein fused to the N terminus of c-Akt, which is myristoylated, and hence v-Akt is predominantly found at the plasma membrane, which may give raise to its oncogenicity (49). The expression of constitutively active PKB, gagPKB, has been described as activating p70S6k in Rat-1 cells (32) and COS1 cells (33). Nevertheless, the ability of phorbol esters to stimulate p70S6k without any discernible activation of PKB indicated that PKB-independent pathways for activation of p70S6k must exist in T cells. To determine the role of PKB in p70S6k activation in T cells, rIL-2-deprived Kit225 cells were co-transfected with a gagPKB expression vector together with an expression vector encoding HA epitope-tagged p70S6k. p70S6k activity was analyzed in anti-HA tag immune complexes with 86 ribosomal subunits as a substrate. Co-expression of constitutively active PKB induced a strong activation of p70S6k that was comparable with increases in p70S6k activity seen by co-expression of rCD2p85 construct that does not regulate D-3 phosphoinositides (29) or three V14Rho, which does not regulate stress-activated protein kinases (50, 51), did not induce ATF-2/LexA-C2-controlled gene expression. Therefore, the GTPases V12Rac and V12Cdc42 are active and can stimulate Rac/Cdc42-regulated signaling pathways in Kit225 T cells.

IL-2 Regulates the Transcription Factor Elk-1 in Kit225 Cells in a PI 3-Kinase-dependent Fashion—In T cells, a PI 3-kinase-sensitive pathway for regulating the activity of Erk kinase (Mek) and the Erks has been reported to co-exist alongside the PI 3-kinase/p70S6k pathway (10). While p70S6k is thought to exert its mitogenic function by controlling translation initiation and protein synthesis, the MAP kinase Erk is implicated in the phosphorylation and activation of certain transcription factors. One well characterized cellular substrate for Erks in fibroblasts and T cells is the transcription factor Elk-1 (40, 52, 53). We therefore tested the ability of IL-2 to regulate Elk-1 transcriptional activity in Kit225 cells. To monitor Elk-1 transcriptional activity, a fusion protein comprising the C terminus of Elk-1 linked to the LexA repressor (41) was co-transfected into Kit225 cells with a LexA operator-controlled CAT reporter gene. The data in Fig. 4A demonstrate that IL-2 can regulate Elk-1 transcriptional activity in Kit225 cells. To confirm that Elk-1 transactivation is induced by a Mek/Erk-sensitive pathway, we investigated the ability of the well characterized inhibitor of Mek activation, PD098059 (54), to prevent IL-2-mediated activation of Elk-1. Treatment of Kit225 cells with the PD098059 component inhibited stimulation of Elk-1 transcriptional activity triggered by rIL-2 (Fig. 4B). Moreover, rIL-2-induction of Elk-1 activity was prevented by the PI 3-kinase inhibitor wortmannin in a dose-dependent manner (Fig. 4C), which corroborates earlier studies indicating that Erk activation by IL-2 requires PI 3-kinase function (10). Treatment of Kit225 cells with rapamycin did not affect Elk-1 transactivation in Kit225 cells (Fig. 4D). To assess whether constitutively active PI 3-kinase and the in vivo production of D-3 phosphoinositides could induce MAP kinase signaling pathways in T cells, the ability of rCD2p110 to induce transcriptional activation of Elk-1 was analyzed. LexA-Elk-1 transcriptional activity was low in quiescent Kit225 cells but could
The data were quantified using a PhosphorImager and are presented as ng/ml PdBu for 5 min. Before stimulation, Kit225 cells were co-transfected with HA-Erk2 plasmids and vector plasmid (empty) or plasmids encoding for rCD2p110, rCD2p110-R/P, or Ha-ras as indicated. Erk assays were performed as described under “Experimental Procedures.” The data were quantified using a PhosphorImager and are presented as the ratio of [32P]phosphate incorporated into S6 to [35S]-conjugated protein A bound to Erk (expressed in arbitrary units). The data are from a representative experiment. Similar results were obtained in two more experiments.

be instigated by co-expression of active Ha-v-ras and by stimulation with phorbol esters, whereas expression of rCD2p110 did not stimulate Elk-1 transactivation (Fig. 4E). However, rCD2p110 signals could potentiate phorbol ester induction of the transcriptional activity of Elk-1. This potentiating effect was not observed in cells expressing the kinase-dead rCD2p110-R/P and was thus dependent on the integrity of the lipid kinase and the cellular production of D-3 phosphoinositides. Moreover, gagPKB cannot mimic the effects of PI 3-kinase on the Erk/Elk-1 pathway (Fig. 4E).

PI 3-Kinase Signals Synergize with Phorbol Esters to Induce Erk Activity in Kit225 Cells—To assess the effect of membrane-localized PI 3-kinase on Erk activity directly, rIL-2-deprived Kit225 cells were co-transfected with expression vectors encoding rCD2p110 and HA epitope-tagged p42 Erk2, and cells were stimulated with phorbol esters or left untreated. Co-expression of rCD2p110 did not stimulate Erk2 activity, although Erk2 could be activated by co-expressing the activated Ras, Ha-v-ras (Fig. 5). These results thus confirm the data in Fig. 4E indicating that PI 3-kinase signals are not sufficient to activate the Erk/Elk-1 pathway. The data in Fig. 5 demonstrate that active PI 3-kinase markedly potentiated the level of Erk2 activation triggered by phorbol esters, an effect that was not observed in cells expressing the kinase-dead rCD2p110-R/P. PI 3-kinase signals did not enhance IL-2 activation responses on Erk (data not shown). Taken together, the results in Figs. 4E and 5 clearly demonstrate that although PI 3-kinase signals are not sufficient for Erk/Elk-1 activation, they can synergize with phorbol esters to induce a maximal response. These results are concordant with a model where PI 3-kinase signals bifurcate to activate the oncogenic form of this kinase causes thymic malignancies. Therefore, PKB has a pivotal role in controlling T cell proliferation/differentiation. The present data identify one function for PI 3-kinase signals to substitute for IL-2 in PKB activation, PKB could be a direct target for PI 3-kinase signals during IL-2 signal transduction. PKB/c-Akt is highly expressed in the thymus (57), and the oncogenic form of this kinase causes thymic malignancies. Therefore, PKB has a pivotal role in controlling T cell proliferation/differentiation. The present data identify one function for PKB in T cells; PKB action is sufficient to stimulate p70S6K. Moreover, PI 3-kinase signals are sufficient for activation of p70S6K, which stresses the close link between PI 3-kinase and PKB in regulating p70S6K activity in T cells. Questions regarding the selectivity of the inhibitors that were first used to define a role for PI 3-kinase in T cell biology have challenged the involvement of this enzyme in the regulation of p70S6K in T cells (28). The present data resolve this controversy and provide unequivocal evidence that PI 3-kinase can function as an upstream regulator of p70S6K in T cells.

RESULTS
The present study has used a membrane-targeted, constitutively active, catalytic subunit of PI 3-kinase as a tool to identify direct targets of PI 3-kinase action in IL-2 signal transduction pathways. We demonstrate that the serine/threonine kinase PKB/Akt can be activated by the cytokine IL-2 via a PI 3-kinase-dependent pathway. Importantly, PI 3-kinase signals alone are sufficient to activate PKB in T cells, demonstrating that PI 3-kinase acts as an upstream regulator of this serine/threonine kinase in lymphoid cells. PKB contains an N-termi-

FIG. 5. PI 3-kinase signals are not sufficient to stimulate Erk2 activity but can synergize with phorbol esters to give an increase in Erk2 activity in Kit225 cells. HA-Erk2 activity was analyzed from extracts of untreated cells (•) or cells stimulated with 50 ng/ml PdBu for 5 min. Before stimulation, Kit225 cells were co-transfected with HA-Erk2 plasmids and vector plasmid (empty) or plasmids encoding for rCD2p110, rCD2p110-R/P, or Ha-ras as indicated. Erk assays were performed as described under “Experimental Procedures.”

FIG. 6. A schematic representation of the IL-2-regulated signaling pathways that involve PI 3-kinase. Binding of IL-2 to its receptor activates PI 3-kinase, PKB, p70S6K, and the Ras/Raf/Erk effector pathway. PI 3-kinase signals are sufficient to stimulate PKB and p70S6K. Activated PKB is sufficient to propagate p70S6K activation. Hence, the available evidence suggests that IL-2 activates PI 3-kinase, which subsequently leads to PKB activation, which in turn stimulates p70S6K. Activation of p70S6K by IL-2, PI 3-kinase, and PKB is sensitive to rapamycin, which indicates that the target of rapamycin, the Frap, is required for p70S6K activation either as a downstream target of PKB (1) or in a parallel pathway (2). p70S6K can also be stimulated by phorbol esters via classical or novel PKC isoforms (2PcK), whereas PKB cannot. p70S6K appears to exert its mitogenic function by regulating translation initiation and protein biosynthesis. PI 3-kinase signals are not sufficient to stimulate the MAP kinase Erk and its cellular target, the transcription factor Elk-1. However, PI 3-kinase signals can synergize with phorbol esters to induce Erk or Elk-1 activation. Erk and Elk-1 activation is not inhibited by rapamycin, and activated PKB does not potentiate phorbol ester induction of Elk-1 transcriptional activity. Hence, PI 3-kinase signals bifurcate to activate the PKB/rapamycin-sensitive p70S6K pathway and independently contribute to the Mek/Erk/Elk-1 pathway. PI 3-kinase signals are implicated in the regulation of the enzyme. Since PI 3-kinase signals are sufficient for activation of p70S6K, which stresses the close link between PI 3-kinase and PKB in regulating p70S6K activity in T cells, Questions regarding the selectivity of the inhibitors that were first used to define a role for PI 3-kinase in T cell biology have challenged the involvement of this enzyme in the regulation of p70S6K in T cells (28). The present data resolve this controversy and provide unequivocal evidence that PI 3-kinase can function as an upstream regulator of p70S6K in T cells.
markedly in their cellular mechanisms for kinase activation. Nevertheless, the present data show a striking conservation of the PI 3-kinase/PKB/p70S6k link in human T cells and simian fibroblasts. The conservation of the PI 3-kinase/PKB/p70S6k signaling cascade in T cells implies a physiological importance of this pathway, which has guaranteed its evolutionary conservation.

The role of PI 3-kinase as an upstream regulator of the Erk kinase pathways can also vary depending on the cell system; expression of an active PI 3-kinase is sufficient for Erk activation in Xenopus oocytes (27) but not in fibroblasts or monoblasts (28–31). The present data show directly that PI 3-kinase can have a positive regulatory role in Erk activation in T cells (see Fig. 6). However, PI 3-kinase signals alone fail to stimulate Erk signaling pathways but markedly potentiate Erk responses in combination with phorbol esters. Erk regulation of downstream nuclear targets is hereby analyzed using the transactivation capacity of the ternary complex factor Elk-1, a well characterized substrate for Erks in fibroblasts and Jurkat T cells (40, 53). We establish that Elk-1 is regulated by IL-2 via Mek- and PI 3-kinase-sensitive pathways. Furthermore, as observed in direct Erk activation assays, PI 3-kinase signals potentely enhance phorbol ester induction of Elk-1 transcriptional activity. PI 3-kinase signals may thus be required for activation of MAP kinase pathways in IL-2-dependent T cells, but they are not sufficient and hence are one component of a more complex signaling network. We have not yet explored the PI 3-kinase effector pathways involved in Erk activation, although previous data have excluded the involvement of the Frap/p70S6k pathway, since activation of Erk is not sensitive to rapamycin inhibition. Moreover, PKB, which is a potent activator of p70S6k, cannot mimic the effects of activated PI 3-kinase on the Erk/Eilk-1 pathway. These data best fit a model in which PI 3-kinase regulation of MAP kinases and PKB/p70S6k are part of the PI 3-kinase/Eilk-1 pathway. These data best fit a model in which PI 3-kinase regulation of MAP kinases and PKB/p70S6k are part of the PI 3-kinase/Eilk-1 pathway. Moreover, several other candidate in vivo targets for D-3 phosphoinositides have been proposed including members of the novel PKC family (58) and the atypical PKC family, PKC-λ (59) and PKC-ζ (60), which has recently been implicated as a regulator of Mek and Erk activity in COS cells (61).

p70S6k plays a key role in cellular growth control mechanisms by coordinating protein biosynthesis via phosphorylation of the S6 subunit of 40 S ribosomes or via regulation of the activity of the eukaryotic initiation factor 4E (62). Expression of an activated PKB can stimulate p70S6k activity in T cells, indicating that PKB substrates are part of the p70S6k activation pathways. Moreover, given the ability of PI 3-kinase signals to stimulate PKB and p70S6k, it seems probable that PKB mediates the PI 3-kinase activation of p70S6k in T cells. The immunosuppressive drug rapamycin inhibits the cell cycle progression and proliferation of T lymphocytes and has been shown previously to block IL-2 activation of p70S6k. Rapamycin forms a complex with the intracellular protein FKBP12, which subsequently provides a high affinity inhibitor of Frap. The Frap kinase is a member of the PI 3-kinase family of enzymes (47) and plays an established, but poorly defined, role as an upstream regulator of p70S6k (24, 25). Rapamycin prevents the activation of p70S6k induced in T cells by the constitutively active PI 3-kinase, cRDP2110, (data not shown) or by active PKB (32, 33), thus indicating that PI 3-kinase or PKB activation signals cannot bypass the role of Frap in p70S6k activation pathways. A simple interpretation of these data is that PI 3-kinase and PKB activation of p70S6k is mediated by Frap, although the possibility cannot be excluded that Frap regulates p70S6k by an essential signaling pathway operating in parallel with PI 3-kinase/PKB signals (Fig. 6). Frap controls p70S6k activation by regulating the phosphorylation of key residues in the enzyme (63, 64). Nevertheless, p70S6k is not a direct substrate for Frap, and some intermediate p70S6k kinase(s), as yet uncharacterized, must be invoked to explain the role of Frap in p70S6k activation. Although the evidence that PKB mediates PI 3-kinase effects on p70S6k is compelling, these data do not exclude the possibility that there are PKB-independent mechanisms for p70S6k activation of T cells. In this context, the present data show that activation of PKC by phorbol esters stimulates p70S6k without any discernible stimulatory effect on PKB.

Recent studies showing that cytokine activation of serine kinases is important for the regulation of apoptosis (65, 66) have focused attention on cytokine-induced serine kinase cascades. PI 3-kinase and PKB have been implicated in the prevention of apoptosis in other cell systems (67, 68). The present study demonstrates that PI 3-kinase can couple the IL-2R to a selective subset of serine/threonine kinase pathways in T cells, and in this respect, the PI 3-kinase/PKB link is intriguing, since PKB mediates activation of the Frap/p70S6k pathway but may also induce other kinase cascades that bifurcate from the PKB/p70S6k pathway including glycogen synthase kinase-3 (GSK3) signaling pathways (69). Therefore, PI 3-kinase and/or PKB have the potential for pleiotropic functions in T cells, and their downstream effectors may include additional serine/threonine kinases evoked by IL-2R engagement.

Finally, PI 3-kinase is activated by members of the cytokine receptor family such as the IL-2R, the IL-4 receptor, the IL-13 receptor, and the IL-13 receptor. Signaling pathways regulated by PI 3-kinase can hence have an impact on lymphocyte biology at multiple points. Accordingly, it is important to establish the function of this enzyme in lymphoid cells. The IL-2R is a prototypical member of this hematopoietin receptor family. The present results directly define PI 3-kinase function in T cells and position PKB for the first time in a physiologically relevant cytokine-induced signal transduction pathway in lymphoid cells. The model described herein may also be applicable to serine/threonine kinase pathways regulated by other receptors that activate PI 3-kinase in T cells.

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REFERENCES

Phosphatidylinositol 3-Kinase Targets in Lymphoid Cells

A negative role for phosphoinositide 3-kinase in T-cell antigen receptor function
Karin Reif, Susan Lucas and Doreen Cantrell

Background: A delicate balance between positive and negative regulatory mechanisms during T-cell activation determines the specificity and magnitude of an immune response. Phosphoinositide 3-kinase (PI 3-kinase) is activated by a diverse set of receptors that determine T-cell function, including the T-cell antigen receptor (TCR), the costimulatory receptor CD28, and negative regulators of T-cell activation such as CTLA-4. PI 3-kinase is also regulated by the haematopoietic cytokines that determine T-cell differentiation and lymphocyte proliferation. PI 3-kinase can thus dynamically influence the outcome of the immune reactions at various stages. In this study, we investigated the importance of PI 3-kinase in TCR-directed T-cell activation using activated or inhibitory versions of PI 3-kinase.

Results: Certain aspects of TCR responses such as the induction of transcriptional activity of AP1 and serum response factor were not affected by expression of the mutant forms of PI 3-kinase. We found, however, that PI 3-kinase profoundly influenced the transactivation capacity of 'nuclear factor of activated T cells' (NF-AT) elicited by the TCR: expression of an activated form of PI 3-kinase inhibited TCR-mediated NF-AT responses, whereas expression of a dominant negative mutant of PI 3-kinase potently enhanced TCR-controlled NF-AT induction. These effects of PI 3-kinase were not mediated by previously identified PI 3-kinase effectors, such as protein kinase B , a positive regulator of PI 3-kinase, or the GTPase Rac, and are therefore likely to involve a novel, as yet unknown, effector molecule.

Conclusions: Our results establish that PI 3-kinase can both positively and negatively regulate T-cell function, and uncover a previously unrecognized function for PI 3-kinase in T cells as a selective negative regulator of TCR-signalling events and therefore as a determinant of T-cell homeostasis.

Background
During T-cell activation, both positive and negative regulatory signalling cascades are vital for a balanced immune response and immune homeostasis. T-cell activation is orchestrated by the T-cell antigen receptor (TCR) and a plethora of accessory molecules such as integrins, the tyrosine phosphatase CD45, the coreceptors CD4 and CD8, and the costimulatory receptor CD28 [1-6]. These regulatory receptors thus determine the outcome of TCR engagement by modulating TCR function and influencing the thresholds of TCR occupancy required to initiate a T-cell immune reaction.

Phosphoinositide 3-kinase (PI 3-kinase) is activated during T-cell activation but its function in T cells is as yet poorly understood [7,8]. PI 3-kinase comprises a regulatory p85 subunit and a catalytic p110 subunit which phosphorylates phosphatidylinositol 4,5-bisphosphate generating phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3) [9,10]. Elevation of cellular levels of PI(3,4,5)P3 correlates with PI 3-kinase action. Studies of PI 3-kinase activation in T cells established that the TCR and CD28 can act in concert to give a substantial rise in PI(3,4,5)P3 levels [11]. Intriguingly, PI 3-kinase is also associated with inhibitory receptors such as CTLA-4 [12,13] or CD5 [14,15]. Moreover, PI 3-kinase activity is regulated by cytokines such as IL-2/IL-4 which have a critical role in downregulating immune hyperactivity [16]. It is thus clear that receptors that activate PI 3-kinase can have major functions as negative regulators of the immune system.

Signal transduction events from the TCR are subject to important autoregulatory and pararegulatory negative-feedback control mechanisms: it is increasingly recognized that receptors on T cells such as CTLA-4, inhibitory MHC class I molecules and cytokine receptors can generate terminating signals, which are extremely important in immune homeostasis [16-20]. There is only a primitive understanding of the effector molecules that downmodulate TCR signal transduction, particularly in the context of
negative-feedback mechanisms generated by the TCR itself. As PI 3-kinase can cause pleiotropic function, it could be critical for defining and revising the biological responses from the TCR by eliciting negative signals.

We have employed genetic approaches using activated or inhibitory mutants of PI 3-kinase to analyze the normal cellular functions of the enzyme in T-cell activation governed by the TCR. We examined the importance of PI 3-kinase in TCR-induced transcription factor responses because transcription factors coordinate and implement various activating or inhibitory input messages and are ultimately responsible for fine-tuning the genetic program. Here, we report that signals downstream from PI 3-kinase impair TCR-controlled transcription from the NF-AT (‘nuclear factor of activated T cells’) response element, whereas these signals do not interfere with the signalling pathways that lead to gene transcription mediated by AP1 or serum response factor (SRF). We show that the serine/threonine protein kinase B (PKB; also termed c-Akt) is activated by PI 3-kinase, but that active PKB cannot substitute for active PI 3-kinase on NF-AT induction because only TCR responses were prevented and those of calcium ionophore and phorbol ester were unaffected (Fig. 1c). TCR induction of NF-AT still occurred in the presence of wild-type rCD2, ‘kinase-dead’ rCD2p110-R/P or rCD2p85 (Fig. 1b,c), showing that the inhibitory effect of rCD2p110 on TCR/NF-AT responses requires the lipid kinase activity of PI 3-kinase [22].

The induction of the transcription factor NF-AT by triggering of the TCR or by the combined action of calcium ionophore and phorbol ester (phorbol 12,13-dibutyrate; PdBu) was studied. TCR induction of NF-AT was completely abrogated by expression of the activated PI 3-kinase chimera, rCD2p110 (Fig. 1b). The repressive effect of the active PI 3-kinase on NF-AT induction was selective because only TCR responses were prevented and those of calcium ionophore and phorbol ester were unaffected (Fig. 1c). TCR induction of NF-AT still occurred in the presence of wild-type rCD2, ‘kinase-dead’ rCD2p110-R/P or rCD2p85 (Fig. 1b,c), showing that the inhibitory effect of rCD2p110 on TCR/NF-AT responses requires the lipid kinase activity of PI 3-kinase [22].

It was noted that expression of rCD2p85 could induce a low level of NF-AT activity in the absence of stimulation. We have observed previously that rCD2p85 acts as a moderate inhibitory mutant of PI 3-kinase signalling pathways, and this could explain the potentiating effect of rCD2p85 on NF-AT transcriptional activity (see Fig. 5). Signalling pathways are initiated and modulated differentially in divergent cell types, therefore the magnitude or quality of a response is dependent upon cell context. We examined the effects of the constitutively active PI 3-kinase on TCR/NF-AT responses in the murine T-cell line EL4. The data in Figure 1d show that TCR-mediated induction of NF-AT was also suppressed in EL4 cells expressing the activated rCD2p110 chimera; however, suppression was not as complete as in Jurkat cells. This incomplete inhibition might occur because EL4 cells exhibit partial constitutive activation of calcium/calcineurin signalling pathways [23], which is one of the crucial signals required for NF-AT regulation [24].

