Complex Interactions of the Domains of the p85 adaptor subunit of Phosphoinositide 3-kinase.

by

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In loving memory of my mother
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**ABBREVIATIONS**

Accepted abbreviations in this thesis are listed in *Biochem. J.* (1992) **281**, 11-12. In addition the following abbreviations are used:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abl</td>
<td>Abelson murine leukaemia virus</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphocytic leukaemia</td>
</tr>
<tr>
<td>BCR</td>
<td>Breakpoint cluster region</td>
</tr>
<tr>
<td>BH</td>
<td>BCR homology</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton's tyrosine kinase</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dicroism</td>
</tr>
<tr>
<td>CDK2</td>
<td>Cyclin-dependent kinase 2</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myelogenous leukaemia</td>
</tr>
<tr>
<td>CSF1</td>
<td>Colony stimulating factor</td>
</tr>
<tr>
<td>c-Src</td>
<td>cellular Src</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DOS</td>
<td>Daughter of Sevenless</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Phase Liquid Chromatography</td>
</tr>
<tr>
<td>Gab1</td>
<td>Grb-2 associated binder</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase Activating Protein</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GPLR</td>
<td>G-protein linked receptor</td>
</tr>
<tr>
<td>GRB-2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-Transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>HP-SEC</td>
<td>High performance Size exclusion chromatography</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum coherence</td>
</tr>
</tbody>
</table>
IgG  Immunoglobulin
Ins  Inositol
InsP3  Inositol 1,4,5-trisphosphate
IPTG  Isopropyl-2-D-thiogalactopyranoside
IRS  Insulin receptor substrate
JAKs  Janus Kinases
JNK  Jun-N-terminal kinase
Kd  Equilibrium Dissociation constant
MAPK  Mitogen-activated protein kinase
NGF  Nerve growth factor
NMR  Nuclear magnetic resonance
NP-40  NonidetP-40
n-Src  neuronal Src
PCR  Polymerase Chain Reaction
PDGF  Platelet-derived growth factor
PDVF  Polyvinylidene fluoride
PH  Pleckstrin homology
PI3K  Phosphoinosdiote 3-Kinase
PtdIns4K  Phosphoinositol 4-Kinase
PtdIns5K  Phosphoinositol 5-kinase
PITP  Phosphatidylinositol Transfer Protein
PKC  Protein kinase C
PLC  Phospholipase C
PLL  Poly-L-lysine
PMSF  Phenylmethylsulphonylfluoride
PTB  Phosphotyrosine binding domain
PtdIns(3)P  Phosphatidylinositol-3-phosphate
PtdIns(3,4)P2  Phosphatidylinositol-3,4-bisphosphate
PtdIns(3,4,5)P3  Phosphatidylinositol-3,4,5-trisphosphate
PtdIns(3,5)P2  Phosphatidylinositol-3,5-bisphosphate
PtdIns(4)P  Phosphatidylinositol-4-phosphate
PtdIns(4,5)P2  Phosphatidylinositol-4,5-bisphosphate
PtdInsTP  Phosphatidylinositol transfer protein
PTK  Protein tyrosine kinase
PTK  Protein tyrosine kinase
PTP  Protein tyrosine phosphatase
pTyr  Phosphotyrosine
RPTK  Receptor protein tyrosine kinase
RPTP  Receptor protein tyrosine phosphatase
RU  Resonance Units
RP-HPLC  Reversed Phase High Performance Liquid Chromatography
SAPK  Stress activated protein kinase
SE-AUC  Sedimentation equilibrium analytical ultracentrifugation
SH  Src homology
Ship  SH2-containing inositol 5-phosphatase
SHP  SH2-containing protein tyrosine phosphatase
SOS  Son of Sevenless
SPR  Surface Plasmon Resonance
STAT  Signal Transducers and Activators of Transcription
TCA  Trichloroacetic acid
TGFβ  Transforming growth factor-β
TLC  Thin layer Chromatography
TM  Transmembrane
TNF  Tumour necrosis factor
TRITC  Tetramethylrhodamine B isothiocyanate
WASP  Wiskott Aldrich syndrome protein
1-d  1-dimensional
2-d  2-dimensional
### Abbreviations for amino acids

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Three Letter Abbreviation</th>
<th>One-letter Symbol</th>
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<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
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</tr>
<tr>
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<td>R</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gin</td>
<td>Q</td>
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<tr>
<td>Glutamic acid</td>
<td>Glu</td>
<td>E</td>
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<td>I</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
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<tr>
<td>Phenylalanine</td>
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ABSTRACT

This thesis describes studies of signal transduction in mammalian systems which are designed to help understand how molecules interacting at the cell surface regulate intracellular responses. In recent years, it has become clear that phosphoinositides (PI) are key components of growth factor triggered signal transduction cascades, and this thesis describes work to elucidate the structure and function of members of the Phosphoinositide 3-kinase (PI3K) family of enzymes. The p85 adaptor subunit of PI3K contains several domains including SH3 (Src homology domain 3), SH2 (Src homology domain 2) and BH (Breakpoint cluster region homology). This thesis describes work which examines the relationship between the structure and function of these domains. As part of the study, a number of deletions and point mutations were introduced into the p85 cDNA. The encoded proteins were expressed either in bacteria (E.coli), insect (Sf9) and mammalian (COS7) cells, and the structure and function of the intact p85 and its isolated domains were examined using biochemical and biophysical techniques.

To investigate the effects of the mutations on the regulation of the enzymatic complex, specific studies involving size-exclusion chromatography of the recombinant proteins, identification of associated proteins using affinity chromatography, biosensor analysis to establish binding affinities, and lipid and protein kinase assays of the recombinant enzymatic complex were used.