**Results**

**PI 3-kinase signals block TCR-induced transcription from NF-AT response elements**

Immediate responses to TCR triggering include the activation of transcription factors such as AP1, SRF and NF-AT [21] which are ultimately important for cytokine gene expression. Here, we have analyzed the consequences of PI 3-kinase activation on transcription-factor regulation by the TCR. The Jurkat T-cell line was transfected with reporter genes responsive to different transcription factors together with a constitutively active form of PI 3-kinase, rCD2p110, comprising the extracellular and transmembrane domains of the rat CD2 (rCD2) cell surface antigen fused to the amino-terminal domain of the p110α catalytic subunit of PI 3-kinase [22]. As controls, cells were similarly transfected with rCD2p110-R/P, a chimera mutated at position 1130 thus catalytically inactivating the kinase activity of the fusion protein [22]; rCD2p85, a chimera comprising the rCD2 extracellular and transmembrane domains linked to the amino terminus of the p85α regulatory subunit of PI 3-kinase [22]; and wild-type rCD2. Expression of the rCD2-P1-3-kinase chimeras was confirmed by flow cytometry (Fig. 1a).

The induction of the transcription factor NF-AT by triggering of the TCR or by the combined action of calcium ionophore and phorbol ester (phorbol 12,13-dibutyrate; PdBu) was studied. TCR induction of NF-AT was completely abrogated by expression of the activated PI 3-kinase chimera, rCD2p110 (Fig. 1b). The repressive effect of the active PI 3-kinase on NF-AT induction was selective because only TCR responses were prevented and those of calcium ionophore and phorbol ester were unaffected (Fig. 1c). TCR induction of NF-AT still occurred in the presence of wild-type rCD2, ‘kinase-dead’ rCD2p110-R/P or rCD2p85 (Fig. 1b,c), showing that the inhibitory effect of rCD2p110 on TCR/NF-AT responses requires the lipid kinase activity of PI 3-kinase [22].

It was noted that expression of rCD2p85 could induce a low level of NF-AT activity in the absence of stimulation. We have observed previously that rCD2p85 acts as a moderate inhibitory mutant of PI 3-kinase signalling pathways, and this could explain the potentiating effect of rCD2p85 on NF-AT transcriptional activity (see Fig. 5). Signalling pathways are initiated and modulated differentially in divergent cell types, therefore the magnitude or quality of a response is dependent upon cell context. We examined the effects of the constitutively active PI 3-kinase on TCR/NF-AT responses in the murine T-cell line EL4. The data in Figure 1d show that TCR-mediated induction of NF-AT was also suppressed in EL4 cells expressing the activated rCD2p110 chimera; however, suppression was not as complete as in Jurkat cells. This incomplete inhibition might occur because EL4 cells exhibit partial constitutive activation of calcium/calcineurin signalling pathways [23], which is one of the crucial signals required for NF-AT regulation [24].

**PI 3-kinase signals do not impair TCR-mediated induction of AP1- or SRF-activated gene transcription**

We next explored the role of PI 3-kinase signals on TCR-mediated induction of the transcriptional activity of the c-fos serum response element (SRE) or AP1-driven enhancer elements which are both central to the regulation of immediate early genes. Activation at the SRE is mediated by p21Ras/Raf/Erk (MAP kinase) pathways that can be initiated in T cells by triggering of the TCR or by phorbol ester (Fig. 2a). Expression of rCD2p110 had no stimulatory effect on SRE activity but more importantly, it did not compromise induction of SRE activity by TCR cross-linking or treatment with phorbol ester. The transcriptional activity of AP1 complexes induced by triggering of the TCR or treatment with phorbol ester plus ionomycin was also unaffected by coexpression of rCD2p110 (Fig. 2b). Expression of a constitutively active
PI 3-kinase functions as a negative regulator of TCR-mediated NF-AT induction. (a) Flow cytometry of Jurkat T cells transiently transfected with either rCD2, rCD2p85, rCD2p110, or rCD2p110-R/P expression vectors (30 μg plasmid DNA) and stained with the anti-rCD2 OX34 monoclonal antibody followed by fluorescein-conjugated rabbit anti-mouse IgG (black line). As control, each population of transfected cells was incubated with anti-mouse IgG as the primary antibody (dotted line). (b,c) Jurkat cells or (d) EL4 cells were transfected with the NF-AT.CAT reporter plasmid together with empty vector or plasmids encoding rCD2p110, rCD2p110-R/P, rCD2p85, or rCD2 as indicated. Cells were either left untreated (Control) or stimulated with (b) anti-CD3 monoclonal antibody UCHT-1, (c) PdBu and ionomycin (lono), or (d) plate-bound anti-CD3 monoclonal antibody 2C11 for (b,c) 13 h or (d) 16 h and then assayed for CAT activity. The CAT activity is presented as percentage conversion. The data are from a representative experiment, and in (d), two experiments are shown. Similar results were obtained in seven (b,c) or three (d) more experiments.

PI 3-kinase therefore has a selective modulatory effect on the repertoire of transcription factor pathways activated by the TCR: signals downstream of PI 3-kinase negatively regulate the TCR signalling pathways that lead to NF-AT-mediated transactivation but do not affect the TCR-mediated induction of SRE and AP1-responsive elements.

PKB is stimulated by PI 3-kinase but does not inhibit transactivation by NF-AT
PI 3-kinase signals are able to activate the serine/threonine kinase PKB in fibroblasts [25,26]. This PI 3-kinase pathway is important in regulating cell-survival responses in fibroblasts and neuronal cells [27,28]. The negative effects of active PI 3-kinase on TCR-mediated NF-AT responses and its lack of effect on TCR-induced SRE and AP1 activation prompted us to examine the effects of rCD2p110 on PKB activity in Jurkat cells. TCR triggering led to an increase in PKB activity (Fig. 3a). Moreover, expression of the membrane-localized PI 3-kinase, rCD2p110, was sufficient to stimulate PKB activity to a level comparable to that initiated by triggering of the TCR/CD3 complex (Fig. 3a). PI 3-kinase action thus does not completely antagonise signalling from the TCR but can also positively regulate proximal TCR signal transduction events. We next investigated whether PKB was involved in transmitting the inhibitory effects of PI 3-kinase on TCR signalling. Expression of a constitutively active PKB, gagPKB, did not mimic the effect of active PI 3-kinase in the abrogation of TCR-mediated NF-AT induction (Fig. 3b). PKB is thus not a candidate for mediating the inhibitory effect of PI 3-kinase on TCR signalling.

Expression of V12Rac does not abrogate transactivation by NF-AT but does induce SRF and AP-1 activity
We have shown recently that the active PI 3-kinase, rCD2p110, can initiate signalling pathways mediated by the GTPase Rac that lead to actin cytoskeleton rearrangements in fibroblasts [22]. Rac can deliver signals for transcriptional activation including stimulation of stress-activated protein kinases [29-31]. We tested the possibility that Rac might
Expression of active PI 3-kinase does not interfere with TCR-induced transcriptional activity from the SRE or from AP1 elements. Jurkat cells were transfected with (a) SRE.CAT or (b) AP1.CAT reporter plasmids together with empty vector or plasmids encoding rCD2p110, or rCD2p110-R/P as indicated. Cells were left untreated (Control) or stimulated overnight with UCHT-1, PdBu, and PdBu plus ionomycin as indicated and subsequently assayed for CAT activity. The CAT activity is presented as percentage conversion. The data are from a representative experiment. Similar results were obtained in four (a) or three (b) more experiments.

mediates the effects of PI 3-kinase on NF-AT activity. Expression of constitutively active Rac, V12Rac, did not substitute for PI 3-kinase in the inhibition of TCR-mediated NF-AT responses (Fig. 4a). Moreover, V12Rac expression stimulated transcription regulated by SRF (Fig. 4b) and AP1 (Fig. 4c), as did expression of Dbl (Fig. 4b,c), a guanine nucleotide exchange factor for Rho family GTPases that activates endogenous Rac and Rho [32]. Expression of rCD2p110 failed to elicit transcriptional activity from the SRE or from AP1 elements indicating that signals from PI 3-kinase do not apparently initiate Rac-mediated transcription-factor pathways in T cells. In addition, previous studies have shown that expression of an inhibitory Rac mutant, N17Rac, suppresses rather than potentiates TCR-induced NF-AT signal transduction cascades [33]. These data demonstrate that effector pathways distinct from PKB- or Rac-governed signalling cascades must mediate the effects of PI 3-kinase on TCR function.

Expression of inhibitory mutants of PI 3-kinase enhances TCR-induced NF-AT responses

It has been known for some time that TCR activation of NF-AT is transient and subject to as yet uncharacterized negative-feedback regulation [34-36]. The inhibitory effects of active PI 3-kinase on TCR-mediated transactivation by NF-AT coupled with the fact that the TCR...
activates PI 3-kinase raise the possibility that PI 3-kinase may elicit deactivation of TCR-provoked NF-AT responses. To test this hypothesis, we examined the effect of p85Δ, an inhibitory mutant of PI 3-kinase, on TCR-mediated induction of NF-AT. The p85Δ mutant corresponds to a p85 regulatory subunit lacking the binding site for the p110 catalytic subunit and therefore prevents regulation and recruitment of the active enzyme complex to the plasma membrane [37]. Expression of p85Δ led to a small but reproducible basal induction of NF-AT activity, and strikingly synergized with TCR stimulation to induce NF-AT transcriptional activity (Fig. 5a,b). In contrast, p85Δ had no effect on TCR-mediated induction at the SRE in parallel experiments (Fig. 5c). The potentiating effect of p85Δ is consistent with the notion that PI 3-kinase signals antagonize the TCR signals that initiate NF-AT transcriptional activity.

**Discussion**

As a delicate balance between positive and negative signals is obligatory for an immune response, it is important to characterize the full spectrum of intracellular molecules that integrate these input signals. Although substantial progress has been made to delineate positive events, much less is known about effectors that negatively modulate or terminate T-cell activation. The data presented here reveal a previously unrecognized function for PI 3-kinase as a selective negative regulator of TCR responses. The importance of this negative signalling role for PI 3-kinase is underscored by its selective mode of action: PI 3-kinase signals are sufficient to stimulate PKB, a serine/threonine kinase involved in the regulation of survival responses in several cell systems; in contrast, PI 3-kinase signals abolish TCR-controlled NF-AT transactivation but allow TCR-mediated induction of AP1 or SRE. Interestingly, an active allele of PKB did not abrogate NF-AT activity triggered by the TCR, nor did expression of an activated form of the GTPase Rac, a second well characterized effector of PI 3-kinase, abolish NF-AT activity. This finding implicates an as yet uncharacterized effector pathway as the mediator of PI 3-kinase regulation of the TCR-induced NF-AT response (Fig. 6).

The transcriptional activation of NF-AT is a complex process that involves translocation of the cytosolic NF-AT component from the cytoplasm to the nucleus, where it associates with its nuclear component, which contains Fos/Jun (AP-1) family members that facilitate the DNA-binding ability and transcriptional activity of NF-AT [38]. The precise mechanism for NF-AT inhibition by PI 3-kinase awaits analysis. However, NF-AT induction by the TCR requires the coordinate action of signalling pathways mediated by the phosphatase calcineurin, the GTPase p21Ras, and may involve the adaptor protein SLP-76 and Vav [3,4]. PI 3-kinase could thus exert its regulatory effects on NF-AT induction at multiple sites.
Cytokine gene expression upon activation of T cells is a transient phenomenon subject to critical negative-feedback control. NF-AT family members govern the transcription of a number of genes including those for the cytokines IL-2, IL-3, and IL-4, and for CD40 ligand and Fas ligand [38,39]. In this respect, it has been recognized for several years that there are negative-feedback signalling mechanisms for the TCR-mediated induction of NF-AT [34-36]. These downregulating mechanisms are likely to be responsible for the transience of cytokine gene expression during T-cell activation. The data presented in this paper, showing the reciprocal effects of activated and inhibitory mutants of PI 3-kinase on TCR-delivered NF-AT responses, are consistent with a model in which PI 3-kinase has a negative-feedback or modulatory role in the induction of NF-AT activity by the TCR (Fig. 6). TCR triggering itself activates PI 3-kinase and the inhibitory PI 3-kinase signals described herein might act as a physiologically relevant homeostatic control mechanism in TCR function that temporally restricts cytokine production to a limited period of T-cell activation.

Moreover, TCR-induced cytokines, such as IL-2 and IL-4, act as growth factors in autocrine loops to drive T cells through the cell cycle [40,41]. These growth factors are powerful activators of PI 3-kinase [42-44] and a PI 3-kinase-mediated NF-AT inhibitory pathway would ensure that cytokine production is restricted to cells that have not yet received a signal to proliferate. There is little reason for a cell that is committed to proliferate to continue to produce growth-regulatory cytokines (Fig. 6). Intriguingly, it has been reported that NF-ATp/NF-AT1-deficient mice show enhanced immune responses [39,45], which suggests that NF-AT family proteins are involved in downregulating immune responses. PI 3-kinase downregulation of NF-AT function would thus not only terminate the positive growth regulatory signals generated by NF-AT, but would also modulate the negative feedback functions of NF-AT.

There could also be broader implications for the terminating effects of PI 3-kinase on TCR function described in this study; molecules such as CTLA-4 that negatively regulate T-cell activation [17–19] also associate with PI 3-kinase [12]. Previous studies have ascribed the negative effects of CTLA-4 signalling to the association of this receptor with the tyrosine phosphatase SHP-2 [46]. However, there is no direct evidence that SHP-2 acts to downregulate T-cell functions and it is noteworthy that the p85 subunit of PI 3-kinase and SHP-2 interact with the same phosphotyrosine motif, Tyr-Val-Lys-Met, in the cytoplasmic domain of CTLA-4 via their SH2 domains [12,46]. PI 3-kinase could thus be a candidate for mediating the negative effects of CTLA-4 on T-cell action. In addition, PI 3-kinase could play a role in the negative signalling pathways initiated by cytokines such as IL-2 and IL-4. These issues are obviously complicated...
**Conclusions**

During T-cell activation and proliferation, PI 3-kinase is activated by multiple receptors, such as the TCR, CD28, IL-2R, IL-4R or CTLA-4, that can positively or negatively regulate these processes. Hence, PI 3-kinase is likely to have pleiotropic functions. In these studies, we have defined one further function of PI 3-kinase in modulating TCR-controlled transcription factor responses, in that it selectively downregulates TCR-mediated induction of NF-AT transcriptional activity. This identifies PI 3-kinase as a determinant of negative signalling pathways in T-cell activation and lymphocyte homeostasis.

**Materials and methods**

**Plasmids**

The plasmids for the following DNA constructs have been described previously: NF-AT.CAT (CAT, chloramphenicol acetyl transferase) and AP1.CAT [33], SRE.CAT [47], HA-PKB and gagPKB [48], pBS5Δ [37], VI2Rac and Dbl [22]. The rCD2p85, rCD2p110 and rCD2p110-R/P constructs, as well as the rCD2 cDNA have been described previously [22] and were subcloned into the pEF-BOS expression vector.

**Cell culture and transient transfections**

Jurkat T cells (JHM 2.2) [49] were maintained in RPMI 1640 supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 µg/ml.
streptomycin, and 100 units ml⁻¹ penicillin; EL4 murine thymoma cells were also maintained in this medium but with the addition of 10 μM β-mercaptoethanol [50]. Jurkat and EL4 T cells were transfected by electroporation with 20–40 μg plasmid DNA. Amounts of plasmid DNA were kept constant per cuvette by adding vector plasmid. Jurkat cells (1.0–1.5 × 10⁶ cells in 0.5 ml) were pulsed at 310 V and 960 μF. EL4 cells (1.0–1.5 × 10⁶ cells in 0.29 ml) were pulsed at 250 V and 960 μF. The following amounts of plasmid were used unless indicated otherwise: 15 μg of the following plasmids: pEF-empty, pEF-rCD2p110, pEF-rCD2p110-R/P, pEF-rCD2p85, pEF-rCD2, or pSG5- gagPKB; 10 μg of the following plasmids: pSG5-HA-PKB, pODSRha-p85A, pEF-V12Rac, pCMV-Dbi; 15 μg of NF-AT.CAT and 2 μg of API.CAT. For gene reporter assays, cells were stimulated 3 h to 6 h after transfection. Inductions were as follows, unless indicated otherwise: 50 ng ml⁻¹ phorbol 12,13 dibutyrate (PdBu; Calbiochem), 500 ng ml⁻¹ ionomycin (Ca²⁺ salt; Calbiochem). For gene expression assays, cells were kept constant per cuvette by adding vector plasmid. Jurkat cells were transfected with 20–40 μg plasmid DNA. Amounts of plasmid DNA were kept constant per cuvette by adding vector plasmid.

**Immunofluorescence staining and flow cytometry**

Cells transfected with rCD2 chimeras were subjected to immunofluorescent staining and flow cytometry using 0.6 μg ml⁻¹ oxazolone monoclonal antibody, and for control staining mouse IgG (Sigma), followed by fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (DAKO) using standard protocols.

**Gene expression analysis**

Cells were harvested and lysed in 200 μl lysis buffer (0.65% NP40, 1.0–1.5 × 10⁶ cells in 0.5 ml) were pulsed at 310 V and 960 μF. EL4 cells with 10 μg ml⁻¹ plate-bound murine CD3 antibody 2C11.

**PKB kinase assays**

Jurkat cells were stimulated with 5 μg ml⁻¹ UCHT-1 monoclonal antibody and lysed (120 μM NaCl, 50 mM Hepes pH 7.4, 10 mM NaF, 1 mM EDTA, 40 mM p-glycerophosphate pH 7.5, 1.0–1.5 × 10⁶ cells in 0.5 ml) were pulsed at 310 V and 960 μF. EL4 cells (1.0–1.5 × 10⁶ cells in 0.29 ml) were pulsed at 250 V and 960 μF. The following amounts of plasmid were used unless indicated otherwise: 15 μg of the following plasmids: pEF-empty, pEF-rCD2p110, pEF-rCD2p110-R/P, pEF-rCD2p85, pEF-rCD2, or pSG5-gagPKB; 10 μg of the following plasmids: pSG5-HA-PKB, pODSRha-p85A, pEF-V12Rac, pCMV-Dbi; 15 μg of NF-AT.CAT and 2 μg of API.CAT. For gene reporter assays, cells were stimulated 3 h to 6 h after transfection. Inductions were as follows, unless indicated otherwise: 50 ng ml⁻¹ phorbol 12,13 dibutyrate (PdBu; Calbiochem), 500 ng ml⁻¹ ionomycin (Ca²⁺ salt; Calbiochem). TCR crosslinking was achieved by stimulating Jurkat cells with 5 μg ml⁻¹ of the human CD3 antibody UCHT1 or by stimulating EL4 cells with 10 μg ml⁻¹ plate-bound murine CD3 antibody 2C11.

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**References**


Negative role for PI 3-kinase in TCR events


The protein interactions of the immunoglobulin receptor family tyrosine-based activation motifs present in the T cell receptor \( \zeta \) subunits and the CD3 \( \gamma,\delta \) and \( \epsilon \) chains

Immunoglobulin family tyrosine-based activation motifs (ITAM), which define the conserved signaling sequence \( \text{EXYXXL/I} \), couple the T cell antigen receptor (TCR) to cellular proteins including protein tyrosine kinases (PTK) and adapter molecules. The TCR is a multichain complex with four invariant chains CD3 \( \gamma \), \( \delta \) and \( \epsilon \) chains of the CD3 antigen each have a single copy of the ITAM. There were also subtle differences in the hierarchy of reactivity of these adapters for the CD3 \( \gamma,\delta \) and \( \zeta \) ITAM that show that the \( \zeta \), CD3 \( \gamma \), \( \delta \) and \( \epsilon \) ITAM have different binding properties. The present study thus shows that the different ITAM of the TCR/CD3 complex can interact with different cytosolic effectors, indicating that differential ITAM phosphorylation during T cell activation could be a mechanism to generate signaling diversity by the TCR complex.

1 Introduction

T cell activation is controlled by the antigen receptor (TCR)/CD3 complex which comprises the polymorphic TCR \( \alpha \) and \( \beta \) subunits in a noncovalent association with the invariant chains of the TCR complex: the TCR \( \zeta \) chains and the \( \gamma,\delta \) and \( \epsilon \) chains of the CD3 antigen complex. The capacity of the TCR to transduce signals across the T cell membrane is mediated by the cytoplasmic domains of the subunits of the CD3 antigen and the \( \zeta \) chains. The intracellular tails of the CD3 and \( \zeta \) molecules contain a common motif, \( \text{E}_{X1}\text{YXXL/I} \), termed an immunoglobulin receptor family tyrosine-based activation motif (ITAM) [1]. This motif is crucial for TCR coupling to intracellular tyrosine kinases and hence absolutely required for all subsequent TCR signaling responses [2-5]. Both tyrosines within the ITAM are required for ITAM function since the function of the TCR/CD3 ITAM is to recruit and activate the protein tyrosine kinase (PTK) ZAP-70 [6]. The association of ZAP-70 with the TCR complex is mediated by the interaction of ITAM with SH2 domains [6]. ZAP-70 has two SH2 domains and binds preferentially with high affinity to a doubly phosphorylated ITAM [7, 8]. The \( \gamma,\delta \) and \( \epsilon \) chains of the CD3 antigen each have a single copy of the ITAM, whereas the \( \zeta \) subunit has three copies of the ITAM. Transfection of chimeric receptors where the receptor cytoplasmic tail comprises either the \( \zeta \) or \( \epsilon \) ITAM are sufficient for activation of TCR signal transduction pathways leading to IL-2 production, calcium mobilization and tyrosine phosphorylation pathway activation [2, 6, 9]. These data indicate that the \( \epsilon \) and \( \zeta \) ITAM can have a similar function and it was suggested accordingly that the presence of multiple ITAM within the TCR acts to amplify signal transduction by the TCR. However, it is increasingly recognized that there can be diversity in TCR signal transduction responses. In particular, recent reports describing partial tyrosine phosphorylation of the TCR complex and recruitment, but not activation, of ZAP-70 during the induction of T cell anergy have focused attention on the mechanisms that the TCR might use to generate different signals [10, 11]. The ITAM may represent a source of variability in TCR signaling. The CD3 and \( \zeta \) ITAM can be tyrosine-phosphorylated during T cell activation [12] and all have the duplicated YXXL sequence. However, they differ quite markedly in their intervening amino acid sequences and thus have the potential to interact with different cytosolic effectors. In this context, it was reported that the pattern of cellular tyrosine phosphorylation induced by chimeric receptors with cytoplasmic domains comprising CD3 \( \epsilon \) or TCR \( \zeta \) is different, indicating that the different ITAM might have unique functions [2]. This viewpoint was strengthened further by a recent report exploring the interaction between ZAP-70 and the \( \zeta,\zeta',\zeta'' \).
2.1 Cells, antibodies and synthetic oligopeptides

Human peripheral blood-derived T cells were prepared as described [13]. The following antibodies were used in this study: anti-CD3 mAb UCHT1 was kindly given by Prof. Peter Beverley (University College, London, GB); CST-1 antisera was generously provided by Dr. Joseph Bolen (ICRF, London, GB) with either both tyrosines unphosphorylated or both tyrosines phosphorylated in the autophosphorylation site of the human epidermal growth factor receptor (EGFR; PVPEYNQS) [14]; TRK-Y490, the binding site for SHC (LENPQYFSDA) [15]; PDGFR-Y751, the binding site for the SH2 domain of ZAP-70 [20, 21] (g.g., TRK-Y490 binds She and Grb-2, and PDGFR-Y751 binds p85 [22]). The control tyrosine phosphopeptides were selected on the basis of their binding properties for adapter proteins previously suggested to bind to the TCR complex, She, and Grb-2 and Grb-751. The other proteins detected in the EGFR-Y1068 precipitates include Grb-2 and PDGFR-Y1009 and HMT-Y324 are binding sites for the SH2 domains of Syp and src kinases, respectively. The TRK-Y490 and PDGFR-Y751 control phosphopeptides were selected on the basis of their binding properties for adapter proteins previously suggested to bind to the TCR complex, She, and the 85-kDa regulatory subunit of PI-3' kinase [20, 21] (g.g., TRK-Y490 binds She [15] and PDGFR-Y751 binds p85 [22]). The control tyrosine phosphopeptides recruited distinct patterns of [35S]methionine-labeled proteins from the T cell lysates, indicating that the protein phosphorylation of tyrosine phosphopeptides can be highly selective (Fig. 1b).

2.2 Immunoprecipitation and Western (protein) blotting

T cells (2 x 10^7 at each time point) were pelleted, lysed for 20 min on ice in 1 ml immunoprecipitation buffer containing 1% NP40, 150 mM NaCl, 50 mM Hepes pH 7.5, 10 mM iodoacetamide, 1 mM phenylmethysulphonylfluoride, 1 mM sodium orthovanadate and 1 mg/ml each of antipain, chymostatin, leupeptin and pepstatin and immunoprecipitated as described [18]. Precipitations using synthetic peptides were performed using 75 nM-25 μM peptide as indicated coupled to Affigel-10 activated-ester agarose (Bio-Rad, Hercules, CA). Coupling procedures followed the manufacturer's recommendations.

Peptide precipitates or acetonprecipitated protein from whole cell lysates were resolved by SDS-PAGE and electroblotted at 0.35A, 60V for 5 h onto PVDF (polyvinylidene difluoride) membranes. Filters were blocked overnight at 4°C with 5% non-fat milk protein in PBS, washed in PBS/0.05% Tween-20 and probed with primary antibodies as indicated. Horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG were used as second-stage reagents as required to allow detection by enhanced chemiluminescence (ECL, Amersharm, Little Chalfont, GB). ECL bands were quantified by laser densitometry of autoradios as recommended by manufacturer.

3 Results

3.1 The binding interactions of the γ and CD3 ITAM with [35S]methionine-labeled T cell proteins

Affinity matrices comprising peptides phosphorylated on both tyrosine residues and corresponding to the ITAM, ITAM, and ITAM of the human TCR/CD3 complex were used to purify proteins from peripheral blood-derived T lymphoblasts biosynthetically labeled with [35S]methionine (Fig. 1). The data in Fig. 1a show the [35S]methionine-labeled proteins precipitated with each of the different TCR/CD3 ITAM peptides, whereas the data in Fig. 1b show the binding properties of a number of tyrosine-phosphorylated peptides with previously defined SH2 domain specificities which we used as control in these experiments: EGFR-Y1068, TRK-Y490, PDGFR-Y751, PDGFR-Y1009 and HMT-Y324 [17, 19]. EGFR-Y1068 binds Grb-2 and PDGFR-Y1009 and HMT-Y324 are binding sites for the SH2 domains of Syp and src kinases, respectively. The TRK-Y490 and PDGFR-Y751 control phosphopeptides were selected on the basis of their binding properties for adapter proteins previously suggested to bind to the TCR complex, She, and the 85-kDa regulatory subunit of PI-3' kinase [20, 21] (g.g., TRK-Y490 binds She [15] and PDGFR-Y751 binds p85 [22]). The control tyrosine phosphopeptides recruited distinct patterns of [35S]methionine-labeled proteins from the T cell lysates, indicating that the protein phosphorylation of tyrosine phosphopeptides can be highly selective (Fig. 1b).

EGFR-Y1068 is an autophosphorylation site of the human EGFR that binds the SH2 domain of the 26-kDa adapter protein Grb. Fig. 1b clearly shows a predominant 26-kDa protein bound to EGFR-Y1068. The other proteins detected in the EGFR-Y1068 precipitates include Grb-2 SH3 domain-binding proteins of 150 and 75 kDa (Fig. 1b). The 150-kDa protein is the p21 ras guanine nucleotide-exchange protein.
Precipitates, as could a 58-kDa molecule (Fig. 1b). HMT-Y324 is a consensus binding site for the SH2 domain of p56lck and p59fyn [17]. Western blot analysis confirmed that HMT-Y324 could bind and preclear both of these src family kinases from T cell lysates (data not shown). In repeated experiments, a 56-kDa protein corresponding to p56lck could be detected in the [35S]methionine-labeled HMT-Y324 complexes, but no 59-kDa labeled proteins were seen, suggesting that p59fyn is not effectively metabolically labeled under these conditions (Fig. 1b). The data in Fig. 1a show the [35S]methionine-labeled proteins precipitated with each of the different TCR/CD3 ITAM peptides. A 70-kDa [35S]methionine-labeled protein associated with all the ITAM peptide complexes that was clearly a specific ITAM binding protein, since it was not observed in any of the control non-ITAM phosphorylated peptide precipitates (Fig. 1b) or in the nonphosphorylated ITAM peptide controls (Fig. 1a and data not shown). The 70-kDa [35S]methionine-labeled protein was equally represented and predominant in all the ITAM precipitates. There were some differences in the patterns of other proteins detected in the ITAM precipitates; notably, there was a doublet of proteins migrating at approximately 45 and 50 kDa in the CD3γ and δ ITAM precipitates (Fig. 1a). This doublet was not seen with the ζ or ε ITAM, nor did it co-migrate with She. There was also a 150-kDa protein present specifically in the ζ1 and ζ2 ITAM precipitates (Fig. 1a). The identity of this protein is not known, but Western blot analyses show that it is not phospholipase Cγ1, a known TCR effector (data not shown).

Figure 1. Analysis of metabolically labeled ζ and CD3 γ, δ and ε ITAM-associated proteins. (a) T cells (4 x 10⁷) were incubated for 4 h with 5 mCi [35S]methionine in 20 ml methionine-free RPMI 1640 medium with 10% dialysed FCS. Precipitates were prepared from NP40 lysates (2 x 10⁶ cells) using 20 μM of the unphosphorylated ζ ITAM and equivalent concentrations of doubly phosphorylated ζ1, ζ2, ζ3, CD3 γ, δ and ε ITAM peptides or Affigel 10 alone. (b) Precipitates were prepared from NP40 lysates (2 x 10⁶ cells) using 25 μM of the unphosphorylated ζ1 ITAM and equivalent concentrations of doubly phosphorylated ζ1 ITAM, EGFR-Y1068 (EGFR-P), PDGFR-Y751 (PDGFR-P1), PDGFR-Y1009 (PDGFR-P2) or HMT-Y324 as indicated. Precipitates were resolved by 7–17% gradient SDS-PAGE under reducing conditions. Molecular mass markers are given in kDa.

3.2 The interactions of the ζ and CD3 ITAM with cellular tyrosine kinases

One criticism of this approach to identify effector molecules on the basis of metabolic labeling experiments is that this technique only identifies proteins that can be metabolically labeled, i.e. those having a relatively high turnover and which interact with relatively high stoichiometry. To examine further the molecules selected by the ITAM, the protein complexes isolated with the different ITAM peptides were subjected to Western blot analysis with antisera to the tyrosine kinases ZAP-70, p59fyn or p56lck. These Western blot analyses showed that the 70-kDa protein tyrosine kinase ZAP-70 was detected in each of the ITAM precipitates, as could a 58-kDa molecule (Fig. 1b). HMT-Y324 is a consensus binding site for the SH2 domain of p56lck and p59fyn [17]. Western blot analysis confirmed that HMT-Y324 could bind and preclear both of these src family kinases from T cell lysates (data not shown). In repeated experiments, a 56-kDa protein corresponding to p56lck could be detected in the [35S]methionine-labeled HMT-Y324 complexes, but no 59-kDa labeled proteins were seen, suggesting that p59fyn is not effectively metabolically labeled under these conditions (Fig. 1b). The data in Fig. 1a show the [35S]methionine-labeled proteins precipitated with each of the different TCR/CD3 ITAM peptides. A 70-kDa [35S]methionine-labeled protein associated with all the ITAM peptide complexes that was clearly a specific ITAM binding protein, since it was not observed in any of the control non-ITAM phosphorylated peptide precipitates (Fig. 1b) or in the nonphosphorylated ITAM peptide controls (Fig. 1a and data not shown). The 70-kDa [35S]methionine-labeled protein was equally represented and predominant in all the ITAM precipitates. There were some differences in the patterns of other proteins detected in the ITAM precipitates; notably, there was a doublet of proteins migrating at approximately 45 and 50 kDa in the CD3γ and δ ITAM precipitates (Fig. 1a). This doublet was not seen with the ζ or ε ITAM, nor did it co-migrate with She. There was also a 150-kDa protein present specifically in the ζ1 and ζ2 ITAM precipitates (Fig. 1a). The identity of this protein is not known, but Western blot analyses show that it is not phospholipase Cγ1, a known TCR effector (data not shown).

Figure 2. T cells (2 x 10⁷) were lysed and proteins were precipitated with peptides coupled to Affigel 10. The data show precipitates prepared with 15 μM of the doubly phosphorylated ζ1, ζ2, ζ3, CD3 γ, δ and ε peptides as indicated or acetone-precipitated total cell lysate (5 x 10⁶ cells). Samples were resolved by SDS-PAGE on a 10% gel and transferred to PVDF membranes and probed with ZAP-70, p59fyn or p56lck antibodies.

Sos [23]. The 75-kDa protein seen in these precipitates is the recently characterized SLP-76 [24] and the 38-kDa protein is unknown. PDGFR-Y751 is a binding site for the SH2 domain of the p85 subunit of PI-3' kinase, p85 and the associated 110-kDa catalytic subunit of the PI-3' kinase are the major [35S]methionine-labeled proteins associated with PDGFR-Y751 (Fig. 1b). PDGFR-Y1009 is a binding site for the 68-kDa tyrosine phosphatase Syp. The data (Fig. 1b) show that a 68-kDa protein is predominant in the PDGFR-Y1009 precipitates. TRK-Y490 is a binding site for the adapter She, and Western blot analyses with She antisera confirmed that TRK-Y490 could affinity-purify the majority of both the 46- and 52-kDa She isoforms expressed in T cells (Fig. 3). [35S]Methionine-labeled molecules corresponding to the 52-kDa, but not the 46-kDa isofrom of She could be detected in the TRK-Y490 precipitates, as could a 58-kDa molecule (Fig. 1b). HMT-Y324 is a consensus binding site for the SH2 domain of p56lck and p59fyn [17]. Western blot analysis confirmed that HMT-Y324 could bind and preclear both of these src family kinases from T cell lysates (data not shown). In repeated experiments, a 56-kDa protein corresponding to p56lck could be detected in the [35S]methionine-labeled HMT-Y324 complexes, but no 59-kDa labeled proteins were seen, suggesting that p59fyn is not effectively metabolically labeled under these conditions (Fig. 1b). The data in Fig. 1a show the [35S]methionine-labeled proteins precipitated with each of the different TCR/CD3 ITAM peptides. A 70-kDa [35S]methionine-labeled protein associated with all the ITAM peptide complexes that was clearly a specific ITAM binding protein, since it was not observed in any of the control non-ITAM phosphorylated peptide precipitates (Fig. 1b) or in the nonphosphorylated ITAM peptide controls (Fig. 1a and data not shown). The 70-kDa [35S]methionine-labeled protein was equally represented and predominant in all the ITAM precipitates. There were some differences in the patterns of other proteins detected in the ITAM precipitates; notably, there was a doublet of proteins migrating at approximately 45 and 50 kDa in the CD3γ and δ ITAM precipitates (Fig. 1a). This doublet was not seen with the ζ or ε ITAM, nor did it co-migrate with She. There was also a 150-kDa protein present specifically in the ζ1 and ζ2 ITAM precipitates (Fig. 1a). The identity of this protein is not known, but Western blot analyses show that it is not phospholipase Cγ1, a known TCR effector (data not shown).

Figure 2. T cells (2 x 10⁷) were lysed and proteins were precipitated with peptides coupled to Affigel 10. The data show precipitates prepared with 15 μM of the doubly phosphorylated ζ1, ζ2, ζ3, CD3 γ, δ and ε peptides as indicated or acetone-precipitated total cell lysate (5 x 10⁶ cells). Samples were resolved by SDS-PAGE on a 10% gel and transferred to PVDF membranes and probed with ZAP-70, p59fyn or p56lck antibodies.
precipitates (Fig. 2). A comparison of ZAP-70 levels associated with the ITAM peptide precipitates to ZAP-70 levels in cell extracts indicated that the doubly phosphorylated ITAM could bind approximately 90–95% of the cellular pool of ZAP-70 (Fig. 2). ZAP-70 did not bind to the control tyrosine phosphopeptides peptides or to nonphosphorylated ITAM peptides (data not shown). No p56lck was detected in the ITAM complexes (Fig. 2). p59fyn is a PTK that has been previously characterized to bind to the TCR ITAM [25]. Western blot analysis of ITAM precipitates detected low levels of p59fyn (approximately 1% of the cellular p59fyn pool) in the ζ1, ζ2 and CD3 γ, δ complexes, but not in ζ3 or CD3 ε complexes (Fig. 2). A ZAP-70-related tyrosine kinase, Syk, can also bind to ITAM motifs [26]. In the present study, Syk was not detected by Western blot analyses in the cell lysates isolated from peripheral blood T lymphoblasts (data not shown). The ITAM are predicted to bind to Syk and the failure to detect Syk in ITAM complexes probably reflects the fact that Syk is expressed at extremely low levels in peripheral blood-derived T cells, particularly in comparison to ZAP-70 [26].

3.3 A comparison of Shc, PI-3'kinase, Grb-2 and ZAP-70 binding to the ζ and CD3 ITAM

It has been described that the adapter Shc binds to the ζ ITAM but not the CD3 ε ITAM [20]. Similarly, the p85 subunit of PI-3' kinase has been described to bind to the ζ ITAM [21]. In the [35S]methionine-labeling experiments, no labeled proteins corresponding to Shc or p85 could be detected in the ζ or ε ITAM precipitates, even though these molecules were readily detected in the appropriate control precipitates. A low level of a 26-kDa [35S]methionine-labeled protein that co-migrated with Grb-2 was detected in the ITAM precipitates. However, by comparison with Grb-2/EGFR-Y1068 binding, if the 26-kDa protein in the ITAM precipitates from [35S]methionine-labeled cells was Grb-2, then the ITAM/Grb-2 interaction must be of low stoichiometry. Thus, in further experiments, more sensitive Western blot analyses using chemiluminescence detection methods were used to compare ZAP-70, Shc, p85 and Grb-2 interactions with the various ζ and CD3 ITAM. In these experiments, the efficiency of ITAM binding was judged on the basis of the efficiency of the ITAM to recruit the proteins from T cell lysates. Of equal importance in these experiments was a comparison of the efficiency of Shc, p85 and Grb-2 binding to ITAM with the binding of these adapter proteins to previously defined high-affinity phosphotyrosine binding sites; e.g. Shc/TRK-Y490 binding, p85/PDGFR-Y751 binding or Grb-2/EGFR-Y1068 binding. The data in Fig. 3 show Shc, p85, Grb-2 and ZAP-70 binding to different concentrations of the ITAM peptides. As described previously with ITAM binding studies with GST fusion proteins of ZAP-70 SH2 domains [81], there is a hierarchy of ZAP-70 binding to the various ITAM. In the present series of experiments, the hierarchy of the ZAP-70/ITAM interaction was ζ1 = γ = δ > ζ3 > ζ2 = ε. The hierarchy of Shc binding to the different ITAM was γ = δ > ζ3 = ζ1, but no effective binding to ζ2 or ε was seen. For p85, the hierarchy was ζ3 = γ = δ > ζ1 = ζ2, but no effective ε binding was seen. For Grb-2, the hierarchy was ζ1 = γ = δ with poor binding of Grb-2 to ζ2, ζ3 and no binding to ε. The interactions of the ITAM with Shc, Grb-2 and p85 were relatively selective. For example, other SH2 domain-containing proteins found in T cell lysates such as Vav, phospholipase Cγ1, or hematopoietic tyrosine phosphatase (HCP) were not present in the ITAM complexes (data not shown).

4 Discussion

ZAP-70 could bind to nanomolar levels of the ITAM, whereas Grb-2 and p85 binding could only be detected with 100-fold higher concentrations of the ITAM. Similarly, Shc binding to the ζ ITAM could only be detected with micromolar levels of ITAM, whereas Shc could bind to tenfold lower levels of the γ and δ ITAM. It is also note-
worthy that only a small percentage of the total cellular pool of She, p85 or Grb-2 could bind to the ITAM, whereas the appropriate positive control tyrosine phosphopeptides could effectively preclude their binding partner from T cell lysates. For example, 20 μM ITAM peptide could effectively recruit 95% of cellular ZAP-70 but only 1% of Grb-2, 1–2% of She, or 1–2% of p85. This did not reflect technical difficulties in the binding of tyrosine phosphopeptides to these molecules, because equivalent levels of EGFR-Y1068 could effectively bind 90–95% of Grb-2, TRK-Y490 could bind 90–95% of cellular She and PDGFR-Y751 could bind 90–95% of p85. The present data do not resolve whether the high binding efficiency of the ITAM for ZAP-70 means that they have a much higher affinity for ZAP-70 than they do for She, p85 or Grb-2. An alternative possibility is that the selectivity of the ITAM for ZAP-70 could reflect the fact that the cellular concentrations of this kinase are much higher than the concentrations of the other molecules. The question of ITAM affinity for different proteins could be resolved with binding experiments in vitro with purified proteins and ITAM peptides. Quantitation of the relative cellular concentrations of this kinase are much higher than the concentrations of the other molecules. The question of ITAM affinity for different proteins could be resolved with binding experiments in vitro with purified proteins and ITAM peptides. Quantitation of the relative cellular concentrations of different proteins is much more difficult to determine, but equally important in predicting ITAM binding properties. For example, the ITAM may bind to purified She, p85 or Grb-2 fusion proteins in isolation but in vivo, they would be exposed to a complex mixture of these molecules. The pattern of ITAM interactions would thus be influenced by differences in the relative levels of kinases and adaptors in different populations of T cells. This could be one mechanism for signaling variations between TCR complexes expressed in different cells.

The idea that the ζ and ε ITAM might not be functionally equivalent was indicated previously in a study that compared the interaction of bacterially expressed ZAP-70 SH2 domains with the tyrosine-phosphorylated ζ, ε ITAM peptides [8]. These experiments directly measured ITAM affinities for purified ZAP-70 SH2 domain fragments, whereas the present experiments measure the ability of the different ITAM to recruit the full-length ZAP-70 protein from total T cell lysates which would contain a mixture of competing proteins. The two sets of experiments are thus not directly comparable. Nevertheless, the major conclusion from both studies is that the doubly phosphorylated CD3 and ζ ITAM do not bind ZAP-70 equivalently. The present data also show differences in the abilities of the different ITAM to bind p59fyn and the adapter proteins She, p85 and Grb-2. Western blot analysis of doubly phosphorylated ITAM precipitates could detect p59fyn very weakly in the ζ1 and more strongly in the ζ2 and CD3 γ, δ complexes, but not in ζ3 or CD3 ε complexes. Previous studies have shown that p59fyn can be co-precipitated with a receptor chimera containing the cytoplasmic domain of the CD3ε subunit. The failure to see direct binding of p59fyn to the doubly phosphorylated CD3ε ITAM implies that the interaction between endogenous CD3ε and p59fyn is not mediated by direct binding of p59fyn to the doubly phosphorylated CD3ε ITAM. Previous analyses with different phosphoforms of the ζ1 ITAM have shown that singly phosphorylated ITAM have different binding affinities for adapter molecules and kinases than the doubly phosphorylated ITAM [27]. For example, we have previously noted that p59fyn preferentially binds to a ζ1 ITAM singly phosphorylated on the C-terminal tyrosine residue, whereas She binds preferentially to a ζ1 ITAM singly phosphorylated on the N-terminal tyrosine residue. Thus, variations in the ratio of singly phosphorylated to doubly phosphorylated ITAM would change their binding specificity. It should also be considered that the ITAM will not normally function in isolation, and the present strategy of examining the binding interactions of the isolated ITAM peptides does not measure the impact of any cooperative interactions between the ITAM. It is also quite possible that the binding specificity of the ITAM might be influenced by structures in the receptor cytoplasmic domains that are outside the ITAM motif. There may well be considerably more heterogeneity in the binding of different adapters and kinases to the TCR complex than revealed by the current analyses of the binding properties of the doubly phosphorylated ITAM. Nevertheless, the present data show that there are quantitative differences in the abilities of the doubly tyrosine-phosphorylated CD3 and ζ ITAM to bind ZAP-70 and qualitative differences in the biochemical properties of the doubly phosphorylated ITAM with regard to their interactions with molecules such as She, Grb-2, p85 and p59fyn. These differences were particularly marked between the CD3 ε ITAM and the CD3 γ and δ ITAM. The presence of multiple ITAM within the TCR has been suggested to amplify signal transduction by the TCR. The qualitative and quantitative differences in the interactions of the various TCR/CD3 ITAM with cellular proteins described herein indicate that any differences in the ratio of phosphorylation of the ITAM could contribute to the generation of signaling diversity by the TCR.