This study presents data and describes proposals which clarify the possible contributions of the SH3 and BCR domains to the function of the p85 adaptor subunit of PI3K. The results presented here show how the SH3 and BCR domains associate with other regions of p85 in vitro, and mechanisms are proposed to explain interactions of p85 with other proteins involved in signal transduction in vivo. Finally based on the data presented, mechanisms by which this enzyme is activated and regulated downstream of transmembrane receptors, are proposed.
The term signal transduction is used to describe the conversion of an input signal received at the extracellular surface of the plasma membrane of a cell, into an intracellular signal that ultimately alters gene expression in the nucleus. The pathways involved in signal transduction are thought to consist of a series of biochemical events that alter cell metabolism. The study of these signalling events is of vital importance in understanding the highly complex systems that control cell metabolism and growth.

Pathways controlling cell growth and differentiation in multicellular organisms are, in part, regulated by polypeptide growth factors, differentiation factors and hormones. Many of these ligands cannot diffuse across the plasma membrane, but instead interact with cell surface receptors, which is the first event in signal transduction. The binding of a ligand to its receptor then elicits a cascade of events including protein phosphorylation, stimulation of second messenger generating systems, ion fluxes and enhanced DNA synthesis. Given this fundamental role in normal patterns of cellular regulation, it is perhaps not unusual to find that breakdown, or subversion, of intercellular signalling pathways is implicated in several disease states including cancers.

Following agonist stimulation, an emerging theme suggests that these signalling pathways may actually be regulated by stoichiometric complexes of cellular proteins. A convergence of genetic, biochemical and structural data has focused attention on conserved protein modules that regulate signal transduction through their ability to mediate protein-protein interactions. These conserved modules represent common regulatory features of many distinct signalling pathways which are used to build up complex networks of interacting proteins.

1.1 Growth Factor Receptors and their ligands

Most receptors serve to link extracellular ligand signals to the cytoplasm of target cells. They are composed of three major domains; an extracellular domain connected via a single membrane-spanning domain to a cytoplasmic domain (reviewed in Ullrich and Schlessinger, 1990). The extracellular domain is responsible for ligand binding and transmission of the biological signal to the cytoplasmic domain, whose role is to transmit the biological signal to intracellular target proteins. Exceptions are the steroid
receptor superfamily which are entirely cytoplasmic and the G-protein linked receptor superfamily which span the membrane seven times.

All known growth factor receptors are classified both by the classes of ligands they bind and their own structural organisation. Known receptor superfamilies include: the steroid receptors, which function essentially as ligand-dependent transcription factors and have lipid soluble ligands (Evans, 1988), the tumour necrosis factor (TNF) receptor superfamily, which are involved in regulation of cytotoxicity, apoptosis and proliferation reviewed in (Bazan, 1989; Smith et al., 1994), and the serine/threonine kinases superfamily of receptors which interact with a family of structurally related, dimeric ligands that includes the three isoforms of transforming growth factor β (TGFβ). Ligand stimulation, activates the SMAD family of signal transducer proteins which relay signals from activated serine/threonine kinase receptors to the nucleus (Massague et al., 1997). TGFβ and related growth factors are involved in growth inhibition, induction of differentiation of cartilage and adipocytes, tumour suppression and chemotaxis. The immunoglobulin superfamily of receptors have a common fold, containing immunoglobulin domains and are generally involved in cell surface recognition. Finally the cytokine receptor superfamily which includes receptors for many interleukins, colony-stimulating factors and interferons and are characterised by the presence in their extracellular domains of one or two copies of a conserved domain of about 200 amino acids (Bazan, 1989) Of relevance to this thesis, are two other receptor superfamilies, the Receptor tyrosine kinases (RTKs) and the G-protein-coupled receptors (GPLRs), (Figure 1.1) which will be discussed in more depth in the following sections.

1.1.1 Receptor Tyrosine kinases

1.1.1.1 Structural features of RTKs

Although the members of the receptor tyrosine kinase (RTK) superfamily can be classified into at least 14 different subgroups based on the details of their structural organisation (Figure 1.2), all share several structural and functional features. The extracellular domain, which interacts with the polypeptide ligand, is connected via a single hydrophobic membrane-spanning domain to an intracellular region that contains a protein tyrosine kinase (PTK) catalytic domain (reviewed in Ullrich and Schlessinger, 1990). The RTK superfamily is sub classified according to the structural motifs present in the extracellular domain and the presence or absence of an insert within the kinase domain (Figure 1.2).
1.1.1.2 Function of the RPTK domains

The extracellular domain is responsible for the binding of the receptor to its corresponding growth factor. The variety of structural motifs that are found in this region reflect the different strategies adopted by different family members for the specific and high-affinity recognition of structurally different ligands.

The primary role of the transmembrane domain is to anchor the receptor in the plasma membrane, thereby making a link between the extracellular environment and the internal compartment of the cell. However, there is evidence that the transmembrane domain can also influence receptor function. For example, a single amino acid substitution of Val 664 to Glu in the transmembrane domain of the c-Neu/c-ErbB2 RTK produces an oncogenic receptor (Bargmann and Weinberg, 1988) which dimerises in the absence of ligand and is constitutively activated (Sternberg and Gullick, 1990).

The juxtamembrane sequences separate the transmembrane region of RTKs from the catalytic domain. This region differs most between RTK subclasses but is conserved within members of the same subclass. The juxtamembrane domain is thought to be involved in a phenomenon termed transmodulation, which is the ability of one type of receptor to affect the activity of a second receptor of another type (Schlessinger, 1988; Ullrich and Schlessinger, 1990). For example, phosphorylation of the epidermal growth factor receptor (EGF-R) receptor, in particular on threonine 654 in the juxtamembrane region by protein kinase C (PKC), results in the loss of high affinity EGF binding (Livneh et al., 1987) and attenuation of the kinase activity of the EGF-R (Davis, 1988).