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5 References


SH3 Domains of the Adapter Molecule Grb2 Complex with Two Proteins in T Cells: The Guanine Nucleotide Exchange Protein Sos and a 75-kDa Protein That Is a Substrate for T Cell Antigen Receptor-activated Tyrosine Kinases*

In T lymphocytes activated via the T cell antigen receptor (TCR), the SH2- and SH3-containing adapter molecule Grb2 forms a complex with the Ras guanine nucleotide exchange protein Sos and tyrosine phosphorylated proteins. The interaction of Sos with Grb2 is mediated via the Grb2 SH3 domains. In this study, it is shown that a 75-kDa protein is also complexed with the Grb2 SH3 domains in T cells, but not in Rat-1 fibroblasts. The identity of the p75 protein is not known, but immunoblot analysis with phosphotyrosine antibodies indicated that it is rapidly tyrosine-phosphorylated in TCR-activated T cells. This characteristic clearly distinguishes p75 from Sos since Sos is not a phosphotyrosine protein. In vitro binding studies indicated that the p75 phosphotyrosine protein binds to a glutathione S-transferase fusion protein of intact Grb2, but not to a Grb2 fusion protein mutated in its SH3 domains. p75 can also bind to the single COOH-terminal Grb2 SH3 domain, whereas Sos has an in vitro binding preference for the NH2-terminal Grb2 SH3 domain. Collectively, these data indicate that in T cells, two proteins can complex with the Grb2 SH3 domains: Sos and a p75 molecule that is tyrosine-phosphorylated in TCR-activated cells. The significance of p75 association with Grb2 is not clear, but by analogy with Sos, p75 is a potential candidate for a Grb2 effector protein. Data are presented showing that the interaction of the Grb2 SH2 domains with tyrosine phosphoproteins may be regulated by conformational restraints imposed by different molecules complexing with the Grb2 SH3 domains. It is thus possible to speculate that the interaction of either p75 or Sos with the Grb2 SH3 domain may influence the interaction of the Grb2 SH2 domain with tyrosine phosphoproteins.

T lymphocyte activation is controlled by the multichain T cell antigen receptor (TCR),1 which controls a cascade of biochemical events, the earliest of which is activation of intracellular protein-tyrosine kinases (1, 2). One protein-tyrosine kinase-controlled signaling pathway originating from the TCR regulates the activity of the p21\textsuperscript{ras} guanine nucleotide-binding protein (3, 4). The protein-tyrosine kinase link between receptors and Ras in many cells involves guanine nucleotide exchange proteins such as Sos, the homologue of a Drosophila Ras GDP/GTP exchange protein, the "son of sevenless" gene product (5). Sos complexes with the adapter protein Grb2/Sem5, which is composed of one SH2 domain and two SH3 domains. The SH3 domains of Grb2 bind to the carboxyl-terminal proline-rich domain of Sos (6), whereas the SH2 domain binds to tyrosine phosphorylation sites in a number of proteins such as the EGFR, Shc, IRS-1, and Syk (6–13). The interactions between the Grb2 SH2 domain and these tyrosine phosphoproteins is proposed to be important in determining the cell localization/function of Sos.

Recently, it was shown that the Grb2 SH2 domain interacts with two substrates for TCR-activated protein-tyrosine kinases: Shc and a 36-kDa membrane-localized protein (12, 14). Sos has been identified as one molecule that interacts with the Grb2 SH3 domain in fibroblasts. However, recent studies of the in vitro binding characteristics of Grb2 have shown that the Grb2 SH3 domain can interact and regulate another protein, the cytoskeletal GTPase dynamin (15). These data raise the possibility that Grb2 SH3 domains can couple to effector molecules other than Sos and thus may function as an adapter molecule in more than one signaling pathway. In this paper, we have characterized the proteins that complex with Grb2 SH3 domains in T lymphocytes. In this study, we established from in vitro experiments that the Grb2 SH3 domain can bind a number of cytosolic proteins including Sos and a group of molecules that are tyrosine-phosphorylated in response to the triggering of the TCR. Studies of the proteins associated with Grb2 SH3 domains in vivo have determined that the predominant Grb2 complexes in T cells are between Grb2 and Sos or between Grb2 and a 75-kDa protein that is tyrosine-phosphorylated in TCR-stimulated cells. By analogy with Sos, p75 is a potential downstream effector molecule for the adapter protein Grb2 in T lymphocytes.

MATERIALS AND METHODS

Antibodies, Peptides, and Fusion Proteins—Monoclonal antibodies (mAbs) against CD3ε (UCHT-1) (16) and the 9E10 Mye epitope (17) were purified from hybridoma supernatants by protein A affinity chromatography. The phosphotyrosine monoclonal antibody 4G10 and the polyclonal antibody mSosl were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The mAb against Grb2 was purchased from Affiniti (Nottingham, United Kingdom). The CD3 mAb OKT3 was purchased from Ortho Pharmaceuticals. The Fab' fragment of OKT3 was a gift from Narin Osman.

The PDGFR-Y751 phosphotyrosine peptide has the sequence DYPFML (G), and the EGFR-Y1068 phosphotyrosine peptide has the sequence PVPEYNQS. The Sos-Pro peptide has the sequence SGKT-DEVVPVPPPVPR, which corresponds to amino acid residues 1144–
RESULTS

Identification of Proteins That Complex with Grb2 SH3 Domains in T Cells—We have used two peptides that bind to either the SH2 or SH3 domains of Grb2 to affinity purify Grb2 and associated proteins from T cell lysates. Briefly, a synthetic proline peptide corresponding to a sequence in the carboxyl-terminal tail of mSos1 (Sos-PRO) that binds to the SH3 domain of Grb2 (5, 15) was used to precipitate Grb2 from T lymphocytes. Grb2 was also purified from T cell lysates using an affinity matrix of a phosphopeptide corresponding to the tyrosine autophosphorylation site Tyr-1068 in the carboxyl-terminal tail of the EGFR. The data in Fig. 1a show a Grb2 Western blot of the cellular proteins purified with the EGFR-Y1068 peptide or the Sos-derived proline peptide and indicate that these two peptides isolate equivalent levels of Grb2 from T cell lysates. The specificity of the interaction of the EGFR-Y1068 peptide with Grb2 is indicated by the failure to precipitate Grb2 with the PDGF-Y751 phosphotyrosine peptide (DYVPMGL) derived from the PDGF cytoplasmic domain. The EGFR-Y1068 motif binds to the Grb2 SH2 domain and thus allows the copurification of Grb2 and proteins that complex with the Grb2 SH3 domains. Western blot analysis with an Sos antiserum indicates that it is thus possible to copurify Sos with Grb2 from T cell lysates when the EGFR-Y1068 motif is used as an affinity matrix (Fig. 1b). The specificity of this interaction is indicated by the failure of the PDGF-Y751 motif to purify Sos (Fig. 1b). Moreover, the Sos-derived proline peptide, which competes with endogenous proteins for the Grb2 SH3 domain, also fails to purify Sos (Fig. 1b). These data confirm that the EGFR-Y1068 motif can be used to purify Grb2 and proteins associated with Grb2 SH3 domains from T cell lysates. The Sos-PRO peptide also affinity purifies Grb2, but prevents the copurification of Grb2 SH3-binding proteins.

To assess whether Sos is the only intracellular protein that can associate with the Grb2 SH3 domain, metabolic labeling experiments were performed in which EGFR-Y1068 peptide complexes were isolated from [35S]methionine-labeled (Fig. 1c) or 32P-labeled (Fig. 1d) T lymphocytes. The relative molecular masses of the proteins found in EGFR-Y1068 precipitates from [35S]-metabolically labeled T cells are 150, 75, 38, and 26 kDa (Fig. 1c). In addition, a spectrum of phosphoproteins with molecular masses of 150, 125, 75, and 38 kDa is present in EGFR-Y1068 complexes purified from [32P]-labeled T lymphocytes (Fig. 1d). In the complexes isolated with the Sos-PRO peptide beads from [35S]methionine-labeled T cell lysates, polyproteins with molecular masses of ~160, 130, 120, 80, 70, 50, 38, and 26 kDa are detected (Fig. 1c). The most striking observation from the [35S]methionine labeling studies is that the most abundant protein present in EGFR-Y1068 and Sos-PRO complexes is a 26-kDa protein that, on the basis of its electrophoretic mobility, is likely to be Grb2. In other respects, the patterns of proteins isolated with the EGFR-Y1068 and Sos-PRO peptides are distinct and nonoverlapping.

The relative molecular mass of the 150-kDa [35S]methionine-labeled protein in the EGFR-Y1068 protein complexes corresponds in size to Sos, which is known to associate with the Grb2 SH3 domain. Sos is post-translationally modified in TCR-activated T cells, which results in a reduction in its electrophoretic mobility (Fig. 1b). Similarly, the 150-kDa [35S]methionine-labeled protein also undergoes a reduction in its electrophoretic mobility in TCR-activated cells (Fig. 1c). The usual explanation for such gel shift behavior of a protein is hyperphosphorylation, although no increase in 32P incorporation into the proteins corresponding to Sos is observed in the 32P-labeled protein experiment in Fig. 1d. The additional proteins that copurify with Sos (Fig. 1c and d) can also bind to the Grb2 SH3 domain and hence be affinity-purified with the EGFR-Y1068 motif because of their association with Grb2. Alternatively, these proteins can bind directly to the EGFR-Y1068 phosphopeptide or indeed be associated with Sos. To establish whether the proteins detected in Grb2 complexes from these in vivo labeling experiments...
Fig. 1. EGFR-Y1068 affinity resin copurifies endogenous Grb2, Sos, and other cellular proteins from T cell lysates. a and b, shown is an immunoblot analysis of proteins affinity-purified from quiescent T lymphocytes (-) or from T lymphocytes activated with the TCR complex agonist UCHT-1 (+) with EGFR-Y1068 (EGF-P), Sos-PRO, and PDGF-R-Y751 (PDGF-P) peptides coupled to Affi-Gel 10 beads or beads alone (control). Precipitated proteins (isolated from 2 x 10^6 cells/lane) were analyzed by SDS-10% PAGE, immunoblotted with Grb2 mAb (a) or mSos1 antiserum (b), and visualized by ECL detection. The migration of the molecular mass standards is indicated to the left in kilodaltons. c, [35S]methionine-labeled T lymphocytes were untreated (-) or stimulated with UCHT-1 (+) and lysed in 50 mM NaCl, 50 mM Hepes, pH 7.4, 1% Brij 96, 10 mM NaF, 10 mM iodoacetamide, 5 mM oxid orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml concentrations of each of the small peptide inhibitors leupeptin, pepstatin A, and chymostatin. Cellular proteins were precipitated with EGFR-Y1068 or Sos-PRO peptide-coupled beads or beads alone (control) and analyzed by SDS-PAGE. d, T lymphocytes were labeled with [32P]orthophosphate as described under "Materials and Methods." T cells were incubated in medium (0 min) or stimulated with the TCR agonist UCHT-1 for the times indicated. Cell lysates were incubated with either Affi-Gel 10 beads alone (control) or EGFR-Y1068 peptide-coupled beads. Affinity-purified proteins in both c and d were subjected to SDS-7-17% PAGE and visualized by autoradiography. The migration of the molecular mass standards is indicated to the left in kilodaltons.

rectly bind to Grb2, Western blot studies were performed on EGFR-Y1068-Grb2 complexes isolated from either T cell lysates or from T cell lysates activated with either the intact Grb2myc or the Grb2mycSH3 fusion protein (Fig. 2). The data show that the EGFR-Y1068 peptide precipitates contain 75- and 150-kDa proteins that can bind to intact Grb2myc, but not to the SH3 mutant Grb2mycSH3 fusion protein (Fig. 2a). The 150-kDa protein is almost certainly Sos since it co-migrates with the protein reactive with the Sos-specific antiserum. The association between Grb2 and Sos or p75 is not dependent on cell activation (Fig. 2a).

p75, like Sos, appears to be a protein that complexes with the Grb2 SH3 domains. First, it is only detected in the complexes purified with the EGFR-Y1068-Grb2 complex and not with complexes isolated with the PDGFR peptide motif (data not shown). Second, p75 is only detected when the PVDF membranes are probed with intact Grb2myc protein, but not with the Grb2mycSH3 mutant (Fig. 2a). Third, p75 is only present in the Grb2 complexes isolated with the EGFR-Y1068 motif and not in the Grb2 complexes purified with the Sos-PRO peptides (Fig. 2a). In fibroblasts, it has been described that Sos binds to the Grb2 SH3 domain. The data in Fig. 2b compare the Grb2-binding proteins in T cells and Rat-1 fibroblasts. Grb2 complexes purified from fibroblasts with the EGFR-Y1068 affinity matrix contain a 150-kDa protein reactive with Grb2 in the Western blot analyses, but do not contain a protein of 75 kDa (Fig. 2b).

In the biosynthetic labeling experiments, multiple proteins could be copurified with the Sos-Grb2 proline peptide complex (Fig. 1c). These Sos proline peptide-purified proteins do not apparently bind directly to Grb2 since they do not bind to the Grb2 fusion protein in the Western blot analyses (Fig. 2). Accordingly, we concluded that although the Sos proline peptide appears to have a preferential affinity for Grb2, it also binds to additional proteins.

75-kDa Protein That Binds to Grb2 SH3 Domain Is Substrate for TCR-activated Protein-tyrosine Kinases—In the [32P]orthophosphate labeling experiments in Fig. 1d, a 75-kDa protein is seen in the EGFR-Y1068-Grb2 complexes, which suggests that the 75-kDa protein that complexes with the Grb2 SH3 domain might be a phosphoprotein. Previous studies have shown that the Grb2 SH2 domain interacts with two proteins that become tyrosine-phosphorylated in response to engagement of the TCR: Shc and a 36-kDa membrane-associated protein (12, 14). To determine whether the Grb2 SH3 domain can also interact with substrates for TCR-activated protein-tyrosine kinases, a panel of full-length or truncated GST-Grb2 fusion proteins (as described above) were used to purify phosphorylating proteins from T cell lysates as assessed by Western blot analysis with phosphotyrosine mAbs. The data in Fig. 3a
show that the intact Grb2 fusion protein can precipitate multiple proteins that are rapidly and transiently tyrosine-phosphorylated in TCR-stimulated cells. Proteins with the following relative molecular masses were detected: 130, 110, 100, 75, 50, 36, and 34 kDa. The reactivity of many of these tyrosine phosphoproteins is abrogated by mutation of the Grb2 SH3 domains, and they are not purified with the Grb2pSH3 fusion protein (Fig. 3b). Thus, in TCR-activated T cells, the major TCR-induced protein-tyrosine kinase substrate isolated with the Grb2pSH3 mutant is a 36-kDa protein previously shown to interact with the endogenous Grb2 SH2 domain in T lymphocytes (12). The other tyrosine phosphoproteins identified in the Grb2 fusion protein complex must bind to Grb2 via its SH3 domains. This conclusion is supported by the observation that these proteins can be precipitated with isolated fusion proteins solely composed of the NH₂- and COOH-terminal Grb2 SH3 domains (Fig. 3b).

One of the major tyrosine phosphoproteins that associates with full-length Grb2 or the carboxyl-terminal Grb2 SH3 domain is a 75-kDa protein that is of similar size to the Grb2 SH3-binding protein shown to coprecipitate with endogenous Grb2 in EGFR-Y1068-Grb2 complexes. We therefore examined whether the 75-kDa protein purified with the endogenous Grb2 complexes becomes tyrosine-phosphorylated in TCR-activated T lymphocytes by performing Western blot analysis with phosphotyrosine antibodies on Grb2 complexes isolated with the EGFR-Y1068 peptide. As shown in Fig. 3c, in the EGFR-Y1068 peptide precipitates a 75-kDa tyrosyl protein from TCR-stimulated T cells, but not from quiescent T cells. This tyrosine phosphoprotein co-migrates with the Grb2-binding protein in
**SH3 Domains of the Adapter Molecule Grb2**

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phosphotyrosine

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phosphotyrosine

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**Sos3**

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**Fig. 3. 75-kDa protein that binds to Grb2 SH3 domains is substrate for TCR-activated protein-tyrosine kinases.** T lymphocytes were untreated (−) or stimulated with UCHT-1 mAbs for 2 min (+) or the times indicated. Cellular proteins were purified as described below and resolved by SDS-10% PAGE. Protein complexes were analyzed by immunoblotting with the phosphotyrosine mAb 4G10. The migration of the molecular mass standards is indicated to the left in kilodaltons. a, proteins were isolated from 2 × 10^6 T cells with the fusion protein of full-length GST-Grb2 or glutathione S-transferase alone (control) immobilized on glutathione-agarose beads; c, proteins were purified from 2 × 10^6 T cells/lane with EGFR-Y1068 (EGF-P) or Sos-PRO affinity resin or Affi-Gel 10 beads alone (control) as indicated; b and d, proteins were precipitated from T cell lysates with fusion proteins encoding glutathione S-transferase alone (control), full-length GST-Grb2 (Grb2), double SH3 mutant GST-Grb2 49L/203R (Grb2pSH3), the isolated amino-terminal GST-huGrb2 SH3 domain (residues 1-58) (N-SH3), and the carboxyl-terminal GST-huGrb2 SH3 domain (residues 159-217) (C-SH3). Figs. 1 and 2 and appears to bind to the Grb2 SH3 domain since it does not coprecipitate with the Grb2 complexes isolated with the Sos-PRO peptide beads that compete out Grb2 SH3-binding proteins. The data in Fig. 3b show that when isolated Grb2 SH3 domains are used as fusion proteins to purify cellular proteins, the p75 tyrosine phosphoprotein preferentially recognizes the
In this study, we have obtained evidence that in T cells the adapter protein Grb2 can complex with two proteins via its SH3 domain: the Ras guanine nucleotide exchange protein Sos and a 75-kDa protein that is a substrate for TCR-stimulated protein-tyrosine kinases. The conclusion that p75 complexes with the Grb2 SH3 domains in vivo is based on experiments that use the EGFR-Y1068 peptide to affinity purify endogenous Grb2 and its associated proteins from T cell lysates. The EGFR-Y1068 tyrosine phosphopeptide binds to the Grb2 SH2 domain and purifies Grb2 without disturbing the association of cellular proteins with the Grb2 SH3 domains as judged by the ability of the EGFR-Y1068 peptide to copurify Grb2 and Sos. The p75 protein copurifies with Grb2 and Sos, clearly binding directly to the Grb2 SH3 domain. First, it can bind an intact but not an SH3 mutant Grb2 fusion protein in Western blot analysis. Second, it does not coprecipitate with Grb2 when the SH3 domains are bound to an Sos-derived proline peptide. Third, it can be precipitated from T cell lysates with fusion proteins of Grb2 SH3 domains, but not with a Grb2 fusion protein that is mutated in its SH3 domains. In these latter experiments, it was noted that Sos and p75 differentially bind in vitro to the COOH- or NH2-terminal SH3 domains of Grb2. Sos binds preferentially to the NH2-terminal SH3 domain, whereas p75 has a preference for the COOH-terminal SH3 domain. Previous studies have indicated that in vitro binding of Sos to Grb2 requires both SH3 domains of the molecule. Accordingly, the in vitro binding reactivities of Sos and p75 with isolated Grb2 SH3 domains do not necessarily imply that Sos and p75 bind to different SH3 domains of Grb2 in vivo. Nevertheless, the data do reveal that the binding characteristics of Sos and p75 for the Grb2 SH3 domains are different, which supports the hypothesis that they are distinct proteins. Additional evidence that p75 and Sos are different proteins stems from the observation that p75, but not Sos, is tyrosine-phosphorylated in TCR-activated T cells.

The association of p75 with Grb2 is constitutive, like the Grb2/Sos association, and is apparently not regulated by triggering of the TCR. The SH2 domain of Grb2 can interact with a 36-kDa TCR-induced tyrosine phosphopeptide that is located at the plasma membrane in T cells. The interaction between the Grb2 SH2 domain and these tyrosine phosphoproteins is proposed to recruit Grb2 and its SH3-associated proteins to the plasma membrane. Grb2 could play a role in recruiting p75 to the cell membrane, where it comes into contact with the TCR-stimulated protein-tyrosine kinases. Moreover, assuming p75 has some effector function, this recruitment event could bring p75 into proximity with its regulatory target. The identity of p75 is not yet established, although experiments are in progress to purify this protein with the anticipation that its structure may yield some clue as to its function. Previously identified tyrosine phosphoproteins of comparable molecular mass in T cells include the protein-tyrosine kinase Zap70 and the cell-surface molecule CD5, but Western blot analysis has excluded these two possibilities (data not shown). It does seem, however, as though p75 may be cell lineage-restricted since it was detected in Grb2 complexes isolated from T cells, but not from Rat-1 fibroblasts.

In a recent analysis of the tyrosine-phosphorylated proteins associated with Sos-Grb2 complexes isolated with Sos antisera only, the p36 protein-tyrosine kinase substrate that binds to the Grb2 SH2 domain (and not a p75 tyrosine phosphoprotein) was detected. These data imply that Sos and p75 may be in mutually exclusive Grb2 complexes, but unfortunately, our Sos antibodies have a relatively low affinity and thus cannot be used for the type of precloning experiment that would allow quantitation of the relative stoichiometry of the Sos-Grb2 versus p75-Grb2 complexes. The Sos protein peptide (SKGTPDEVFPVPPFPVR) used in this study is equivalent to one of three proline-rich sequences contained in the COOH terminus of Sos that have been shown to compete for Grb2 binding. One intriguing observation is that the Sos protein peptide or the GST-C-Sos fusion protein can interact with Grb2 SH3 domains and purify equivalent levels of endogenous Grb2 from
cells. However, the p36 TCR-induced tyrosine phosphoprotein that binds to the Grb2 SH2 domain was only seen in the Grb2 complexes isolated with the GST-C-Sos fusion protein, but not in Grb2 purified with the Sos-derived peptide. The interpretation of this experimental observation is that the additional proline-rich sequences may interact with the Grb2 SH3 domains in a way that leads to conformational changes in the Grb2 molecule and its SH2 domain and therefore to different interactions of the Grb2 SH2 domain with tyrosine phosphoproteins. The observation that at least two proteins can complex with the Grb2 SH3 domain thus takes on a new significance since it is possible to speculate that the interaction of either p75 or Sos with the Grb2 SH3 domains may influence the interaction of the Grb2 SH2 domain with tyrosine phosphoproteins. To date, at least five protein-tyrosine kinase substrates have been shown to bind to the Grb2 SH2 domain, including p36, Shc, EGFR, Syp, and IRS-1 (7-12, 14). These tyrosine phosphoproteins are not necessarily present in the same cells, although in T cells, p36 and Shc are coexpressed. Future studies will establish whether the interaction of these proteins with Grb2 is regulated by their tyrosine phosphorylation status alone or whether the binding of different proteins to the Grb2 SH3 domains also has a regulatory effect on the specificity of Grb2 SH2/phosphotyrosyl interactions.