The catalytic domain is the best conserved domain within the RTK superfamily and contains several residues that form a typical ATP-binding site motif, which is conserved in all protein kinases (Hanks et al., 1988). The kinase domain catalyses the transfer of the γ-phosphate of ATP to tyrosine residues on substrate proteins. Mutation of a conserved lysine residue in the ATP-binding site motif has been shown to abolish kinase activity in the EGF, insulin and platelet derived growth factor (PDGF) receptors both in vivo and in vitro. Deletion mutants of RTK's have revealed that the intrinsic kinase activity was dispensable for their expression and targeting to the cell surface (reviewed in Schlessinger and Ullrich, 1992; van der Geer and Pawson, 1994), however, the kinase activity is absolutely required for signal transduction of both early and delayed responses to growth factors including mitogenesis and transformation.

The kinase domains of the PDGF and FGF subclass receptors are separated into two parts by insertions of up to 100 predominantly hydrophilic amino acid residues, known
as the kinase insert region. Although it has been demonstrated that the kinase insert region is not required for catalysis (Taylor et al., 1989), it contains a number of autophosphorylation sites, for example tyrosines 740, 751 and 771 in the human PDGFβ receptor (Fantl et al., 1992; Kashishian et al., 1992; Kazlauskas and Cooper, 1989). These phosphorylated tyrosines are known binding sites for downstream effectors (reviewed in Pawson and Schlessinger, 1993 and section 1.2), thus it appears that the role of the kinase insert region is to modulate RTK interactions with various cytoplasmic substrate and effector proteins.

The carboxy-terminal tail sequences are among the most divergent between all known receptor tyrosine kinases (Yarden and Ullrich, 1988). Several autophosphorylation sites have been mapped to this region of neu and the EGF receptor (Hazan et al., 1990; Margolis et al., 1989), the insulin receptor (Torqvist and Avruch, 1988), the nerve growth factor (NGF) receptor, (Obermeier et al., 1993; Obermeier et al., 1993) and the PDGF receptor (Ronnstrand et al., 1992). As with the kinase insert region, many of these autophosphorylation sites appear to be required for interactions with downstream effectors (section 1.2.1 and 1.2.2). Several studies also suggest that the carboxy-terminal tail sequences may negatively regulate receptor tyrosine kinase signalling functions (Haley et al., 1989; Roussel et al., 1987; Velu et al., 1989).

1.1.1.3 Growth Factor Activation of RTK's

Growth factors and cytokines exert their effects by binding to cell surface receptors, resulting in the transmission of this signal across the plasma membrane and activation of signalling pathways inside the cell. The currently accepted model proposes that ligand binding drives the dimerisation or oligomerization of receptor molecules (Schlessinger, 1988; Ullrich and Schlessinger, 1990; Williams et al., 1988) (Figure 1.3). A steady-state equilibrium is thought to exist at the cell surface between inactive receptor monomers and active dimers. Ligand binding to the extracellular domain stabilises receptor dimers, thus shifting the equilibrium in favour of dimer formation. There are a number of mechanisms by which receptor dimers are formed. For example, dimerization may be driven by a dimeric ligand, which would therefore contain two receptor binding sites, or by ligand-induced conformational change in the extracellular domain of the receptor that expose interaction sites for other receptor monomers. Also two receptor binding sites may exist on one ligand as in the case of growth hormone (Cunningham et al., 1991; de Vos et al., 1992).

The EGF receptor was the first RTK to be shown to dimerize upon ligand binding (Yarden and Schlessinger, 1987). EGF-induced receptor dimerization is probably mediated by a conformational change (Greenfield et al., 1989) in the extracellular
domain that stabilises the interaction of two occupied receptor molecules. PDGF is a dimeric molecule, and the dimerization of its receptor is thought to be mediated by ligand-induced bridging of two PDGF receptors (Hart et al., 1988; Heldin et al., 1988; Heldin and Westermark, 1989; Heldin and Westermark, 1989). The two known forms of PDGF (A and B types) are able to form AA or BB homodimers, or AB heterodimers. Similarly, there are two forms of the PDGF receptor (α and β) that are displayed on the cell surface as either αα or ββ homodimers or αβ heterodimers. Quantitative binding studies indicate that the various dimeric forms of the PDGF receptor exhibit differential binding affinities for the different dimeric forms of the PDGF ligands. Hence, receptor dimerization can expand the repertoire of receptor ligand interactions, and may even extend the diversity of intracellular signals generated.

The insulin receptor, on the other hand, is displayed on the cell surface as a disulphide-bridged homodimer of α and β insulin receptor subunits. Insulin appears to activate its receptor by inducing a conformational change within a pre-existing dimeric structure (White et al., 1984).

One consequence of receptor dimerisation is the juxtaposition of the cytoplasmic domains of the dimerised receptors that results in a transient activation of the tyrosine kinase catalytic domain, possibly induced by a conformational change. In turn, this allows transphosphorylation of specific tyrosine residues within the intracellular domain of the RTK, most of which lie outside the catalytic domain. Activation of kinase activity appears to be essential for a biological response. A variety of mutagenesis studies on several receptors have shown that abolition of this kinase activity, does not affect ligand binding, but results in the loss of many downstream signalling events (Rozengurt, 1986; Ullrich and Schlessinger, 1990). Unlike the PDGF receptor, dimerisation of the EGF receptor is sufficient to activate its intrinsic tyrosine kinase activity, but autophosphorylation of a tyrosine residue within the kinase domain of the PDGF receptor is required for its activation. Intermolecular phosphorylation may also occur between two related receptors, such as the transphosphorylation of c-erbB2 by the EGF receptor (King et al., 1988) and the transphosphorylation between FGF or PDGF receptor subtypes (Bellot et al., 1991; Kelly et al., 1991).

1.1.1.4. Formation of signalling complexes

The tyrosine residues that become phosphorylated following growth factor stimulation of RTKs are located at many positions within the intracellular domain, and are specific for each receptor. Distinct phosphotyrosine-containing motifs, comprising the autophosphorylation site and amino acid residues immediately carboxy-terminal to the phosphotyrosine (pTyr), act as specific and high-affinity recruitment sites for downstream intracellular signalling molecules (Figure 1.3). A requirement for these
protein-protein recognition events is the presence of distinct domains in the downstream signalling molecules, which mediate the recognition of specific tyrosine-phosphorylated sequences (Cantley et al., 1991; Cohen et al., 1995).

The first class of protein domains to be recognised that specifically bind tyrosine-phosphorylated targets were the SH2 domains (section 1.2.1). Receptor autophosphorylation serves as a switch for assembling SH2 domain-containing molecules into receptor-bound signalling complexes (Pawson and Schlessinger, 1993). (Figure 1.3). SH2 domains are present in many intracellular proteins, and more than one SH2 domain can be present in a single protein (Figure 1.4 and Table 1.1). Another recently identified pTyr binding domain is the PTB domain (section 1.2.2), which is thought to represent an alternative mechanism for mediating the assembly of signalling complexes during tyrosine kinase signalling (van der Geer and Pawson, 1995). Other conserved recognition modules have been identified that mediate complex formation between signalling molecules. One of the best studied is the SH3 domain (reviewed in Musacchio et al., 1992), which mediates protein-protein interactions through the recognition of proline-rich motifs (Gout et al., 1993; Ren et al., 1993) (section 1.2.5). Many SH2 domain-containing proteins also contain SH3 domains (Table 1.1 and Figure 1.4.). Another domain, known as the pleckstrin homology (PH) domain (Musacchio et al., 1993), often co-exists in the same protein with SH2 and SH3 domains. The amino acid sequence of PH domains is less well conserved than those for SH2 and SH3 domains, which may be reflected in their broader binding specificity. PH domains are believed to be involved in both protein-protein and protein-lipid interactions (Figure 1.4) (section 1.2.7).

1.1.1.5 RPTK Substrates and Targets
There are two main classes of RTK targets. Firstly, there are enzymes whose activity can be altered directly by phosphorylation by the receptor kinase or which can gain access to their substrates by the translocation to the plasma membrane which is a consequence of receptor binding. Examples include phospholipase C-γ (PLC-γ) (section 1.4.2), the Ras GTPase activating protein (RasGAP) (section 1.2.5.5), the non-receptor tyrosine kinase c-Src, (section 1.3.3.1) and the tyrosine-specific phosphatase SH-PTP2 (Syp) (section 1.3.3.3). Secondly, there are proteins that lack an obvious catalytic domain. These proteins commonly contain SH2 and SH3 domains and have become known as adapters because they are believed to serve as intermediaries between RTKs and downstream signalling molecules. Examples include Nck, c-Crk, SHC and Grb2 (section 1.3.1). In this category, there are also docking proteins such as the insulin receptor substrate 1 (IRS-1) (Yenush and White, 1997) (section 1.3.2), that, when phosphorylated can bind multiple SH2 domain-containing
proteins following phosphorylation and thus act as an RPTK surrogate, triggering signalling via SH2 domain containing protein-mediated pathways. Binding of SH2 domain-containing proteins to activated RTKs can result in activation of an associated activity, therefore not all the proteins that bind to RTKs are necessarily for the kinase activity. An example is Grb2 which is not tyrosine phosphorylated upon stimulation of cells with growth factors that bind to RTKs. Instead, receptor binding induced translocation of Grb2 from the cytosol to the plasma membrane appears to be sufficient for the transmission of downstream signals (section 1.3.1.1). Proteins such as Grb2 can be called targets of RTKs, rather than substrates.

1.1.1.6 Mechanisms of signal propagation
There are two general mechanisms by which signalling molecules are regulated by interaction with RTKs (Panayotou and Waterfield, 1993; Pawson and Schlessinger, 1993): Firstly, enzymes may undergo a change in activity when they interact with the receptor. This usually arises through a conformational change in the enzyme upon receptor binding, or phosphorylation of specific tyrosine residues in the enzyme by the receptor tyrosine kinase activity (Backer et al., 1992; Shoelson et al., 1993). Secondly, cytosolic enzymes can be physically juxtaposed with their membrane-bound substrates, upstream activators, or downstream effectors as a result of receptor binding and translocation to the plasma membrane (Aronheim et al., 1994; James et al., 1993; Leevers et al., 1994). The specific set of intracellular molecules that binds to an activated receptor mediates which signal transduction pathways will be stimulated in a given cell type. Cross-talk between these pathways may augment or desensitise their respective signals, presumably in order to finely tune cellular responses.

1.1.2 Other receptor subtypes

1.1.2.1 G protein-linked receptors (GPLRs)
G protein-linked receptors are characterised by having seven membrane-spanning domains and are coupled to intracellular responses through a direct interaction with heterotrimeric GTPases or G proteins (Bourne et al., 1990; Kaziro et al., 1991) (Figure 1.1). Despite the variety of the stimuli for GPLR’s, which include light, taste and smell, and common intercellular messengers like aromatic amines, proteins, peptides, lipids and others, the receptors that regulate G proteins are both structurally and functionally homologous. They are of similar size (40-50kDa) and show considerable amino acid sequence similarity. The seven membrane-traversing regions are linked by three cytoplasmic and three extracellular loops (Figure 1.1). The extracellular amino-terminal tail may contain one or more glycosylated residues. The third intracellular loop and the carboxy-terminal tail are rich in serine and threonine residues, which maybe
sites of phosphorylation. Ligand recognition is frequently defined by conserved, charged residues in the transmembrane segments, which form a ligand binding pocket buried deeply in the membrane (reviewed in Baldwin, 1994).