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REFERENCES
Phosphatidylinositoi 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways
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Background: Phosphatidylinositol 3'-hydroxyl kinase (PI 3-kinase) is activated by many growth factor receptors and is thought to exert its cellular functions through the elevation of phosphatidylinositol (3,4,5)-trisphosphate levels in the cell. PI 3-kinase is required for growth-factor induced changes of the actin cytoskeleton which are mediated by the GTPases Rac and Rho. Recently, a role for Rac and Rho in regulating gene transcription has become evident.

Results: Here, we show that membrane targeting of the p110 catalytic subunit, but not the p85 regulatory subunit, of PI 3-kinase generates a constitutively active enzyme that allows us to assess the relative contribution of PI 3-kinase activation to a particular cellular response. Expression of this active PI 3-kinase induced actin reorganization in the form of Rac-mediated lamellipodia and focal complexes, and Rho-mediated stress fibres and focal adhesions. However, expression of active PI 3-kinase did not induce the Ras/Rac/Rho signalling pathways that regulate gene transcription controlled by the c-fos promoter, the c-fos serum response element or the transcription factors Elk-1 and AP-1.

Conclusions: Our results demonstrate that PI 3-kinase induces a selective subset of cellular responses, but is not sufficient to stimulate the full repertoire of Rac- or Rho-mediated responses.

Background
Stimulation of cells with a wide variety of agonists results in the activation of phosphatidylinositol 3'-hydroxyl kinase (PI 3-kinase) and a rapid elevation of the levels of phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P)) and phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2). PI 3-kinase is involved in cellular transformation or signalling by receptor and non-receptor tyrosine kinases in many cells. The form of PI 3-kinase implicated in these processes comprises a regulatory 85 kDa subunit containing two Src homology 2 (SH2) domains and (at its amino terminus) one SH3 domain, and a catalytic 110 kDa subunit [1,2]. In response to extracellular stimuli, PI 3-kinase has been shown to associate physically with several signalling proteins, including growth factor receptors such as the platelet-derived growth factor (PDGF) receptor, non-receptor tyrosine kinases of the Src family, Abl or Crk [3], and, more recently, GTP-binding proteins of the Ras and Rho families [4-6].

Initial studies to identify the cellular functions of PI 3-kinase used mutant receptors whose phosphotyrosine-binding sites for PI 3-kinase were replaced with phenylalanine [7,8], or used a p85 mutant that lacked the p110-binding site [9,10]. These mutants prevent the recruitment of PI 3-kinases into activated receptor complexes, but such dominant interfering approaches cannot distinguish between the requirement for two proteins that bind to the same site [11]. Additional tools for the study of PI 3-kinase effector pathways are inhibitors of PI 3-kinase function, such as wortmannin or the LY294002 compound, which compete for the lipid- or the ATP-binding sites on PI 3-kinase, respectively.

A recent advance in PI 3-kinase signalling studies has come from experiments with activated mutants generated by targeting the p110 catalytic subunit to the membrane. Studies comparing the effects of activating and interfering approaches have concluded that PI 3-kinase signals are necessary and sufficient for activation of p70^S6K [12], a serine/threonine kinase, specific for ribosomal protein S6, which is required for cells to enter S phase after mitogen stimulation [13]. PI 3-kinase has also been shown to be required for activation of the extracellular signal-regulated kinase (ERK) pathway in several cell systems [14,15], although the expression of an activated PI 3-kinase does not seem to be sufficient for activation of the ERKs in mammalian cells [12]. These results demonstrate that PI 3-kinase can either function alone to initiate cellular responses or act in concert with other signalling pathways.

PI 3-kinase has also been shown, by the use of inhibitors, to be involved in the regulation of the actin cytoskeleton by growth factors such as PDGF or insulin [16-18]. Growth
factors trigger membrane ruffling, and the subsequent formation of actin stress fibres, by a mechanism that is dependent on the function of the Rho family GTPases Rac and Rho, respectively [19]. The expression of constitutively active forms of these proteins, V12Rac and V14Rho, can mimic the responses induced by PDGF: V12Rac induces membrane ruffling and focal complex formation, and V14Rho induces actin stress fibres and focal adhesion assembly [19,20]. The activation of PI 3-kinase is necessary for PDGF activation of Rac- and Rho-mediated rearrangements of the actin cytoskeleton [16,17]; indeed, previous studies have suggested that PI 3-kinase may regulate GTP-loading of Rac [21] and, hence, regulate Rho [17,19]. It is unclear, however, whether PI 3-kinase activation is sufficient to induce Rac- and Rho-mediated cytoskeletal rearrangements or whether PI 3-kinase is a single component of more complex activation processes.

Comprehensive studies have recently established an additional role for Rac and Rho in mediating transcriptional regulation of the c-fos serum response element (SRE) [22]. The data concerning a role of PI 3-kinase in regulating SRE transcription has been controversial: some studies state that PI 3-kinase function is required for SRE induction [10,15], whereas others could not define a role for PI 3-kinase in this response [22]. Such discrepancies could be explained by the existence of parallel compensatory pathways. Information concerning the ability of active PI 3-kinase to generate positive signals that regulate Rac/Rho transcription factor responses would thus be of value. A key question is whether PI 3-kinase is a general stimulator of Rac and Rho function in cells.

We investigated the ability of constitutively active PI 3-kinase to initiate Rac/Rho-mediated cytoskeletal rearrangements and Rac/Rho-mediated activation of transcription factor pathways. Our strategy to generate a constitutively active mutant was to localize subunits of PI 3-kinase to the membrane. Here, we show that membrane-targeting of the p110α catalytic subunit of PI 3-kinase generates a constitutively active enzyme that can induce actin reorganization, forming Rac-mediated membrane ruffles and focal complexes and Rho-mediated stress fibres and focal adhesions. However, expression of active PI 3-kinase did not induce Ras/Rac/Rho signalling pathways that can regulate transcription of c-fos, c-fos SRE, or the transcription factors AP-1 or Elk-1. Hence, PI 3-kinase induces a selective subset of cellular responses but is not sufficient to stimulate the full range of Rac- or Rho-mediated responses.

Results

Construction and biochemical characterization of membrane-targeted rCD2–PI 3-kinase chimeras

To localize PI 3-kinase at the membrane, we constructed chimeric molecules in which the cytoplasmic domain of the rat CD2 (rCD2) cell-surface antigen was replaced with either subunit of PI 3-kinase (Fig. 1a): the extracellular and transmembrane domains of the rCD2 antigen were linked to the amino terminus of p85α (rCD2p85) or p110α (rCD2p110). As a control, a rCD2p110 construct with an inactivating point mutation (the arginine at position 1130 replaced with a proline; R1130P) in the ATP-binding site [23], which abolishes its kinase activity (rCD2p110-R/P), was generated. These fusion proteins were transiently expressed in Cos7 cells, and western-blot analysis of the resulting protein chimeras with rCD2 monoclonal antibodies revealed the presence of molecular species of the predicted masses (Fig. 1b). Cell-surface expression of the different rCD2–PI 3-kinase chimeras was confirmed by flow cytometric immunofluorescence analysis with an anti-rCD2 monoclonal antibody (Fig. 1c).

To assess whether rCD2–PI 3-kinase chimeras had lipid kinase activity, the chimeras were immunoprecipitated with an anti-rCD2 monoclonal antibody and subjected to PI kinase assays in vitro. rCD2p110 had strong PI 3-kinase activity in vitro, which was dependent on the integrity of the p110 ATP-binding site — there was no detectable lipid kinase activity in comparable immunocomplexes of rCD2p110-R/P (Fig. 1d). These results show that the fusion of p110 to the rCD2 extracellular and transmembrane domains does not adversely affect the folding of the protein and thus destroy the catalytic activity of the enzyme. There was also low-level PI kinase activity associated with the rCD2p85 chimera. Western-blot analysis confirmed that this reflected an association of rCD2p85 with the endogenous p110α catalytic subunit of PI 3-kinase (data not shown).

To explore the effect of membrane recruitment of the PI 3-kinase subunits on cellular levels of D-3 phosphorylated inositol lipids in intact cells, Cos7 cells were transiently transfected with expression vectors for the different rCD2–PI 3-kinase chimeras. The cells were metabolically labelled with [32P]orthophosphate, the phospholipids were extracted, and the levels of D-3 phosphorylated phosphoinositides were determined (Fig. 1e). Strikingly, expression of rCD2p110 caused a four-fold increase in PI(3,4)P2 levels and approximately a six-fold elevation of PI(3,4,5)P3 levels compared with basal levels in control cells. The stimulatory effects of rCD2p110 were due to the catalytic activity of the membrane-bound p110 protein — expression of equivalent levels of the rCD2p110-R/P mutant did not alter levels of PI(3,4)P2 and PI(3,4,5)P3. Similarly, expression of rCD2p85 did not increase cellular levels of PI(3,4)P2 or PI(3,4,5)P3 (Fig. 1e). We conclude that plasma membrane-targeting of the p110 catalytic subunit generates a mutant that is constitutively active in vitro, whereas membrane-targeting of the p85 subunit has no effect on the levels of D-3 phosphorylated inositol lipids in vitro. Immunocomplexes of rCD2p85 contained PI 3-kinase (data not shown).
Construction and characterization of membrane-targeted rCD2–PI 3-kinase chimeras. (a) Schematic of the rCD2–PI 3-kinase chimeras. Numbers correspond to amino acids in the protein and demarcate the extracellular, transmembrane, and cytoplasmic domains. Amino acid 216 depicts the start of the wild-type p85α or p110α molecules. The location of the kinase domain (green box) and the inactivating point mutation R1130P (black dot) are denoted. rCD2p110 and rCD2p110-R/P contain a carboxy-terminal myc tag (triangle). (b) Western-blot analysis of cell lysates from Cos7 cells transiently transfected with 10 μg of expression vectors encoding rCD2p85, rCD2p110, or rCD2p110-R/P, using anti-rCD2 (0x34) monoclonal antibodies. (c) Flow cytometry of Cos7 cells, from (b), expressing rCD2p85, rCD2p110 and rCD2p110-R/P stained with anti-rCD2 monoclonal antibodies followed by fluorescein-conjugated anti-mouse antibody (shaded in black). As a control, each population of transfected cells was incubated with mouse IgG as primary antibody (shaded in grey). (d) PI kinase activity present in anti-rCD2 immunoprecipitates. Cos7 cells were transiently transfected with 10 μg of empty vector (control), or expression vectors encoding rCD2p85, rCD2p110 or rCD2p110-R/P, as indicated. Immunoprecipitations and PI kinase assays were performed as described in Materials and Methods. [32P]Phosphate incorporation into PI (see inset) was quantified using a Phosphoimager and is expressed as fmol of [32P]PI/P per min. The expression levels of rCD2–PI 3-kinase chimeras in anti-rCD2 immunoprecipitates were assessed in parallel by western-blot analysis using anti-rCD2 monoclonal antibodies. Expression levels of rCD2p110 and rCD2p110-R/P were equal; rCD2p85 expression levels were about 3-fold higher than those of rCD2p110 or rCD2p110-R/P, as quantitated by scanning of protein band intensities from the autoradiograph and densitometry analysis. (e) Elevation of PI(3,4)P2 and PI(3,4,5)P3 levels in intact Cos7 cells by expression of rCD2p110. Cos7 cells were transfected with 5 μg plasmid DNA encoding the indicated constructs, labelled with [32P]orthophosphate, and the levels of PI(3,4)P2 and PI(3,4,5)P3 were determined. The data is expressed as fold induction of basal levels of PI(3,4)P2 and PI(3,4,5)P3 in control cells. Data were normalized to PI(4,5)P2 levels in each dish. In control cells, the values of each polyphosphoinositide were: PI(4,5)P2, 167 362 cpm; PI(3,4)P2, 6 821 cpm; PI(3,4,5)P3, 1 699 cpm. The fold inductions for rCD2p110 in three independent experiments were: for PI(3,4)P2: 4.2-, 4.4- and 14.5-fold; and for PI(3,4,5)P3: 5.8-, 8.6- and 3.4-fold. Expression of rCD2–PI 3-kinase chimeras was checked in parallel by western-blot analysis using an anti-rCD2 monoclonal antibody.

Recent studies have shown that membrane-targeting of the PI 3-kinase catalytic subunit using a CAAAX motif generates a constitutively active enzyme that activates the serine/threonine kinases PKB and p70^S6K [12], which play a critical role in the regulation of protein translation and cell-cycle progression [13]. rCD2p110, but not rCD2p85 or rCD2p110-R/P, was able to fully replace serum for activation of PKB and p70^S6K (data not shown). In contrast, rCD2p110 did not activate the mitogen-activated protein (MAP) kinase ERK1 (data not shown), in agreement with recent studies showing that PI 3-kinase is...
Expression of active PI 3-kinase leads to membrane ruffling and stress fibre formation in Swiss 3T3 cells

Cytoskeletal responses triggered by PDGF require PI 3-kinase activation [16,17]. We used membrane-bound rCD2p110 to determine whether PI 3-kinase signals were sufficient to trigger Rac/Rho cytoskeletal signalling pathways. Serum-starved subconfluent Swiss 3T3 cells were microinjected with an expression vector encoding the active PI 3-kinase rCD2p110, and the pattern of filamentous actin distribution was assessed by immunofluorescence in cells expressing rCD2p110 proteins (Fig. 2). Four hours after injection, polymerized actin was seen to form localized lamellipodia around the cell margin and, moreover, to form actin stress fibres (Fig. 2a). These results were confirmed by time-lapse image recording where subconfluent cells expressing rCD2p110 showed active membrane ruffling (data not shown). Swiss 3T3 cells expressing rCD2p110 also exhibited a polarized cell shape (Fig. 2a,b). To analyze these cytoskeletal rearrangements further, quiescent cells were stained for vinculin distribution, which monitors the formation of Rac-dependent focal complexes and Rho-dependent focal adhesions [24]. In cells expressing the active PI 3-kinase, vinculin could be found localized in discrete punctuate spots around the cell margin forming focal complexes and focal adhesions in the body of the cell (Fig. 2b). Control cells expressing the inactive rCD2p110-R/P chimera did not show these structures (Fig. 2c,d). The changes in cell morphology triggered by V12Rac and rCD2p110 were qualitatively the same (compare Fig. 2a,b with 2c,d), although V12Rac induced the formation of vinculin spots uniformly around the entire periphery of the cell (Fig. 2b), whereas the effects of rCD2p110 were localized to discrete areas of the plasma membrane (Fig. 2b).

Cytoskeletal rearrangements induced by PI 3-kinase are dependent on endogenous Rac and Rho function

Membrane ruffling and focal complex formation induced by rCD2p110 were similar to those described for activators of Rac, whereas the effects of rCD2p110 on actin stress fibres and focal adhesion assembly were identical to those described for activators of Rho [19,24]. To assess directly the role of Rac and Rho in PI 3-kinase-mediated cytoskeletal responses, we used N17Rac, a dominant inhibitory mutant of Rac that prevents activation of endogenous Rac [19]. To inhibit endogenous Rho function, cells were microinjected with Clostridium botulinum C3 transferase, which ADP-ribosylates and inactivates Rho [19]. The microinjection of N17Rac proteins abolished membrane ruffles and stress fibre formation in cells expressing active rCD2p110 (Fig. 2e, compare the right-hand cell with the left-hand cell). Hence, cytoskeletal rearrangements triggered by active PI 3-kinase are dependent on the function of Rac proteins. Expression of C. botulinum C3 transferase inhibited rCD2p110-induced assembly of actin stress fibres but not the increase in filamentous actin forming not sufficient to activate the ERKs in mammalian cells [12]. These data indicate that membrane-localized rCD2p110 is an effective tool to investigate the cellular functions of PI 3-kinase.
membrane ruffles (Fig. 2f). Strikingly, in cells where Rho function was blocked, the active PI 3-kinase induced a pattern of vinculin staining indistinguishable from that seen in cells expressing activated V12Rac (compare Fig. 2g with 2h): focal complexes around the cell margin, but no vinculin aggregation indicative of Rho-mediated focal adhesions, could be detected (compare Fig. 2g with 2h). Collectively, these data show that PI 3-kinase can stimulate both Rac-dependent pathways for the formation of focal complexes and membrane ruffling, and Rac/Rho pathways that induce stress fibre and focal adhesion assembly.

rCD2p110 does not induce SRE-, c-fos-, or Elk-1-dependent gene expression

An additional function for the GTPases Rac and Rho has been demonstrated recently: to regulate the transcriptional activity of the c-fos serum response factor (SRF) [22]. We therefore examined whether PI 3-kinase is a universal regulator of Rac- and Rho-function by assessing the ability of rCD2p110 to induce changes in transcriptional activity of the c-fos SRE. In these experiments, we investigated cellular responses to the expression of constitutively active mutants of Rac and Rho, and to regulators of endogenous Rho signalling pathways: serum and Dbl [25], a guanine nucleotide exchange protein that regulates Rho family GTPases. SRE activity was monitored with a SRE-CAT (chloramphenicol acetyl transferase) reporter gene, which is known to be the target of a signalling cascade initiated by the GTPases Cdc42, Rac and Rho [22]. Accordingly, expression of V14Rho or V12Rac stimulated the expression of the SRE-CAT reporter gene to a level equivalent to the response induced by serum (Fig. 3a). However, no SRE-CAT induction could be observed in cells expressing the constitutively active PI 3-kinase alone or in conjunction with suboptimal levels of serum. Furthermore, expression of rCD2p110 alone did not stimulate c-fos promoter activity (Fig. 3c), nor could it synergize with serum for this response (data not shown). The expression of Dbl induced SRE activity (Fig. 3b), but SRE responses triggered by the expression of Dbl or serum were prevented by co-expression of C3 transferase, indicating that SRE regulation by these stimuli is mediated by endogenous Rho signalling pathways. Thus, transcriptional activation of the SRE can be regulated by signalling pathways initiated by serum or by Dbl and mediated by the GTPase Rho, but these responses cannot be induced by a constitutively active PI 3-kinase.

Transcriptional activation from the SRE requires SRF binding. At the c-fos SRE, SRF forms a ternary complex with TCF (ternary complex factor), which cannot bind the SRE by itself. One member of the TCF is Elk-1, which is a substrate for both MAP kinase families, the ERKs [22,26] and the stress-activated protein (SAP) kinase (also known as c-jun N-terminal kinase; SAPK or JNK) [27-29]. As Rac has been shown to be involved in regulating the JNK/SAPK signalling pathway [30,31], we investigated whether activated rCD2p110 could induce Elk-1 activation. To monitor Elk-1 transcriptional activity, a fusion protein comprising the carboxyl terminus of Elk-1 linked to the LexA repressor [32] was co-transfected into NIH 3T3 cells with a LexA operator-controlled CAT reporter gene. LexA–Elk-1 transcriptional activity was low in quiescent fibroblasts, but could be induced by co-expression of active Ha-v-ras, by stimulation with serum, or by UV irradiation (Fig. 3d). However, expression of rCD2p110 did not stimulate Elk-1 activity (Fig. 3d), indicating that a constitutively active PI 3-kinase cannot stimulate the UV-activated stress kinase JNK. This observation was confirmed by direct kinase assays of JNK in vitro (data not shown).

Expression of the Fos protein is required for the promoter activity of many genes. Fos is a component of the AP-1 complex, and hence increased cellular levels of Fos proteins can be monitored by assessing whether the activity of the transcription factor AP-1 becomes upregulated. The AP-1 complex also contains the transcription factors c-Jun and ATF2, and is a target for members of both MAP kinase families, SAPKs and Erks, and their upstream regulators of the Ras and Rho family of GTPases. We determined the ability of activated Ras and activated PI 3-kinase to induce transcriptional activity of AP-1 complexes: Ha-v-ras led to expression of an AP-1–CAT reporter gene and synergized with serum for optimal induction of AP-1–CAT (Fig. 3e); expression of rCD2p110 did not stimulate AP-1 transcriptional activity, nor could it synergize with Ras or serum-derived signals. The failure of rCD2p110 to activate SRE, c-fos, Elk-1 or AP-1 transcription collectively show that PI 3-kinase signals are not sufficient to stimulate MAP kinase effector pathways activated by the GTPases of the Ras and Rho family.

Discussion

A chimeric molecule in which the cytoplasmic domain of the rCD2 cell-surface antigen was replaced with the p110α subunit of PI 3-kinase allows plasma-membrane localization of the catalytic subunit of PI 3-kinase. The membrane-bound rCD2p110 induces accumulation of cellular levels of PI(3,4)P2 and PI(3,4,5)P3 equivalent to those seen after mitogenic stimuli: in cells transiently transfected with rCD2p110, there is an approximate five-fold increase in cellular levels of PI(3,4,5)P3. As shown in Figure 1c, 15–20 % of the total population of transiently transfected Cos7 cells express the rCD2p110 chimera. When normalized to the transfection efficiency, this gives an increase in PI(3,4,5)P3 levels in cells expressing rCD2p110 of approximately 25–30-fold — well within the range described for growth-factor induced elevations of cellular levels of PI(3,4,5)P3. That the rCD2p110 fusion protein is a useful tool for the investigation of the cellular responses triggered by PI 3-kinase was confirmed in
coexpression studies: rCD2p110 activated PKB/Akt and p70^S6k but not the ERKs (data not shown).

Here, we show that PI 3-kinase signals are sufficient to induce cytoskeletal changes that include actin polymerization, resulting in Rac-mediated formation of membrane lamellipodia and focal adhesions and Rho-induced formation of actin stress fibres and focal complexes. Moreover, we show that endogenous Rho and Rac functions are required for these processes, respectively. It is now recognized that Rho family GTPases play an important role in the activation of transcription factors and hence are contributing to the regulation of cell growth. The ability of PI 3-kinase signals to initiate the Rac/Rho-mediated responses in the context of the actin cytoskeleton raises the issue of whether PI 3-kinase is a universal activator of Rac/Rho signal transduction pathways. The present data show that expression of constitutively active PI 3-kinase cannot induce Ras/Rac/Rho signalling pathways that regulate gene transcription from SRE, c-fos, Elk-1 or AP-1 reporters. PI 3-kinase signals also could not synergize with serum to induce gene expression from these CAT reporters. In contrast, activation of endogenous GTPases with serum or by expression of Dbl could activate Rho-mediated stress fibre formation [33] and Rho-mediated SRE transcriptional activation (Fig. 3b). Similarly, studies
have shown that Dbl can induce Rac-mediated actin cytoskeleton responses and Rac-mediated JNK/SAPK activation [30,31]. Our results demonstrate that PI 3-kinase induces a selective subset of cellular responses but is not sufficient to stimulate the full range of Rac- or Rho-coordinated pathways. PI 3-kinase is therefore not a universal activator of Rac/Rho-mediated signalling cascades. To explain this phenomenon, it is necessary to evoke diverse activator of Rac/Rho-mediated signalling cascades.