G proteins are composed of three polypeptide subunits an α subunit that binds and hydrolyses GTP, and a βγ dimer which forms a stable, non-covalently bound complex that associates with membranes (Hamm and Gilchrist, 1996). To date, twenty different α subunits, five β subunits and twelve γ subunits have been identified. The Gα subunits are the most diverse in structure and function and can be divided into 4 subtypes according to their amino acid sequence homology; these are Gαs, Gαi, Gαq and Gα12. This family of G proteins is distinct from the family of small G proteins whose most notable member is the proto-oncogene Ras (Bourne et al., 1990).

At present, it is not yet fully understood how ligand-binding activates GPLRs and transduces a signal. Heterotrimeric G proteins act as molecular switches that can be turned on and off via the GTPase cycle. When GDP is bound, the Gα subunit associates with the γ subunit to form an inactive heterotrimer. Following ligand stimulation, the heptahelical receptor becomes activated by a mechanism that seems to involve a conformational change. Receptor activation leads to a decreased affinity of Gα for GDP by an unknown mechanism, then Gα binds GTP and consequently dissociates from both the receptor and the βγ dimer. This activated state of Gα persists until the GTP is hydrolysed to GDP by the intrinsic GTPase activity of the Gα subunit, and the Gα and Gβγ subunits reassociate in what has been proposed to be a two-step process (Neubig et al., 1994). Thus, binding and hydrolysis of GTP drive the transition between the activated GTP-bound state and the inactive GDP-bound state. The rate of GTP hydrolysis may be regulated by the interaction of the Gα subunit with its effector or target molecules (Bourne et al., 1991). Examples of such effectors are phosphodiesterases, adenylyl cyclase, phospholipase A2, ion channels, and phospholipase C (PLC) isoforms (reviewed in Birnbaumer, 1992). While prominence was originally given to the α-subunits of G proteins as the pathway for downstream signalling, roles for βγ subunits have also emerged. It has been suggested that α and βγ subunits can activate different PLC isoforms, possibly via their PH domains (section 1.2.7), and lead to the production of the second messengers diacylglycerol (DAG) and Inositol 1,4,5-trisphosphate (InsP3) (Katz et al., 1992; Park et al., 1993). Furthermore, purified type I and type II adenylyl cyclases are inhibited and activated respectively by direct interaction of βγ subunits (Taussig et al., 1993). The target molecules not only propagate the GPLR-derived signal, but may also be involved in its down regulation (Berstein et al., 1992; Bourne et al., 1991). For example, the Gαq subunit has a very low intrinsic GTPase activity which is enhanced by its interaction
with PLCβ1. Thus PLC β1 acts as a GTPase activating protein that inactivates GoαQ in a similar manner to the GTPase activating protein for Ras (RasGAP) (Berstein et al., 1992).

1.1.2.1.1 Novel mechanisms of RTK signal generation
As well as activation by a specific ligand, RTKs have recently been shown to be involved in signal integration of a variety of stimuli, including calcium-dependent responses in neuronal cells (Rosen and Greenberg, 1996) or cellular stress responses to stimuli such as UV radiation (Knebel et al., 1996). There is also evidence for a role for RTKs in signalling via G-protein coupled receptors. It has been reported that EGFR and the neu oncoprotein become rapidly tyrosine-phosphorylated upon stimulation of Rat-1 cells with the GPLR agonists endothelin-1, lysosphatic acid and thrombin, suggesting there is an intracellular mechanism for transactivation (Daub et al., 1996). These results demonstrate a role for RTKs as downstream mediators in GPLR-derived mitogenic signalling, and suggest the existence of a ligand-independent mechanism of RTK activation through intracellular crosstalk.

1.1.2.2 Tyrosine kinase coupled receptors
The cytokine receptor superfamily includes receptors for many interleukins, colony stimulating factors, interferons and certain other factors and hormones (reviewed in (Daub et al., 1996; Kishimoto et al., 1994; Mui and Miyajima, 1994). Like the RTKs, cytokine receptors comprise an extracellular ligand binding domain, a single transmembrane domain and an intracellular domain. However, unlike RTKs the intracellular domains of cytokine receptors lack intrinsic enzymatic activities. However, despite this structural difference between cytokine receptors and RTKs, their mechanism of activation appears to be similar. In many cases, the ligand-binding subunit(s) form signalling complexes with transmembrane signal-transducing molecules that are structurally related to cytokine receptors but that are themselves unable to bind ligands. Although receptor subunit heterodimerisation appears to be the most common mechanism of cytokine receptor activation, there are examples of cytokine receptors that are activated by homodimerisation, for example the receptor for growth hormone (GH). Analysis of co-crystals of GH in complex with the extracellular domain of the GH receptor revealed that each ligand binds two receptor molecules simultaneously (Cunningham et al., 1991; de Vos et al., 1992; Ultsch et al., 1991). As for RTKs, activation of cytokine receptors by homo or heterodimerisation leads to receptor phosphorylation and activation of downstream signal transduction pathways. Because cytokine receptors have no intrinsic kinase activity, they employ constitutively bound cytoplasmic tyrosine kinases, the JAKs, for this purpose (Darnell et al., 1994).
Phosphorylation of cytokine receptors by activated JAKs creates binding sites for the SH2 domains of the STAT transcription factors (Boulton et al., 1994; Yi et al., 1993).

The T cell receptor is composed of several transmembrane proteins with very short cytoplasmic sequences, which are associated with a large number of invariant subunits also lacking intrinsic enzymatic activities, but capable of interacting with cytoplasmic tyrosine kinases (Cambier and Jensen, 1994; Cunningham et al., 1991; de Vos et al., 1992; Weiss and Littman, 1994) (Figure 1.1). Tyrosine kinases of the Src family bind to the T cell receptor even in its resting state (Iwashima et al., 1994; Letourneur and Klausner, 1992). The precise mechanism that triggers antigen receptor activation and subsequent phosphorylation of the invariant subunits is unclear. However it has been suggested that antigen binding causes receptor aggregation that allows intersubunit interactions and cross-phosphorylation of tyrosine kinases in the Src families.

1.2 Protein modules

Numerous proteins involved in signal transduction possess protein modules which are found within the same polypeptide chain and allow the formation of a network of protein-protein and protein-phospholipid complexes that can, in principle, disseminate signalling information to a wide range of cellular processes. The resulting interactions are important for signalling from the cell surface to the nucleus, for protein trafficking and subcellular localisation, for the control of cell architecture and cell-cell interactions and for cellular responses to infection. The evolutionary conservation of these domains gives clues to the function of the protein which contains them. Genetic analysis provided initial evidence for a biological function of these modules. Biochemical and structural studies have indicated that they mediate an extremely complex and versatile network of interactions which may co-ordinate cellular responses to the environment. How these domains attain specificity in a cell awaits further studies.

1.2.1 SH2 Domains

Src homology 2 (SH2) domains are modules of approximately 100 amino acids, which were originally identified in the cytoplasmic tyrosine kinases v-fps and v-Src, by virtue of their effects on both catalytic activity and substrate phosphorylation (Pawson, 1988; Sadowski et al., 1986). SH2 domains are found in many intracellular signalling proteins and have been shown to recognise distinct phosphotyrosine (pTyr)-containing motifs on activated receptors and cytoplasmic phosphoproteins (Anderson et al., 1990; Matsuda et al., 1990; Valius and Kazlauskas, 1993).
1.2.1.1 SH2 domain binding

SH2 domain interactions with pTyr containing motifs are both transient and dynamic, and are characterised by fast rates of both association and dissociation. This has considerable implications for the regulation of complex formation, as it facilitates competition between different domains, and/or access for specific tyrosine phosphatases that may down-regulate the response. These interactions can be mimicked using isolated SH2 domains and short pTyr containing peptides of 5-10 amino acids in length \textit{in vitro}. SH2 domains bind to their cognate pTyr containing peptides with relatively high affinity ($K_D=10-100\text{nM}$), and to pTyr containing peptides of random sequence with an approximately 1,000-fold lower affinity (Felder \textit{et al.}, 1993; Panayotou \textit{et al.}, 1993; Piccione \textit{et al.}, 1993). They have no detectable affinity for non-phosphorylated peptides. Most of the binding energy therefore derives from interactions of the SH2 domain with pTyr, although the amino acids surrounding the pTyr can increase the affinity by up to three orders of magnitude (Piccione \textit{et al.}, 1993).

Mapping of SH2 binding sites has revealed that SH2 specificity is largely determined by the three residues immediately carboxy-terminal to the pTyr, (pTyr-X-X-X, where X-X-X varies for different SH2s). For example, the consensus binding site for the two SH2 domains of the regulatory subunit of phosphoinositide 3'-kinase (PI3K) is p-Tyr-(Val/Met)-X-Met. Site-directed mutagenesis of the platelet-derived growth factor $\beta$ receptor (PDGF$\beta$R) at sites containing the consensus sequences $Y^740MDM$ and $Y^751VPM$ severely disrupts the binding of PI3K to PDGF$\beta$R mutants. Mutation of the two tyrosine residues (Y) to phenylalanine (F) results in the abolition of PDGF-stimulated DNA synthesis (Cantley \textit{et al.}, 1991; Fantl \textit{et al.}, 1992), however this mutant retains considerable mitogenic activity when expressed in some cell types (Kazlauskas \textit{et al.}, 1992). In addition, the binding site for GAP, another SH2 domain containing protein, can be mutated in the PDGF$\beta$R with no apparent effect on PDGF stimulated mitogenesis (Fantl \textit{et al.}, 1992; Kazlauskas \textit{et al.}, 1992), suggesting that SH2-mediated interactions are not absolutely required for all cellular responses, and it is likely that some may be redundant.

1.2.1.2 SH2 domain structure

The structure of SH2 domains from several proteins have been determined by either x-ray crystallography or NMR, and reveal an overall similarity in three dimensional structure, in which a central anti-parallel $\beta$ sheet is flanked by two $\alpha$ helical regions with loops of variable sizes linking these secondary structural elements (Kuriyan and Cowburn, 1993) and Cowburn 1993). The amino- and carboxy-termini are close together on the face opposite the phosphotyrosine-binding site, allowing the domain to
be inserted into a protein with a minimum of structural alteration and with its functional site exposed towards the solvent. The three-dimensional structure of several SH2 domains in complex with pTyr-containing peptides has also been determined, and showed that the phosphopeptide binding site is bipartite. The pTyr residue is buried within a deep pocket in the SH2 domain (Eck et al., 1993; Waksman et al., 1992; Waksman et al., 1993) which is lined by basic residues (Figure 1.5). Residues within this pocket are among the most conserved between various SH2 domains and includes an arginine residue which forms hydrogen bonds with two oxygen atoms within the phosphate moiety of pTyr, and which is part of a conserved amino acid motif (FLVRES) that is often used to identify SH2 domains.

In some cases, the specificity of SH2 domains may not be absolute for pTyr. There is a report of high-affinity binding of an SH2 domain to a phosphoserine or phosphothreonine containing peptide (Pendergast et al., 1991). The Abl SH2 domain has been shown to bind to peptides derived from the amino terminal region of the BCR protein (Pendergast et al., 1991). In addition, the SH2 domains of PLCγ, RasGAP and c-Src have been shown to interact with phosphoserine residues, although with a reduced affinity compared to phosphotyrosine residues (Muller et al., 1992).