The present study suggests that different Rac and Rho effectors are not necessarily coordinately activated and may be triggered by distinct intracellular signalling pathways. Hence, PI 3-kinase signals can induce Rac/Rho-mediated cytoskeletal changes without activating Rac/Rho-mediated transcription factor pathways. Expression of Dbl activates a wider repertoire of Rac/Rho cellular responses than the membrane-targeted PI 3-kinase. These observations could be explained by several models, described below, which imply spatial restrictions of Rac/effector complexes.

In a simplistic model, the failure of PI 3-kinase-activated Rac to interact with the full range of Rac effector molecules could result from subcellular compartmentalization of different pools of these proteins. For example, pool 1 of Rac/Rho effectors involved in cytoskeletal rearrangements could be constitutively membrane-localized: receptor activation of PI 3-kinase will generate D-3 phosphoinositides in the plasma membrane, which would spatially restrict the regulatory capacity of these lipids to only membrane-localized pools of Rac/Rho effectors. The expression of oncogenic Dbl is not confined to the plasma membrane and Dbl would come into contact with both pools of Rac/effector complexes, including pool 2, which has a more cytoplasmic distribution and transduces transcriptional activation. The discrepancy between the functional effects of Dbl and active PI 3-kinase could then be explained by their different cellular localization. Evidence from a recent study supports this type of model: the pleckstrin homology (PH) domain of Dbl targets the protein to specific cytoskeletal locations, and a plasma membrane-targeted Dbl with a truncated PH domain is not fully active [42].

A second spatial model, in which pool 2 of effector proteins are actively recruited, allows for the potential of additional signals derived from growth factors (spatial model 2; Fig. 4a). Such signals would recruit different pools of Rac effector molecules into the proximity of Rac activated by PI 3-kinase. Hence, PI 3-kinase signals may be able to cooperate with other signals generated from growth factors to allow full induction of Rac/Rho-mediated signalling pathways, in particular the activation of transcription factor responses. In this context, a recent report describes that the adaptor protein Nck binds to the Rac effector kinase Pak1 [43]; this result is consistent with a mechanism that recruits Pak1 into activated growth-factor receptor complexes and hence to sites in the membrane containing Rac activated by PI 3-kinase. Whether this is the case remains to be elucidated, yet PI 3-kinase signals did not concur with serum-derived signals to give activation of gene expression. Therefore, an alternative model to activate Rac/Rho-mediated gene transcription may involve an independent, as yet uncharacterized, mechanism that does not involve PI 3-kinase — autonomous regulation of pool 2 of effector proteins (spatial model 3; Fig. 4b).

The identity of the direct targets for D-3 phosphoinositides that regulate the activation of Rac and Rho is currently unknown. The activity of Rho family GTPases is controlled by the concerted action of guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation inhibitors (GDIs) and GTPase activating proteins (GAPs). Which of these will be targeted by D3 phosphoinositides is not clear but it is tempting to speculate that PH domains, which have been shown to bind phosphoinositol lipids and are present in Rho family GEFs, could provide the link between lipid kinases and Rac/Rho. However, any model for regulation of Rho family GTPases must include a mechanism to explain how growth factors apparently have the ability to activate Rac and Rho by mechanisms autonomous of PI 3-kinase. For example, the lysophosphatidic acid (LPA) receptor can bypass PI 3-kinase and Rac to stimulate Rho-dependent responses by a different route. Finally, as demonstrated by the present report, models for activation of Rac and Rho must be superimposed by models that allow for selective activation of different Rac/Rho effectors by diverse regulatory proteins with the subsequent stimulation of divergent cellular responses.

Conclusions
Rho family GTPases have been shown to be important for the regulation of different cellular responses, including gene transcription, the activation of certain members of the MAP kinase family, and changes in the actin cytoskeleton. Although it is becoming evident that these diverse cellular responses are mediated by different effector proteins that associate with the activated GTPase complexes, little is
known of how growth factor receptors orchestrate these
distinct effector pathways, in particular whether triggering
of the receptor will uniformly activate all cellular pathways
mediated by a certain GTPase.

PI 3-kinase is activated by many growth factor receptors,
leading to an increase in D-3 phosphoinositides in the
plasma membrane. PI 3-kinase-derived signals are suffi­
cient to induce growth factor-like changes of the actin
cytoskeleton. Further induction of Rac/Rho-mediated pathways for activation of
gene transcription requires a second growth
factor-derived signal that will recruit the Rac
effector molecules involved in MAP kinase
activation into the proximity of PI 3-kinase
activated Rac. Y indicates a phosphotyrosine
residue and PH indicates a pleckstrin homology
domain. (b) Spatial model 3: autonomous
regulation of pool 2 of effector proteins. PI 3-
kinase activation triggers membrane ruffles and
stress fibre formation that entangles pool 1 of
Rac/effector complexes situated in
the proximity of the plasma membrane. To trigger
GDP/GTP exchange on Rac GTPases that
couple to pool 2 of effector proteins, an
independent mechanism is evoked by the
activated growth-factor receptor. This may
involve an uncharacterized upstream signalling
molecule (X) that activates Rac at a different
subcellular location where Rac proteins are
networked to pool 2 of effector proteins. Note:
in these models, only guanine nucleotide
exchange factors (GEFs) or GTPase activating
proteins (GAPs) are depicted as regulators of
Rho family GTPase activity. Equally possible is
the regulation of Rho family GT Pases via
guanine nucleotide dissociation inhibitors
(GDI s). Which regulatory mechanism is
engaged is currently not clear, and may involve
a combination of all three sets of Rho family
regulators.

Materials and methods

Construction of chimeric cDNAs

The coding regions of bovine p85α [44] and p110α [2] were attached
to a truncated rat CD2 molecule consisting of the extracellular
and transmembrane domain (EMBL accession number X05111; [45]). The
rCD2 intracellular domain was truncated at the stop transfer sequence
(amino acids 1–212) by the addition of a
Noû site at the 5' end omitting
the methionine of the leader sequence and introducing the peptide
SGR in the coding sequence at the hinge. The Noû-pl10 construct
was tagged at the carboxyl terminus with the Myc epitope [46]. To con­
struct the inactive rCD2p110-R/P, a 533 bp PstI–NcoI cartridge from
Noû-pl110 was replaced with the corresponding cartridge from the
described p110-BanHI plasmid containing the R916P mutation [23]. The
rCD2-Pl 3-kinase chimeras were assembled by subcloning the
mutated cDNAs into the pcDNA3 vector (Invitrogen) (Fig. 1) or a
human cytomegalovirus (CMV) promoter-driven expression vector [47]
(Figs 2,3). All obtained constructs were verified by nucleotide sequenc­
ing and/or restriction enzyme analysis.

Plasmids and reporter constructs

Hα-v-ras [48], V14Rho, V12Rac and C3 transferase [22] vector con­
structs have been described. For the microinjection experiments,
V12Rac was expressed as myc-tagged derivative from the CMV promoter-driven vector (see above). Human Dbl (amino acids 495–826; EMBL accession number X12306) was expressed as myc-tagged derivative from the CMV promoter-driven vector (see above) and kindly provided by M.F. Olson. The reporter plasmids have been described: pMLNV1ex.ekl-1, 2 lexoptk.CAT [32]; AP-1.CAT [49]; wild-type SRE.CAT [50]; c-fos CAT [51].

Cell culture, transfection and metabolic labelling
Cos7 cells, Swiss 3T3 cells and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS).

Cos7 cells were transfected by electroporation (450 V, 250 μF) with 5–10 μg of plasmid DNA. After 47 h cells were collected. When cells were metabolically labelled, cells were washed extensively after 38 h, labelled with [32P]orthophosphate (1 mCi per dish) in F-12 medium containing 0.8% dialysed FCS and 20 mM Hepes for 8 h and phospholipids were extracted. NIH 3T3 cells were transfected by the diethylaminoethyl (DEAE)-Dextran method [50]. DNA concentrations in each experiment were constant (generally 9 μg) by adding vector plasmid. Transfected cells were serum-deprived for 36 h, stimulated by UV irradiation, and are expressed as fmol of PIP per min.

Microinjection experiments
Serum-starved sub-confluent Swiss 3T3 cells were prepared for microinjection as described [55]. Plasmid DNAs were microinjected at 200 μg ml⁻¹ in PBS into the nucleus. After 3–4 h, cells were fixed with 4% paraformaldehyde (w/v). To prevent activation of endogenous Rac and Rho proteins, rCD2p110 microinjected cells were re.injected with recombinant N17Rac proteins (0.7 mg ml⁻¹) and C3 transferase (100 μg ml⁻¹), respectively, and after 30 min cells were fixed. Vinculin actin was visualized by immunofluorescence, essentially as described [55]. Briefly, permeabilized cells were double-labelled for actin and protein expression by first incubating with OX3 monoclonal antibody to detect rCD2–PI 3-kinase chimeras. Then cells were incubated with FITC-conjugated goat anti-mouse antibody, and for detection of actin, with rhodamine-phalloidin (0.1 μg ml⁻¹). For re-injection experiments, recombinant proteins were co-injected together with rat IgG (0.5 mg ml⁻¹) as an injection marker which was visualized using a cascade blue-conjugated goat anti-rat IgG. For vinculin-staining cells were co-injected with Texas Red-lysinated dextran (2 mg ml⁻¹) as an injection marker. Vinculin was localized as described [55]. Recombinant N17Rac proteins and C3 transferase were prepared as described [19].

Gene expression analysis
8–10 h after inductions as indicated, transfected serum-starved NIH 3T3 cells were washed with PBSA and cells were lysed in 150 μl lysis buffer (0.65% Triton-X100, 20 mM Tris pH 7.5, 137 mM NaCl, 15% (v/v) glycerol, 2 mM EDTA, 2 mM PMSF, 20 μg ml⁻¹ apro tin, 2 mM benzamidine, 1 mM sodium orthovanadate, and 2 μg ml⁻¹ of the small peptide inhibitors leupeptin, pepstatin A and chymostatin). The washed immunoprecipitates were resuspended in 10 μl lipid mixture (1 mg ml⁻¹ each Lα-phosphatidylinositol and Lα-phosphatidyly-l-serine, dispersed by sonication in 25 mM HEPES pH 7.4 and 1 mM EDTA). The reaction was initiated by the addition of 40 μl PI kinase reaction buffer (containing 100 mM [γ²³²P]ATP (10 μCi), 125 mM ATP, 12.5 mM MgCl₂, 125 mM NaCl, 25 mM HEPES pH 7.4 and 250 μM adenosine). After 15 min at 25 °C, the reaction was terminated and the samples processed as described [52]. Incorporation of [³²P]PI into PI was quantified using a PhosphorImager (Molecular Dynamics). The [³²P]PI counts obtained for each sample were normalized to the specific activity of the [γ²³²P]ATP used for the assay, and are expressed as fmol of PI per min.

HPLC analysis of inositol phosphates
Phospholipid extractions were based on the method described previously [53]. Briefly, cells were washed with PBSA, collected in 1 ml 2.4 M HC1 and transferred to a polypropylene test tube. CH₃OH₂MeOH (1:2 v/v) (1.5 ml) containing 10 μg phosphoinositides (Sigma) to act as carrier lipids were added followed by 1 ml CH₂Cl₂. After vortexing and centrifugation the lower phase was removed to a fresh tube, the upper phase was re-extracted twice with 1.5 ml of CH₂Cl₂ and the CH₂Cl₂ phases were combined. Dry lipid films were deacylated with 0.5 ml methanolamine at 50 °C for 1 h in the presence of 50 μl n-butanol. The resulting glycerophosphoinositol esters were re-dissolved in 0.5 ml water and extracted once with an equal volume of n-butanol. The aqueous phase was mixed with 1H-Labelled IP2 (Du Pont) as an internal standard [54] and resolved by ion-exchange on HPLC using a S5 SAX column (Phase Separations). The column was eluted at 1 ml min⁻¹ with a linear gradient of Milli Q water versus 2.5 M Na₂HPO₄ (pH 3.8 with NaOH (% B)); times were t = 0, % B = 0; t = 10, % B = 12; t = 70, % B = 20; t = 110, % B = 50, t = 130, % B = 100; t = 140; % B = 0; fractions were collected every 0.5 min. The data are collected as values of [³²P]PI and [³²H]water radioactivity measured using a calibrated H²/³²P dual-label program on a Beckman LS6000 series scintillation counter.

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Note added in proof
Didichenko, Thelen and colleagues (Curr Biol 1996, 6:1271–1278) have recently described the effect of expressing a membrane-targeted PI 3-kinase on protein kinase B (PKB) and p47phox. Their data confirm that membrane-targeting of the p110alpha catalytic subunit of PI 3-kinase leads to elevation of PI(3,4,5)P3 levels in cells and that expression of membrane-localized PI 3-kinase results in the continuous activation of PKB. Interestingly, the authors show that PI 3-kinase signals lead to constitutive phosphorylation of p47phox, a cytosolic factor required for the assembly of the phagocyte NADPH oxidase. As Rac is an essential component of the NADPH oxidase complex, it will be interesting to analyze whether Rac is involved in events leading to the phosphorylation of p47phox.
The regulation and function of p21^ras during T-cell activation and growth

Manolo Izquierdo Pastor, Karin Reif and Doreen Cantrell

The delivery of signals that control the growth of T cells is a key event for effective co-ordination of T-cell-dependent immune responses. It is now recognized that guanine nucleotide binding proteins play an important role in signal transduction by the T-cell receptor (TCR) and cytokine receptors. Here, Manolo Izquierdo Pastor, Karin Reif and Doreen Cantrell review the numerous recent advances in understanding how the p21^ras guanine nucleotide binding protein couples the TCR to the T-cell signalling cascade.

The ability of the T-cell receptor (TCR) and the interleukin 2 receptor (IL-2R) to regulate the guanine nucleotide binding cycle of the low-molecular-weight G protein p21^ras suggests that these proteins might be important in immunological responses. T-cell growth can be divided into two phases: the first phase, termed T-cell activation, is initiated by the interaction of antigen with the TCR, which triggers the G0-G1 transition of the cell cycle, as well as the induction of IL-2 secretion and IL-2R expression. The second phase involves G1-S phase progression and mitosis, and is controlled by IL-2-IL-2R interactions. In this review, we wish to discuss current understanding of the regulation and function of p21^ras in the immune system, using the paradigms of TCR and IL-2R control of T-cell activation and growth.

The regulation of p21^ras by the TCR and IL-2R

Triggering of the TCR- or the IL-2R-coupled protein tyrosine kinases (PTKs) perturbs the guanine nucleotide binding cycle of p21^ras, and results in a rapid and prolonged accumulation of active p21^ras-GTP complexes. The TCR is a multichain complex comprising the polymorphic aP chains, the invariant CD3 \( \gamma \), \( \delta \) and \( \varepsilon \) chains, and the \( \zeta \) (16kDa) subunits. The intracellular tails of the CD3 and \( \zeta \) molecules couple the TCR to intracellular PTKs such as ZAP-70, p59^{ck} and p56^{ck}. This PTK regulation is absolutely required for all subsequent TCR responses, including p21^ras activation. Triggering of the IL-2R also activates p21^ras in a PTK-dependent response. The IL-2R comprises three subunits, \( \alpha \), \( \beta \) and \( \gamma \), of which the \( \beta \) and \( \gamma \) chains are members of the haematopoietin receptor family. Deletion analysis of the IL-2R \( \beta \) subunit has identified a membrane-distal acidic domain that binds src-family kinases such as p56^{ck}, as well as a membrane-proximal serine-rich domain that binds Janus family kinases (JAKs). Both of these domains of the IL-2R \( \beta \) chain are required for p21^ras activation.

The adapter molecule Grb2 and associated proteins in T cells

One mechanism for p21^ras activation is the stimulation of guanine nucleotide exchange proteins such as the homologue of the Drosophila 'son of sevenless' (Sos) gene product. In fibroblasts, growth factors regulate p21^ras by stimulating Sos via a mechanism involving the adapter protein Grb2/Sem5 (Ref. 6). Grb2 comprises one src-homology 2 (SH2) domain and two SH3 domains. The SH2 domain of Grb2 binds to the C-terminal proline-rich domain of Sos, whereas the SH2 domain interacts with tyrosine-phosphorylated molecules that regulate the cell localization/function of this p21^ras exchange protein. Studies of Grb2 in T cells have identified two proteins that, when tyrosine phosphorylated, can potentially bind to the Grb2 SH2 domain. These are the SH2 domains of Grb2 bind to the SH2 domain of Sos, whereas the SH2 domain interacts with tyrosine-phosphorylated molecules that regulate the cell localization/function of this p21^ras exchange protein. Studies of Grb2 in T cells have identified two proteins that, when tyrosine phosphorylated, can potentially bind to the Grb2 SH2 domain. These are the SH2 domain-containing adaptor protein Shc, of which two isoforms of 46 and 52kDa are expressed in T cells, and a membrane-located tyrosine phosphoprotein of 36kDa (Ref. 5).

In TCR-activated cells, both Shc and p36 are tyrosine phosphorylated and, in vitro, can be shown to bind Grb2 SH2 domains. Shc has an SH2 domain that can
Fig. 1. A summary of the immediate membrane-proximal signals triggered by TCR ligation. TCR-coupled tyrosine kinases, such as ZAP-70, phosphorylate a novel adaptor molecule p36, which binds to PLC, thus regulating inositol phospholipid metabolism and calcium/PKC signalling pathways. p36 also binds the SH2 domain of the adaptor protein Grb2. The SH3 domains of Grb2 bind a novel 75 kDa molecule or Sos, a guanine nucleotide exchange protein for p21Ras. p36–Grb2–Sos complexes may thus link the TCR to the p21Ras/Raf-1/ERK kinase cascade. The TCR also regulates p21Ras via inhibition of p21Ras GAPs. Solid lines indicate interactions based on protein phosphorylation. Dashed lines indicate uncharacterized pathways. Abbreviations: TCR, T-cell receptor; PLC, phospholipase C; PKC, protein kinase C; SH2, src-homology domain 2; Sos, Drosophila 'son of sevenless'-like protein; ERK, extracellular-signal-regulated kinase; GAP, GTPase-activating proteins; MKK, mitogen-activated protein kinase kinase.

bind to the tyrosine-phosphorylated TCR ζ chain, hence tyrosine-phosphorylated Shc could potentially recruit Grb2 and Sos to the TCR complex in activated cells. However, no Shc–Grb2–Sos complexes are detected in TCR-activated cells. Moreover, it appears that the Shc interaction with ζ chains is of relatively low affinity as compared with the binding of tyrosine kinases such as ZAP-70 or p59Erk to ξ (N. Osman, D. Cantrell, unpublished). This may ensure that the Shc interaction with ζ is transient and perhaps only sufficient to bring Shc to the TCR complex, where it can be tyrosine phosphorylated, but is insufficient for the formation of stable TCR–Shc complexes. Tyrosine-phosphorylated Shc may then dissociate from the receptor and form a complex with cytoplasmic adaptor molecules. The major tyrosine phosphoprotein to associate with Grb2 SH2 domains in TCR-activated cells is a 36 kDa molecule (p36), which is proposed to be an adaptor that links the TCR-activated PTKs to Grb2–Sos complexes (Refs 5,8). The failure to find tyrosine-phosphorylated Shc binding to Grb2 and Sos in TCR-activated cells may be the consequence of competition between p36 and Shc for the SH2 domain of Grb2. However, it seems unlikely that Shc recruits Sos to the TCR complex or that Shc is involved in TCR coupling to Grb2–Sos or p21Ras. This does not mean that Shc is unimportant for TCR signalling: there is the possibility that Shc can form a complex with other adaptor molecules and hence regulate an effector system distinct from the Sos/p21Ras-pathway in response to TCR ligation (Fig. 1).

In contrast to the failure to see Shc–Grb2–Sos complexes in TCR-activated cells, there are data suggesting that Shc may be important in coupling the IL-2R to p21Ras. Thus, in IL-2-activated cells, there is no tyrosine phosphorylation of p36, rather there is a high level of Shc tyrosine phosphorylation and rapid formation of Shc–Grb2–Sos complexes (Fig. 2).
Intracellular PTKs that are important for TCR signal transduction include the src-family kinases p56\(^{67}\), p59\(^{68}\) and ZAP-70 (Ref. 1), whereas the IL-2R most notably regulates the activity of p56\(^{67}\) (Ref. 3). She is tyrosine phosphorylated both by the TCR- and IL-2R-induced PTKs and, on this basis, it would be predicted that She is a substrate for the src kinases, although a role for IL-2-stimulated JAKs in She phosphorylation cannot be excluded. By contrast, p36 is only tyrosine phosphorylated in response to TCR triggering and a simplistic analysis would suggest that it is probably a substrate for the kinase ZAP-70, which is activated by the TCR but not by the IL-2R. Recently, it was observed that p36 also exists in a complex with phospholipase C in TCR-activated cells, suggesting that p36 may be an adaptor that couples the TCR both to p2\(^{116}\) and to Ca\(^{2+}\)/protein kinase C (PKC) signalling pathways that originate from the TCR (Ref. 10; Fig. 1).

In summary, the current hypothesis for the regulation of p2\(^{116}\) in T cells is that the IL-2R regulates Sos, a guanine nucleotide exchange protein for p2\(^{116}\), via the adaptors She and Grb2 (Fig. 2). The TCR may also regulate the p2\(^{116}\) guanine nucleotide binding cycle via Grb2-Sos, but it is an unknown 36 kDa tyrosine phosphoprotein rather than She that links the TCR to the adaptor Grb2 (Fig. 1). However, it must be emphasized that the purported role of Grb2 and Sos in TCR or IL-2R regulation of p2\(^{116}\) is based on correlative data and remains to be proven by genetic studies. Moreover, two molecules in addition to Sos are found complexed to the SH3 domains of Grb2 in T cells. These two proteins, of 75 and 116 kDa respectively, are constitutively associated with the SH3 domains of Grb2, analogous to the Grb2-Sos association\(^ {8,11}\). p75 and p116 are substrates for TCR-activated, but not IL-2R-activated, PTKs. On the basis of their association with the Grb2 SH3 domains, p75 and p116 are candidates for Grb2 effector molecules. Whether they are involved in p2\(^{116}\) regulation remains to be determined.

**Ras-GTPase-activating proteins (GAPs) and other p2\(^{116}\) regulatory molecules**

It has been suggested that p95\(^{64}\) is a guanine nucleotide exchange protein for p2\(^{116}\) in T cells\(^ {15}\), even though its structure suggests that p95\(^{64}\) is a more likely candidate for a rho or rac exchange protein\(^ {15}\). p95\(^{64}\) is tyrosine
phosphorylated in response to TCR triggering and, in vitro, this appears to stimulate the guanine nucleotide exchange activity of the protein. However, tyrosine phosphorylation of p95\(^{\text{ERK}}\) does not correlate with p21\(^{\text{ERK}}\) activation: for example, in T cells derived from peripheral blood, p59\(^{\text{ERK}}\) is not phosphorylated in response to IL-2 even though IL-2 activates p21\(^{\text{ERK}}\). Conversely, stimulation of T cells via CD28, with the B7-1 ligand, induces a strong, persistent tyrosine phosphorylation of p95\(^{\text{ERK}}\) but does not activate p21\(^{\text{ERK}}\) (Ref. 14). Instead, the failure of CD28 to regulate the p21\(^{\text{ERK}}\) guanine nucleotide binding cycle correlates with the failure of CD28 to induce the tyrosine phosphorylation of p36, Shc or any other protein capable of recruiting the Grb2-Sos complex to the membrane. Moreover, the ability of p95\(^{\text{ERK}}\) to function as a p21\(^{\text{ERK}}\) guanine nucleotide exchange factor is not supported by analyses of p95\(^{\text{ERK}}\) function in fibroblasts, where no evidence for its regulation of p21\(^{\text{ERK}}\) can be detected. Thus, p95\(^{\text{ERK}}\) may well have important functions in hematopoietic cells but possibly not as a guanine nucleotide exchange protein for p21\(^{\text{ERK}}\).

Undoubtedly, the current trend in studies on p21\(^{\text{ERK}}\) regulatory function is to focus on receptor-mediated control of p21\(^{\text{ERK}}\) guanine nucleotide exchange proteins. However, the importance of negative-regulatory p21\(^{\text{ERK}}\) GTPase-activating proteins (GAPs) for p21\(^{\text{ERK}}\) activation should not be ignored. The GTPase activity of p21\(^{\text{ERK}}\) is controlled by GAPs such as p120-GAP and neurofibromin, and experiments have shown that TCR triggering inhibits the activity of such p21\(^{\text{ERK}}\)-GAPs (Ref. 16). The mechanism by which the TCR inhibits these proteins is not understood but it is probable that simultaneous regulation of guanine nucleotide exchange proteins and GAPs by the TCR contributes to p21\(^{\text{ERK}}\) regulation. By contrast, IL-2 activation of p21\(^{\text{ERK}}\) is not associated with any detectable regulation of p21\(^{\text{ERK}}\)-GAP proteins and it is predicted therefore that the IL-2R regulates p21\(^{\text{ERK}}\) via the activation of p21\(^{\text{ERK}}\) guanine nucleotide exchange proteins.

p21\(^{\text{ERK}}\) function in TCR-mediated signal transduction

The function of p21\(^{\text{ERK}}\) in T cells has been explored using transient transfection protocols that examine the consequences on T-cell activation of expressing mutated, constitutively active or dominant inhibitory p21\(^{\text{ERK}}\) molecules. The expression of a dominant negative p21\(^{\text{ERK}}\) suppresses IL-2 gene induction, and a constitutively active p21\(^{\text{ERK}}\) protein can activate transcriptional factors such as AP-1 and synergize with a calcium signalling pathway to activate the IL-2 gene. One point to emphasize regarding the role of p21\(^{\text{ERK}}\) in T-cell activation is that p21\(^{\text{ERK}}\) function is essential, but not sufficient, for a full activation response. For example, IL-2 gene expression requires the coordinate interaction of multiple transcriptional factors, which reflects the convergence of multiple signalling pathways triggered by both the TCR and costimulatory receptors, only some of which involve p21\(^{\text{ERK}}\) (Fig. 3). The regulation of lymphokine expression is one important facet of T-cell activation, but equally important are the signals that regulate the expression and function of lymphokine receptors and adhesion molecules. Indeed, p21\(^{\text{ERK}}\) may have a role in this aspect of TCR function, as demonstrated by observations that p21\(^{\text{ERK}}\) regulates the expression of CD69, a T-cell activation marker.

The transmission of signals from p21\(^{\text{ERK}}\) to the nucleus is proposed to involve the regulation of the activity of mitogen-activated protein (MAP) kinases or extracellular-signal-regulated (ERK) kinases such as ERK1 and ERK2 (Ref. 22). Two intracellular pathways for ERK2 regulation co-exist in T cells: one mediated by p21\(^{\text{ERK}}\) and the other by PKC (Ref. 23). The TCR stimulates both p21\(^{\text{ERK}}\) and PKC, although it appears that p21\(^{\text{ERK}}\) rather than PKC couples the TCR to the regulation of MAP kinases. The MAP kinases are activated by a kinase cascade involving a MAP kinase kinase (MKK) that phosphorylates and stimulates ERK1 and 2 directly. The activity of the MKK is itself controlled by phosphorylation and hence a MAP kinase kinase (MKKK) plays a crucial role in the regulation of ERK1 and 2 (Ref. 26). In some cells, the serine/threonine protein kinase Raf-1 has been identified as the MKKK that plays a key role in coupling p21\(^{\text{ERK}}\), and hence receptors that stimulate p21\(^{\text{ERK}}\), to the MAP kinases. The N-terminal regulatory domain of Raf-1 can interact directly with 'activated' GTP-bound p21\(^{\text{ERK}}\) (Ref. 28). It has also been shown that constitutively active Raf-1 can mimic the effect of activated p21\(^{\text{ERK}}\), stimulating ERK2 and synergizing with calcium signals to induce the IL-2 gene. These observations collectively suggest that Raf-1 is an effector molecule for p21\(^{\text{ERK}}\) in T cells, but they do not rule out that other p21\(^{\text{ERK}}\) effectors may also exist.

p21\(^{\text{ERK}}\) regulation of the IL-2 gene is apparently mediated by nuclear factor of activated T cells (NF-AT1). NF-AT1 is a complex comprising AP-1 and NF-ATp, a member of the c-rel family of transcriptional factors. NF-ATp is cytoplasmic in quiescent cells and translocates to the nucleus in response to calcium/calcineurin-dependent signals. p21\(^{\text{ERK}}\) and calcineurin synergize for NF-AT1 induction and the role of p21\(^{\text{ERK}}\) in NF-AT1 regulation can probably be explained by p21\(^{\text{ERK}}\) effects on AP-1 (Refs 20,32). One level of AP-1 regulation is via induction of fos-family proteins. The phosphorylation of the transcriptional factor Elk1 by ERK2 appears obligatory for induction of the c-fos gene. Accordingly, the role of p21\(^{\text{ERK}}\) in coupling the TCR to ERK2 and the ability of ERK2 to translocate to the nucleus, where it can directly modulate transcriptional factors such as Elk1, could explain the role of p21\(^{\text{ERK}}\) in TCR signal transduction. However, there is as yet no functional proof that ERK2 is the link between p21\(^{\text{ERK}}\) and the induction of lymphokine gene expression. Furthermore, it is known that AP-1 activity also requires jun-family proteins and the regulation of c-jun phosphorylation mechanisms is complicated by the involvement both of positive and negative phosphorylations that control transcriptional activity. Kinases other than ERK1 and ERK2 can phosphorylate c-jun and hence regulate AP-1. One of these, JNK-1, is activated synergistically by the TCR and the costimulatory receptor CD28. p21\(^{\text{ERK}}\) may play a role in the TCR synergy with CD28 for JNK-1 activation and it should also not be excluded that other p21\(^{\text{ERK}}\)-regulated ERKs will exist. NF-AT1 binding sites have been described in the promoters of a number of cytokine genes and...
Fig. 3. Signalling pathways that control T-cell activation. At least three essential signalling pathways are involved in TCR-mediated signalling: those mediated by p21^ras, calcium/calcineurin signals and PKC. The costimulatory molecule CD28 does not regulate these pathways but activates PI 3-kinase and a MAP kinase family member JNK-1 (see also Ref. 44). The nuclear target for each signalling pathway is a transcriptional factor such as AP-1, NF-κB or NF-ATp. Cytokine genes such as IL-2 require the coordinate action of multiple transcriptional factors for transcriptional activation. This explains why a single intracellular signal is insufficient for IL-2 gene induction. The known key convergence points for these intracellular signals are indicated by an asterisk. Abbreviations: NF-ATp, cytosolic component of nuclear factor of activated T cells; PI 3-kinase, phosphoinositide 3-kinase; JNK, Jun kinase; [Ca^{2+}], intracellular calcium; PtdIns(4,5)P_2, phosphatidylinositol (4,5)-bisphosphate; PtdIns(1,4,5)P_3, phosphatidylinositol (1,4,5)-trisphosphate; lns(1,4,5)P_3, inositol (1,4,5)-trisphosphate; DAG, diacylglycerol. For all other abbreviations, see Figs 1 and 2.

p21^ras may therefore play a general role in controlling cytokine expression in lymphocytes.

p21^ras function in IL-2R-mediated signal transduction

Although IL-2R triggering is associated with p21^ras and Raf-1 activation, there are discrepant reports as to whether IL-2-mediated stimulation of ERK2 occurs^38,39. Recently, it has been shown that cell phosphatase activity may be rate limiting for regulation of ERK2. In particular, a dual specificity phosphatase PAC-1 has been identified that dephosphorylates ERK2 in vivo, thus terminating or preventing its activation in T cells^40. PAC-1 expression is upregulated only after T-cell activation, so in the initial stages of TCR signalling it can play no role. However, its upregulation in response to the TCR may explain the transience of TCR-mediated activation of ERK2. IL-2Rs are expressed only in activated T cells and, since the constitutive expression of PAC-1 suppresses activation of ERK2, the presence of PAC-1 in activated (i.e IL-2R expressing) T cells could explain the inability of IL-2 to stimulate ERK2 (Ref. 40). Thus, it is becoming clear that the expression of MAP kinase phosphatases is an important factor that will determine the cellular outcome of p21^ras stimulation of the ERK1 and 2 pathway. However, it is not yet known whether p21^ras couples the IL-2R to a downstream kinase cascade analogous to the role of p21^ras in TCR coupling to ERK2. Lymphocyte cell growth is dependent on cytokine production, which means that p21^ras function would be, albeit indirectly, indispensable for T-cell growth. It remains to be established whether p21^ras function has a more direct impact on T-cell growth by playing an essential role in IL-2R control of mitogenesis.

Concluding remarks

The ability of the TCR and the IL-2R to regulate a common signal transduction molecule such as p21^ras may seem at odds with the observations that there are
distinct patterns of gene expression regulated by the TCR and IL-2R (Ref. 41). However, p21vras signalling may be more complicated than is frequently depicted, and the potential exists for multiple p21vras effectors. The only p21vras pathway identified in T cells to date is the p21vras/Raf-1/MAP kinases cascade, but other pathways may exist. It is also clear that p21vras activation is only one of a complex series of biochemical responses triggered by the TCR or IL-2R. In particular, biochemical signalling pathways specific to either the TCR or the IL-2R exist. One well-documented TCR-mediated signalling response that is not shared by the IL-2R is the activation of phosphatidylinositol hydrolysis, intracellular calcium mobilization and PKC activation. Similarly, the IL-2R but not the TCR activates a signal transduction pathway that involves JAKs as well as signal transducers and activators of transcription (STAT) proteins. The synergistic interaction between p21vras signals and calcium/PKC signals is essential for T-cell activation. The interaction between p21vras signals and the JAK/STAT pathway is likely to be required for T-cell cycle progression and clonal expansion.

Note added in proof: Swan, Perlmuter and colleagues (Swan, K.A. et al. (1995) EMBO J. 14, 276–285) have recently described the effects of expressing a dominant-negative Ras mutant in thymocytes. Their data confirm that p21vras function is important for TCR signal transduction. Furthermore, the involvement of p21vras can distinguish positive and negative selection in thymocytes.

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Regulation of the Adapter Molecule Grb2 by the FceR1 in the Mast Cell Line RBL2H3*

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Antigenic cross-linking of the high affinity IgE receptor (FceR1) on mast cells results in protein tyrosine kinase activation. The object of the present study was to explore the regulation of the SH2 and SH3 domain containing adapter molecule Grb2 by FceR1-stimulated PTK signal transduction pathways. Affinity purification of in vivo Grb2 complexes together with in vitro experiments with Grb2 glutathione S-transferase fusion proteins were used to analyze Grb2 complexes in the mast cell line RBL2H3. The data show that in RBL2H3 cells several different proteins are complexed to the SH3 domains of Grb2. These include the p21 ras guanine nucleotide exchange factor Sos, two basally tyrosine-phosphorylated 110- and 120-kDa molecules, and a 75-kDa protein that is a substrate for FceR1-activated PTKs. By analogy with Sos, p75, p110 and p120 are candidates for p21 ras effector proteins which suggests that Grb2 may be a pleiotropic adapter. Two Grb2 SH2-binding proteins were also characterized in RBL2H3 cells; the adapter Shc and a 33-kDa molecule. Shc is constitutively tyrosine phosphorylated in unstimulated cells and FceR1 ligation induces no changes in its phosphorylation or binding to Grb2. In contrast, p33 is a substrate for FceR1-activated PTKs and binds to Grb2 SH2 domains in FceR1 activated but not quiescent cells. The β subunit of the FceR1 is a 33-kDa tyrosine phosphoprotein, but the p33 Grb2-binding protein described in the present report is not the FceR1 β chain and its identity is unknown. The present report thus demonstrates that there are multiple Grb2 containing protein complexes in mast cells of which a subset are FceR1-regulated. Two other of the Grb2-binding proteins described herein are tyrosine phosphorylated in response to FceR1 ligation; the 75-kDa protein which binds to Grb2 SH3 domains and the 33-kDa protein that associates with the Grb2 SH2 domain. We propose that protein complex formation by Grb2 is an important consequence of FceR1 cross-linking and that this may be a signal transduction pathway which acts synergistically with calcium/PKC signals to bring about optimal mast cell end function.

Antigenic cross-linking of the high affinity immunoglobulin E (IgE) receptor (FceR1) on mast cells and basophils results in expression of the production of cytokines and exocytotic secretion of allergic mediators (1). The stimulation of PTKs by the activated FceR1 is an immediate membrane proximal event crucial for FceR1 signal transduction and hence mast cell end function (2–4). The FceR1 complex is comprised of three subunits, the 45-kDa α and 30-kDa β chains and a homodimer of two disulfide-linked 10-kDa γ chains (5). The intracellular tails of the β and γ subunits contain a common motif, termed the tyrosine-based activation motif (TAM) of the general sequence EXyXyL/I/IXyX/LI/ (6) which is thought to couple the FceR1 to intracellular PTKs. TAMs are found also in the invariant subunits of the B cell and T cell antigen receptors (BCR and TCR) and thus appear to be an evolutionarily conserved sequence motif essential for the activation of lymphocytes (7, 8–11).

Members of the TAM-based receptor family are typically coupled to two subfamilies of PTKs. The FceR1 associates with the src-family tyrosine kinase p56lck and a 72-kDa PTK, Syk (3, 12). The BCR is similarly associated with p66lyn(13) and Syk whereas the TCR is predominantly associated with the src-family p59fyn and a kinase homologous to Syk, Zap70 (14, 15). It has also been reported that FceR1 cross-linking induces tyrosine phosphorylation and activation of the atypical src-like Bruton tyrosine kinase (16) suggesting that a third category of PTKs may contribute to FceR1 signaling. One common response to triggering of the FceR1, TCR, and BCR is tyrosine phosphorylation and activation of phospholipase C-γ1 (2, 17, 18). This permits these receptors to control inositol polyphosphate and diacylglycerol production which in turn modulate intracellular calcium concentration and the activation of PKC, respectively. Calcium flux and PKC play a critical role in TCR signal transduction (19, 20) and are important signals for mast cell activation and the secretion of granule components such as histamine and 5-hydroxytryptamine (21, 22).

A second, essential, PTK-controlled signaling pathway to originate from the TCR involves the guanine nucleotide-binding proteins p21 ras (23). The mechanism of TCR coupling to p21 ras was proposed to involve the guanine nucleotide exchange protein Sos, the homologue of the Drosophila “son of sevenless” gene product (24, 25). Sos is known to complex with the adapter protein Grb2/Sem 5 which is composed of one SH2 domain and two SH3 domains (26, 27). In many cell systems, the SH3 domains of Grb2 bind to Sos whereas the interaction between the Grb2 SH2 domain and tyrosine-phosphorylated molecules such as the epidermal growth factor (EGF) receptor or Shc may regulate the function and cell localization of Sos (27–30).

Studies in T lymphocytes have identified a number of ty-

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Grb2 Regulation by FceR1

- Grb2 binds Sos and 75- and 116-kDa tyrosine phosphoproteins in B cells, whereas the major Grb2 SH2 domain-binding proteins are She and p36. She binds to tyrosine phosphorylated proteins composed of p36-Grb2-Sos complexes by the BCR. In this system both She tyrosine phosphorylation and the formation of She-Grb2-Sos complexes are observed in response to BCR ligation.

- The predominant complexes that can be detected are composed of p36-Grb2-Sos (25, 35). Studies with B cells have generated evidence for regulation of Grb2 complexes by the BCR. In this system both She tyrosine phosphorylation and the formation of She-Grb2-Sos complexes are observed in response to BCR ligation (36, 37). The pattern of PTK activation in response to BCR or FceR1 triggering is similar, served in response to BCR ligation (36, 37). The pattern of PTK activation in response to BCR or FceR1 triggering is similar.

- The data presented here show that the SH3 domain of Grb2 binds Sos and 75-, 120-, and 140-kDa tyrosine phosphoproteins whereas the major Grb2 SH2 domain-binding proteins are She and an unknown 33-kDa protein. Two of the Grb2-binding proteins described herein are tyrosine phosphorylated in response to FceR1 ligation; the 75-kDa protein which binds to Grb2 SH3 domains and the 33-kDa protein that associates with the Grb2 SH2 domain. She is apparently not a substrate for FceR1-activated PTKs and hence its association with Grb2 is not controlled by cell activation. The present report thus demonstrates that there are multiple Grb2-containing protein complexes in mast cells of which a subset are FceR1-regulated.

**Materials and Methods**

**Antibodies**—The anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). The anti-phosphotyrosine antibody FB2 (W. Fantl, University of California) was purified from hybridoma supernatant. Polyclonal anti-She antibody was purchased from TCS Biologicals. Monoclonal anti-Dinitrophenyl (mouse IgG isotype) was purchased from Sigma. Anti-mouse Sos and anti-Grb2 were purchased from Upstate Biotechnology Inc. and Affiniti (Nottingham, United Kingdom), respectively. The monoclonal antibody JRK1, with specificity for the β chain of FceR1 (38), was a generous gift of Dr. Juan Rivera (NIAMS, Bethesda, MD).

**Peptide Reagents and Fusion Proteins**—The EGFR-Y1068 peptide sequence is PVPepYINQS. The Trk-Y1490 peptide sequence is IENpYFSDA. For use in affinity purification these peptides were coupled to Affi-Gel 10 beads (Bio-Rad). The following glutathione S-transferase (GST) fusions were used and have been previously described (28, 32).

- GST-alone, GST-Grb2 (full-length Grb2), GST-μSH3 49L/203R double SH3 mutant, GST-Grb2NSH3 isolated amino-terminal SH3 domain (amino acids 1-58), GST-Grb2C9SH3 isolated carboxy-terminal SH3 domain (amino acids 156-217) and GST-Sos (msSos1 carboxy-terminal residues 1135-1336). The fusion proteins were coupled to glutathione-agarose beads (Sigma) for affinity purification experiments.

**Cell Culture**—The rat basophilic leukemia cell line RBL2H3 was a gift of Dr. Roberto Solari, Glaxo Research and Development, U K. They were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated (56 °C for 30 min) fetal bovine serum and 2 mM glutamine. Only the adherent fraction of the cell cultures was passaged or used in experiments.

**Cell Stimulation and Lysis**—RBL2H3 monolayer cells were detached from the culture flask using a cell scraper, washed once, and primed in suspension with 1 µg/ml IgE anti-dinitrophenol (IgE anti-DNP) in RPMI 1640, 10% fetal bovine serum for 1 h at 37 °C. Receptor cross-linking was effected using 10 µg/ml keyhole limpet hemocyanin (KLH-DNP conjugate) at 37 °C. After stimulation all cells were lysed in a buffer containing 50 mM HEPES (pH 7.4), 1% (w/v) Nonidet P-40, 150 mM NaCl, 20 mM NaF, 2 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml protease inhibitors (leupeptin, pepstatin A, and chymotrypsin), and 1 mM Na3VO4.

**Affinity Purifications and Western Blotting**—Lysates (1.5 x 10^7) RBL2H3 were precleared with Protein A-insoluble suspension (Sigma) for 15 min at 4 °C. Lysates for fusion protein affinity purifications were then precleared with glutathione-agarose bead suspension (Sigma). Lysates for peptide precipitations (Trk-Y1490 and EGFR-Y1068) were cleared with Affi-Gel 10 (Pharmacia). Affinity purifications with specified reagents were carried out for 2 h at 4 °C with constant rotation. The beads were washed three times in 1 ml of lysis buffer and boiled in reducing SDS sample buffer for 10 min. Samples were resolved on 11% SDS-PAGE.

The resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) which was blocked in 5% non-fat milk for 1 h. The membrane was probed with a specific antibody followed by an appropriate second horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham). Reactive bands were detected using the enhanced chemiluminescence system (Amersham).

**Results**

Tyrosine Phosphorylation of Multiple Grb2 Binding Proteins Is an Immediate Consequence of FceR1 Cross-linking—The data in Fig. 1 show a Western blot analysis with anti-phosphotyrosine antibodies of total cell lysates from quiescent and FceR1-stimulated RBL2H3 cells. The experiment demonstrates that there is prominent FceR1-induced tyrosine phosphorylation of proteins at 60, 70, and 55 kDa. This phosphorylation is rapid, detected within 2 min and sustained for a period of 30 min. In control experiments no increase in tyrosine phosphorylation of any proteins in RBL2H3 cells which have been incubated with monomeric IgE but not exposed to cross-linking multivalent antigen was observed. Binding experiments with a GST-fusion protein of wild type Grb2 were carried out in order to establish whether FceR1-induced tyrosine phos-
phoproteins include a subset of Grb2-binding proteins. The data from this experiment are shown in Fig. 2. In precipitates purified from RBL2H3 cell lysates with GST-Grb2, there are multiple tyrosine phosphoproteins in both quiescent and activated cells. Many proteins are observed which are basally tyrosine phosphorylated in unstimulated cells. However, there is an FceR1-induced increase in tyrosine phosphorylation of proteins migrating at 120, 110, 75, 65, and 30–33 kDa.