The specificity of SH2 domain binding has been examined by screening a combinatorial peptide library to determine the optimal peptide ligand for various SH2 domains (Songyang et al., 1993; Songyang et al., 1994). Most SH2 domains fall into one of two broad categories. The first group, known as group I ligand binders, are typified by the SH2 domains of Src and Ick. These SH2 domains bind preferentially to phosphopeptides containing hydrophilic amino acids at the +1 and +2 positions, and have a small hydrophobic pocket which accommodates a hydrophobic residue at the +3 position. Binding of the group I SH2 domains to phosphopeptides has been described as "a two-pronged plug engaging a two-holed socket" (Waksman et al., 1993). In contrast, the SH2 domains PLC-γ1 and the Syp/SH-PTP2 protein-tyrosine phosphatase (PTP) belong to the group II ligand binders. These domains recognise at least five, mainly hydrophobic residues following the pTyr, which fit into an extended hydrophobic groove running across the ligand-binding surface (Lee et al., 1994; Pascal et al., 1994). The Grb2 SH2 domain is unusual in that an asparagine residue at the +2 position has been shown to be critical for binding (Songyang et al., 1993). The three-dimensional structures of the Grb2 SH2 domains in complex with a pTyr containing peptide were determined by both x-ray crystallography and NMR, and showed that the pTyr and the +1, +2 and +3 residues form a β-turn due to the presence of bulky tryptophan side chain at position 121 of the SH2 domain which, brings the asparagine

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residue at Tyr+2 in close contact to the specificity pocket (Rahuel et al., 1996; Wang et al., 1996).

1.2.1.3 SH2 domain function

Activation and autophosphorylation of the EGFR and other receptors creates a binding site for the SH2 domain of Grb2 and recruits the Grb2-SOS complex to the receptor, bringing SOS into proximity with its membrane-bound substrate, Ras. Translocation is also important for the pathways that activate PLC-γ1, PI3K and RasGAP because like SOS, these molecules are cytosolic, and their substrates, phospholipids and Ras respectively, are membrane associated.

Phosphorylation of SH2-containing molecules may recruit other molecules to the activated complex. For example, the phosphorylation of Syp by the PDGFR creates a new binding site for Grb2 on Syp (Lechleider et al., 1993; Li et al., 1994; Valius and Kazlauskas, 1993). Alternatively, phosphorylation by the activated receptor may alter the intrinsic catalytic activity of the SH2 domain-containing protein. For example, phosphorylation of PLC-γ1 by the EGFR increases its activity towards profilin-bound phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2). Hydrolysis of PtdIns(4,5)P2 frees profilin, which then regulates actin polymerisation (Aderem, 1992).

SH2 domain-containing proteins that are downstream of non-RTK receptors function in a similar manner. Most cytokine receptors are constitutively bound to JAK kinases (Darnell et al., 1994), JAKs are members of a family of non-receptor tyrosine kinases, and associate either constitutively or inducibly with membrane-bound receptors, particularly those of the cytokine superfamily. Ligand-binding induces receptor oligomerization and concomitant JAK activation, possibly by cross-phosphorylation. JAK kinases also phosphorylate activated receptors, creating binding sites for a range of SH2 domain-containing proteins, which may also become phosphorylated by JAKs (Boulton et al., 1994; Yi et al., 1993). One class of these SH2 domain-containing proteins is the STAT transcription factors, (Schindler et al., 1992) which become phosphorylated at a tyrosine immediately carboxy-terminal to the SH2 domain, thus inducing dimerization by mutual interactions between this pTyr and the endogenous SH2 domain (Schindler et al., 1992; Shuai et al., 1994). STAT dimers can then translocate to the nucleus where they bind specific promoter elements and induce gene expression (Darnell et al., 1994).

For some SH2 domain-containing proteins, binding to a phosphorylated receptor is the only event required for their activation. For example, the phosphatase activity of Syp is increased more than 50-fold upon binding to a peptide of the same sequence as the Syp
binding site in the PDGF receptor (Sugimoto et al., 1994). Little structural alteration is seen within SH2 domains upon ligand binding (Lee et al., 1994), however ligand binding may induce conformational changes within the rest of the protein, thus altering internal interactions of the SH2 domains with the rest of the protein. In Src, when tyrosine 527 in the carboxy-terminal tail becomes phosphorylated, it interacts with the SH2 domain of Src and down regulates the kinase (Superti Furga et al., 1993) (section 1.3.3.1). A somewhat similar mechanism is apparently utilised by the Syp phosphatase, as limited protease digestion of the carboxy-terminal tail of Syp activates phosphatase activity and mitigates the increase in activity seen upon phosphopeptide binding (Sugimoto et al., 1994).

SH2 domain function can be inhibited by binding to a substrate other than pTyr. A phosphoinositide product of PI3K, PtdIns(3,4,5)P3, has been shown to bind to the SH2 domains of the p85 regulatory subunit of PI3K (Rameh et al., 1995). The amount of PI3K associated with tyrosine-phosphorylated proteins inversely correlates with the amount of PtdIns(3,4,5)P3 in the cell, suggesting that the product of activated PI3K is able to down-modulate PI3K activity by competing for pTyr binding, thus creating a negative feedback loop. In the same study, the SH2 domain of Src has also been shown to bind PtdIns(3,4,5)P3, and this binding was competed for by a phosphopeptide specific for the Src SH2 domain.