The experiment in Fig. 2 explores also the SH domain specificity of FceR1-induced tyrosine-phosphorylated Grb2-binding proteins. Affinity binding experiments were performed with truncated or mutated Grb2 fusion proteins comprising the isolated Grb2 NH2-terminal and COOH-terminal SH3 domains, called GST-Grb2NSH3 and GST-Grb2CSH3, respectively. In addition, GST-Grb2 with mutations in both SH3 domains which leave the SH2 domain intact (GST-Grb2 µSH3) was used. Fig. 2 shows a Western blot analysis with anti-phosphotyrosine antibodies on proteins affinity purified from RBL2H3 lysates using the panel of GST-Grb2 variants in comparison with wild-type GST-Grb2. The data show that a prominent FceR1-induced tyrosine phosphoprotein of 75 kDa was detected in the precipitates isolated with wild type GST-Grb2, GST-Grb2NSH3, or GST-Grb2CSH3 fusion proteins. Both GST-Grb2 SH3 domain fusion proteins also purified tyrosine-phosphorylated proteins of 38, 52–55, 120, and 140 kDa. Cross-linking of the FceR1-induced tyrosine phosphorylation of 33- and 65-kDa proteins which bound to the wild type GST-Grb2 fusion protein. These species did not bind the isolated Grb2 SH3 domains. In addition to the isolated SH3 domains, further characterization of domain specificities was possible using the GST-Grb2 µSH3 molecule. This is a full-length Grb2 construct which has point mutations introduced into each of the SH3 domains but an intact SH2 domain. The FceR1-induced 75-kDa tyrosine phosphoprotein did not bind to the GST-Grb2 µSH3 fusion protein, nor did the 110-kDa tyrosine phosphoprotein. However, FceR1-induced tyrosine phosphoproteins of 33 and 65 kDa bound efficiently to the GST-Grb2 µSH3 protein. As well, a 55-kDa tyrosine phosphoprotein and a doublet of high molecular mass tyrosine-phosphorylated proteins at 120–140 kDa were detected binding to GST-Grb2 µSH3. The latter species were inducibly tyrosine phosphorylated in FceR1-activated RBL2H3 to varying degrees between experiments.

From these binding studies it can be concluded that there are FceR1-induced tyrosine phosphoproteins of 33 and 65 kDa which can bind to Grb2 SH2 domains. In addition, there is an FceR1-induced tyrosine phosphoprotein of 75 kDa which binds to Grb2 SH3 domains. Other tyrosine phosphoproteins that associate with Grb2 SH3 domains and SH2 domains have molecular masses of 110–120 and 120–140 kDa, respectively. The binding experiments truncated and mutated Grb2 fusion proteins show that the proteins migrating in the 110–120 kDa range may in fact be resolved into both basally and FceR1-induced tyrosine-phosphorylated proteins. Similarly, there are multiple tyrosine-phosphorylated proteins at 52–55 kDa binding to the intact GST-Grb2 fusion protein that resolve into both basally phosphorylated proteins and FceR1-induced tyrosine phosphoproteins upon analysis with the truncated and mutant Grb2 fusion proteins.

Tyrosine Phosphoproteins That Associate with the SH3 Domains of Endogenous Grb2 in RBL2H3 Cells—Initial experiments showed that there is a complicated pattern of FceR1-induced tyrosine phosphoproteins which are capable of binding to Grb2 fusion proteins. Therefore, to establish whether there are FceR1-induced complexes between tyrosine-phosphorylated proteins and endogenous Grb2, we used affinity purification protocols to isolate Grb2 and associated proteins from RBL2H3 cell lysates. This technique has been described in detail previously (32) and has been used effectively to examine Grb2 complexes in TCR-activated T-lymphocytes. Briefly, a synthetic tyrosine phosphopeptide corresponding to the auto-phosphorylation site Tyr-1068 in the carboxyl-terminal tail of the epidermal growth factor receptor (EGFR-Y1068) was used to precipitate Grb2 from RBL2H3 cells. The EGFR-Y1068 peptide binds to the Grb2 SH2 domain with nanomolar affinity and thus competitively blocks associations between cellular proteins and the SH2 domains of Grb2. Use of this peptide allows the copurification of Grb2 and proteins complexed with Grb2 SH3 domains. Grb2 was also purified from RBL2H3 cells using a GST fusion protein of a proline-rich fragment from the carboxyl terminus of murine Sos (GST-Sos). The GST-Sos fusion
Grb2 Regulation by FceR1

**Fig. 3.** The EGFR-Y1068 peptide and GST-Sos fusion protein affinity purify Grb2 from RBL2H3 cell lysate. RBL2H3 cell lysates containing 2 × 10^6 cell equivalents were precleared as described and affinity purified with either EGFR-Y1068 coupled to Affi-Gel-10 or GST-Sos fusion protein coupled to glutathione-agarose beads. Post-nuclear proteins were acetone precipitated from 2 × 10^6 RBL2H3 (total lysate). The samples were resolved on 11% SDS-PAGE under reducing conditions before transfer to PVDF and blocking of the membrane. A, the membrane was then probed with 0.5 μg/ml anti-Grb2 for 1 h and developed with anti-mouse Ig-horseradish peroxidase second stage antibody. B, the PVDF membrane was blocked and probed with 1 μg/ml anti-mSosI for 2 h before development with anti-rabbit Ig-horseradish peroxidase second stage antibody.

protein used in these experiments binds the Grb2 SH3 domain and therefore isolates Grb2 and proteins associated with the Grb2 SH2 domain. GST-Sos competitively prevents the copurification of Grb2 SH3-binding proteins. The data in Fig. 3 show an anti-Grb2 Western blot of the cellular proteins purified with GST-Sos or EGFR-Y1068 and indicate that these two reagents isolate equivalent levels of endogenous Grb2. Moreover, a comparison of the levels of Grb2 purified with EGFR-Y1068 or GST-Sos with Grb2 levels in total cell lysates (Fig. 3A) shows that these reagents are capable of affinity purifying the majority of endogenous Grb2 from RBL2H3 cells. The data in Fig. 3B show that it is possible to copurify Sos with Grb2 when the EGFR-Y1068 motif but not GST-Sos is used as an affinity matrix.

To determine whether any of the tyrosine phosphoproteins observed in GST-Grb2 affinity purifications bind to endogenous Grb2 via its SH3 domains, we performed Western blot analysis with phosphotyrosine antibodies on Grb2 complexes isolated with EGFR-Y1068 peptide. As shown in Fig. 4A, the EGFR-Y1068 peptide precipitates a 75-kDa tyrosine phosphoprotein from FceR1-activated but not quiescent RBL2H3 cells. The EGFR-Y1068 peptide also purified two tyrosine phosphoproteins of 110 and 120 kDa which were apparently equally phosphorylated in quiescent and FceR1-activated cells. These tyrosine phosphoproteins appear to bind to endogenous Grb2 via its SH3 domains. They do not coprecipitate with Grb2 which has been isolated using the GST-Sos fusion protein to compete out Grb2 SH3-binding proteins (Fig. 4B). They also comigrate with the 75-, 110-, and 120-kDa tyrosine phosphoproteins detected in the binding experiments using GST-Grb2 single SH3 domain fusion proteins (Fig. 2). Affinity purification with the GST-Grb2 fusion protein produces the characteristic pattern of tyrosine phosphoproteins observed in previous experiments. It should be noted that there was a variable nonspecific binding of a 50–52-kDa doublet of tyrosine phosphoproteins to control

**Fig. 4.** A, the EGFR-Y1068 phosphopeptide purifies tyrosine phosphoproteins from stimulated RBL2H3. RBL2H3 were primed with IgE anti-DNP and stimulated with KLH-DNP for 15 min as described. Lysates were made, and affinity purifications were carried out on 2 × 10^6 cell equivalents/lane with the following reagents: Affi-Gel-10 beads alone, EGFR-Y1068 coupled to Affi-Gel-10, GST-Grb2 fusion protein, and GST alone coupled to glutathione-agarose beads. The samples were resolved under reducing conditions on 11% SDS-PAGE before transfer and blocking as described above. The membranes were then probed with 1 μg/ml 4G10 for 1 h and developed with anti-mouse Ig-horseradish peroxidase. B, binding of 55- and 33-kDa tyrosine phosphoproteins to endogenous Grb2 in RBL2H3. RBL2H3 cells were stimulated and lysed as described previously. Lysates from 2 × 10^6 cells/lane were precleared and affinity purified for 2 h with either GST-Grb2, GST-Grb2μSH3, or GST-Sos. After 11% SDS-PAGE and transfer to PVDF membrane, Western blot analysis was carried out with 1 μg/ml 4G10. The membrane was then stripped and reprobed with 1 μg/ml anti-Shc overnight at 4 °C and developed with anti-rabbit Ig-horseradish peroxidase.
affinity purifications comprising GST alone or Affi-Gel beads alone.

Tyrosine Phosphoproteins of 33 and 55 kDa Associate with Endogenous Grb2 SH2 Domains—The data in Fig. 4B show anti-phosphotyrosine Western blots of the endogenous Grb2 complexes isolated from RBL2H3 cells with GST-Sos. These data indicate that the 120-, 140-, and 65-kDa tyrosine phosphoproteins observed binding to the normal and the double SH3 mutant GST-Grb2 fusion proteins do not coprecipitate with endogenous Grb2. However, an FceR1-induced tyrosine phosphoprotein of 33 kDa was detected in the endogenous Grb2 complexes purified by GST-Sos. In addition, a tyrosine phosphoprotein of 55 kDa was observed to bind to endogenous Grb2 in both quiescent and FceR1-stimulated RBL2H3. These p33 and p55 molecules appeared to bind to Grb2 SH2 domains based on their pattern of binding to the panel of Grb2 fusion proteins. As well, they did not coprecipitate with the Grb2 complexes purified with the EGFR-Y1068 peptide, and their interaction with endogenous Grb2 was competed by the EGFR-Y1068 peptide that binds to the Grb2 SH2 domain (data not shown).

In many cells the adapter molecule Shc is tyrosine phosphorylated in response to receptor stimulation and forms a complex with Grb2 SH2 domains. Western blot analysis with Shc antisera has shown that RBL2H3 cells express two isoforms of Shc. The 55-kDa isoform is predominant and the 46-kDa Shc protein is a minor component of the Shc population in these cells. The data in Fig. 4B show that a tyrosine phosphoprotein of 55 kDa is coprecipitated with endogenous Grb2 by the GST-Sos fusion protein from both quiescent and FceR1-stimulated RBL2H3 cells. This protein had a slightly lower mobility than the nonspecific 50–52-kDa proteins described earlier and could thus possibly represent tyrosine phosphorylated 55-kDa Shc isoform. Accordingly, the blots were reprobed with a Shc antisera. Fig. 4B shows that the 55-kDa Shc isoform binds to the wild type and SH3-mutated Grb2 fusion proteins and also can be copurified with endogenous Grb2 by GST-Sos. The binding of Shc to endogenous Grb2 was not influenced by the activation state of the RBL2H3.

To further analyze the tyrosine phosphorylation of Shc in RBL2H3 cells, we used a tyrosine phosphopeptide Trk-Y490, which corresponds to a high affinity binding site for Shc SH2 domains in the Trk subunit of the nerve growth factor receptor. This was used as a reagent to affinity purify Shc from RBL2H3 cells. The data in Fig. 5A show an anti-Shc Western blot of proteins affinity purified with the Trk-Y490 peptide from RBL2H3 cells. The 55- and 46-kDa Shc isoforms bind to the Trk-Y490 peptide but not to the EGFR-Y1068 peptide used here as a specificity control. The data in Fig. 5B show an anti-phosphotyrosine Western blot of Trk-Y490 peptide precipitated from quiescent and FceR1-triggered RBL2H3 cells. The 55-kDa Shc isoform is tyrosine phosphorylated in both quiescent and stimulated cells. These data demonstrate also that the 55-kDa tyrosine phosphoprotein binding to GST-Grb2 and GST-Sos comigrates with the tyrosine-phosphorylated 55-kDa Shc protein purified with Trk-Y490. The nonspecific 50–52-kDa protein doublet can once again be observed binding to control Affi-Gel beads alone but with a different mobility to either of the Shc isoforms.

The 33-kDa Grb2 SH2 Domain-binding Tyrosine Phosphoprotein Is Not the β Subunit of the FceR1—The accumulated evidence from the experiments in Figs. 1–5 show that FceR1 triggering results in tyrosine phosphorylation of a 33-kDa protein which binds to endogenous Grb2 in an SH2 domain-mediated association. The β subunit of the FceR1 is 30–33 kDa and is known to be tyrosine phosphorylated in response to receptor triggering. Accordingly, we examined whether the p33 Grb2-binding protein was the β subunit of the FceR1 (Fig. 6). Western blot analysis with the FceR1 β chain antibody JRK1 showed that the β chain can be immunoprecipitated with the anti-phosphotyrosine antibody FB2 from FceR1-stimulated but not quiescent cells (Fig. 6). Furthermore, it was possible to observe a hyperphosphorylated population of JRK1-immunoreactive β chain molecules in total lysates from FceR1-activated RBL2H3. This latter population comigrated on SDS-PAGE with the population selected for by the FB2 anti-phospho-
The $p_{33}$ tyrosine phosphoprotein binding the Grb2 SH2 domain in RBL2H3 cells is not the FceR1 $\beta$ chain. Affinity purifications of lysates from $2 \times 10^5$ quiescent or FceR1-stimulated RBL2H3 cells were carried out using GST-Grb2. Post-nuclear protein from $2 \times 10^6$ stimulated or unstimulated RBL2H3 cells was acetone-precipitated. Immunoprecipitations using $2 \mu$g-point of the anti-phosphotyrosine antibody FB2 were carried out on lysates from $2 \times 10^5$ stimulated or unstimulated RBL2H3 cells. The samples were resolved and transferrred as described. The membrane was probed with the mouse monoclonal antibody JRIK1, which is reactive for the FceR1 $\beta$ chain, overnight at $4^\circ C$. The Western blot was then developed with an anti-mouse Ig-horseradish peroxidase second stage antibody. An arrow marks the position of antibody light chain in the anti-phosphotyrosine immunoprecipitations.

Western blot analysis of GST-Grb2 affinity purifications from FceR1-stimulated RBL2H3 was not immunoreactive with the mouse monoclonal antibody JRIK1 and is thus not the $\beta$ subunit of the FceR1. It is apparent, moreover, that the SDS-PAGE mobility of $p_{33}$ is different from the tyrosine-phosphorylated $\beta$ chain.

### DISCUSSION

The data presented here show that in RBL2H3 mast cells, as in many cell systems, the guanine nucleotide exchange factor for $p_{21}$ras, Sos, is complexed to the SH3 domains of Grb2. The data show also that there are FceR1-induced complexes between tyrosine phosphoproteins and the adapter molecule Grb2. These include two basally tyrosine-phosphorylated 110- and 120-kDa molecules and a 75-kDa protein substrate for FceR1-activated PTKs which binds to the Grb2 SH3 domain. Two protein substrates for FceR1-activated tyrosine kinases were observed to bind GST fusion proteins of Grb2 but more importantly were also found in association with endogenous Grb2. A 75-kDa protein which was tyrosine phosphorylated in activated but not quiescent RBL2H3 cells was associated with the SH3 domains of Grb2. We also detected a 33-kDa tyrosine phosphoprotein complexed to Grb2 SH2 domains in FceR1 stimulated but not quiescent cells. Previous studies of Grb2-binding proteins in T cells have illustrated how experiments that identify Grb2-binding proteins solely on the basis of affinity purifications with GST-Grb2 fusion protein experiments suggested that in RBL2H3 lysates there are tyrosine phosphoproteins of 33, 55, 65, 120, and 140 kDa which are capable of binding to Grb2 SH2 domains. The 33- and 65-kDa proteins in particular are substrates for FceR1-activated PTKs as evidenced by their inducible tyrosine phosphorylation. However, only the constitutively tyrosine-phosphorylated 55-kDa and the FceR1-induced 33-kDa tyrosine phosphoproteins were detected in association with endogenous Grb2 SH2 domains. Similarly, only a subset of the tyrosine-phosphorylated proteins identified as Grb2 SH3-binding proteins by the in vitro binding experiments were present in the in vitro Grb2 complexes isolated from RBL2H3 cells: a 75-kDa FceR1-induced tyrosine phosphoprotein and two proteins of 110 and 120 kDa, respectively, which were equivalently tyrosine phosphorylated in quiescent and activated cells and appeared to associate constitutively with Grb2 SH3 domains.

It has been described in many cell systems that tyrosine-phosphorylated Shc forms a complex with Grb2 (28, 39). In the present study, Shc was observed to be tyrosine phosphorylated in quiescent RBL2H3 cells, and no increases in Shc tyrosine phosphorylation were detected in response to FceR1 triggering. This suggests that Shc is not a major substrate for FceR1-activated tyrosine kinases in RBL2H3 cells. In accordance with the lack of FceR1 control of Shc tyrosine phosphorylation, no FceR1-induced changes in Shc-Grb2 complex formation were observed in RBL2H3 cells although Shc was constitutively associated with Grb2 in an SH2 domain-mediated association.

It has been previously noted that there is no significant formation of Shc-Grb2 complexes in response to TCR ligation in T cells (25, 35). In contrast, in B cells ligation of the BCR induces tyrosine phosphorylation of Shc and induces a Shc-Grb2-Sos complex that is proposed to link the BCR to $p_{21}$ras (36, 37). Interestingly, Shc is a reasonably abundant protein in both T cells and mast cells. Moreover, in both cell types cytokines such as interleukin-2 and interleukin-3 can stimulate high levels of Shc tyrosine phosphorylation and induce Shc-Grb2 complexes indicating that there are no technical problems in detecting such complexes in these cells (40–42).

In the absence of inducible tyrosine phosphorylation of Shc, a possible candidate for linking signals transduced by the FceR1 to the Grb2 signaling pathway is a 33-kDa tyrosine phosphoprotein. This $p_{33}$ is tyrosine phosphorylated in response to FceR1 stimulation and binds Grb2 SH2 domains. The $\beta$ subunit of the FceR1 has a similar molecular weight to $p_{33}$ and is tyrosine phosphorylated in response to receptor ligation (17). We have shown, however, that the $p_{33}$ tyrosine phosphoprotein observed to bind the SH2 domain of endogenous Grb2 is not the $\beta$ chain of the FceR1. This does not exclude an indirect interaction between Grb2 and the FceR1. The FceR1-induced $p_{33}$ molecule appears functionally analogous to the TCR-induced $p_{36}$ phosphoprotein observed as the major tyrosine-phosphorylated species to bind Grb2 SH2 domains in activated T cells (25, 35). It remains to be determined, however, whether $p_{36}$ and $p_{33}$ share any structural homology. In this context, studies in a variety of cell systems have identified many tyrosine kinase substrates with apparently diverse functions which can bind to Grb2 SH2 domains. These include adapters such as Shc, tyrosine phosphatases such as R-PTP$\alpha$ and Syp, and growth factor receptors and receptor subunits such as the EGF receptor and IRS-1 (27, 43, 44). Accordingly, it appears that multiple mechanisms have evolved to couple cellular PTKs to Grb2, of which the $p_{36}$ and now $p_{33}$ molecules may be one type.

One well-documented role of Grb2 is to couple tyrosine kinases to the guanine nucleotide exchange factor proteins and hence to $p_{21}$ras signaling pathways (45). We have observed that triggering of the FceR1 in RBL2H3 cells results in activation of $p_{21}$ras and downstream effectors molecules such as Raf-1 and the MAP kinase ERK2. The current data demonstrate that Sos is constitutively associated with Grb2 SH3 domains in RBL2H3 cells. Accordingly, the FceR1-induced 33-kDa tyrosine phosphoprotein that binds to Grb2 SH2 domain may be an adapter that couples the FceR1 to Grb2-Sos complexes thereby permitting FceR1 stimulation of $p_{21}$ras. However, it has been recognized increasingly that Grb2 may link PTKs to molecules
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other than Sos. For example, a second p21ras guanine nucleo­
tide exchange protein C3G binds to Grb2 SH3 domains in PC 12
cells (46). As well, the GTPase dynamin can bind to and be
activated by Grb2 SH3 domains in vitro and in vivo (47). In the
present report, we have shown that in RBL2H3 cells Grb2 SH3
domains do not only bind the p21ras guanine nucleotide ex­
change protein Sos. There are also tyrosine-phosphorylated
proteins of 75, 110, and 120 kDa which associate with endog­
enous Grb2 SH3 domains. By analogy with Sos, C3G, and
dynamin, the p75, pllO , and pl20 molecules are candidates for
alternate Grb2 effector molecules. The p75 molecule is of par­
ticular interest in this context because it is a substrate for
FceRl-activated PTKs. One of the major substrates for FceRlactivated PTKs has been recently cloned and is a 75-kDa pro­
tein termed SPY or H S l, which has an SH3 domain and also
contains proline-rich sequences reminiscent of SH3-binding
domains characterized in molecules such as Sos (48). Western
blot analysis of endogenous Grb2 complexes in both T cells and
RBL2H3 cells demonstrated that H S l is not the Grb2-associated p75 described in this study.® Associations between Grb2
and p75 have not been detected in fibroblasts suggesting that
this molecule has a limited tissue distribution and hence func­
tion (32). In preliminary analysis the FceRl-induced p75 ap­
pears identical to a 75-kDa protein that is a substrate for
TCR-activated PTKs and is constitutively associated with Grb2
SH3 domains in T cells (32). We propose that p75 may be a
common signaling element for the TAM-based receptor family
that includes the TCR and the FceRl. Recently, pl20"^^\ which
is a substrate for TCR-activated PTKs, was shown to bind to
the NHg-terminal SH3 domain of Grb2 fusion proteins (34).
The constitutively tyrosine-phosphorylated 120 kDa protein
described herein to associate with endogenous Grb2 complexes
was not p l20‘^*’’ although, pl20
was detected in the Grb2
fusion protein complexes isolated from RBL2H3 cells and cor­
responded to the 120-kDa FceRl-induced tyrosine phosphopro­
tein seen in these complexes (data not shown).
The role of the FceRl is to regulate the exocytosis of mast cell
granules and to modulate cytokine gene expression. The pres­
ent data have shown that the FceRl induces tyrosine phospho­
rylation of multiple Grb2-binding proteins, strongly suggesting
that this adapter protein is involved in coupling the FceRl to
one or more signal transduction pathways. It has been demon­
strated that calcium and PKC have an important role in FceRlregulated cellular responses (21). We would propose that Grb2coupled signaling pathways may also be important for FceRlinduced mast cell activation.
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