1.2.2 PTB domains

Recently, a distinct protein module also capable of binding to pTyr was found in several proteins, including the adaptor protein Shc, and the insulin receptor substrates IRS-1 and IRS-2 (Blaikie et al., 1994; Kavanaugh and Williams, 1994; van der Geer and Pawson, 1995). This domain has been identified not only in proteins involved in tyrosine kinase signalling, but also in the neuronal proteins Fe65 and X11, and a membrane-associated protein, Numb. This module, termed the phosphotyrosine binding (PTB); (van der Geer et al., 1996) or phosphotyrosine interaction (PI) domain differs from the SH2 domain in that it recognises phosphotyrosine in the amino acid sequence context NPXpY, in contrast to SH2 domains, which recognise residues carboxy-terminal to pTyr. Different PTB domains exhibit selectivity for residues at positions -5 to -8 to the pTyr (Li et al., 1997; van der Geer et al., 1996). The three dimensional structure of the Shc PTB domain in complex with a phosphopeptide derived from the TrkA receptor determined by NMR (Zhou et al., 1995), and an x-ray crystal structure and NMR derived three dimensional structure of the IRS-1 PTB domain in complex with phosphopeptides derived from the insulin receptor (Eck et al., 1996; Zhou et al., 1996) show both PTB domains contain two orthogonal β sheets and
connecting loops, and have very similar folds despite their low sequence similarity. Both have a carboxy-terminal amphipathic α-helix capping one end of the β-sandwich. The amino-terminal residues of the peptide ligand form an additional anti-parallel β strand to the second β-sheet. Interestingly, determination of these structures showed them both to have exactly the same topology as PH domains (Lemmon et al., 1996). Indeed, like the PH domains, the Shc PTB domain has been shown to bind phospholipids (Harlan et al., 1994; Zhou et al., 1995), suggesting that both the PTB and PH domains despite their lack of sequence homology, share a structural fold that is capable of binding to either phospholipids or phosphopeptides.

1.2.3 The c-Met binding (MDB) domain

Recently, another new domain capable of interacting with pTyr has been identified (Weidner et al., 1996). The c-Met receptor was shown to bind an 83 amino acid region in Gab1, a multiple pTyr-containing docking protein that serves as an intermediary in EGF and insulin receptor signalling (section 1.3.2) (Holgado Madruga et al., 1996). Mutation of the two tyrosine residues that become phosphorylated when c-Met is activated prevented Gab1 binding. Unlike SH2 and PTB domains, this domain is rich in proline residues and is the smallest of the phosphotyrosine binding domains. Subsequent sequence analysis and database searches have identified an MDB domain in SICl, a protein in yeast (Nugroho and Mendenhall, 1994) and in Pik1-p30, a cyclin-dependent kinase inhibitor (HP.Parek in press). A three-dimensional structure of a Met domain has not yet been determined. Despite the small number of known Met domains, two conserved arginine residues have been identified that may be important for pTyr binding.

1.2.4 PDZ Domains

PDZ domains (also known as DHR domains or GLGF repeats) are repeat domains comprising approximately 90-residues that are found in a number of proteins implicated in ion-channel formation and receptor clustering, and the linking of receptors to effector enzymes (Doyle et al., 1996; Gomperts, 1996). PDZ domains are thought to be protein-recognition modules. Some PDZ domains recognise proteins containing the consensus tripeptide motif S/TV near their carboxy-termini with high specificity (Saras and Heldin, 1996; Songyang et al., 1997). Other PDZ domains form homotypic dimers. The PDZ domain of a neuronal enzyme, nitric oxide synthase, binds to the PDZ domain of a 95kDa post synaptic density protein (PSD-95), and is implicated in its synaptic association (Brenman et al., 1996). A recent x-ray crystal structure of the PSD-95 PDZ domain shows it consists of a five-stranded antiparallel β-barrel flanked
by three α-helices (Cabral et al., 1996). A groove runs over the surface of the domain, ending in a conserved hydrophobic pocket and a buried arginine, thought to be the binding site for the carboxy-terminal peptide.

1.2.5 SH3 Domains

Many of the proteins involved in the transmission of signals from RTKs contain, in addition to SH2 domains, another conserved motif of 50-75 residues, the SH3 domain (Cohen et al., 1995; Musacchio et al., 1992; Pawson and Schlessinger, 1993) This domain was originally identified through sequence comparison of protein tyrosine kinases such as Src, the oncogene product Crk, phospholipase Cγ, α-spectrin, myosin IB and yeast proteins CDC25 and FUS1 (Lehto et al., 1988; Mayer et al., 1988; Rodaway et al., 1989; Stahl et al., 1988). Unlike SH2 domains or Src-related tyrosine kinases, SH3 domains have been identified in yeast. This may suggest that SH3 domains have evolved independently of tyrosine kinases and SH2 domains, but have been subsequently been acquired by the SH2 domain containing-signalling proteins of metazoan organisms to allow coupling of tyrosine kinase signalling pathways to SH3 domain regulated pathways.

The first indications of the importance of SH3 domains were from mutagenesis studies of the c-Abl and c-Src tyrosine kinases. Mutations and deletions in the SH3 domain of these kinases (Franz et al., 1989; Hirai and Varmus, 1990; Hirai and Varmus, 1990; Jackson and Baltimore, 1989; Kato et al., 1986; Nemeth et al., 1989) can activate their transforming ability, suggesting that the normal function of these particular SH3 domains in vivo is to negatively regulate kinase activity.

SH3 domains are present in a wide variety of proteins (Table 1.1), including many which associate with the cytoskeleton or membranes (Rodaway et al., 1989; Rodaway et al., 1990) such as myosin 1 (Jung et al., 1987) and the α chain of spectrin (Wasenius et al., 1989). SH3 domains are also present in signal transduction enzymes, such as non-receptor tyrosine kinases and PLCγ, two neutrophil NADPH oxidase-associated proteins, p47phox and p67phox (Leto et al., 1990; Rodaway et al., 1989; Rodaway et al., 1990; Volpp et al., 1989) which are absent in patients with distinct forms of chronic granulomatous disease (section 1.2.5.3), and in the transcriptional regulators Vav and Hs1 (Leto et al., 1990; Volpp et al., 1989). The location of the SH3 domains within their host protein varies, as does the number of SH3 domains. Proteins with enzymatic activities, such as Src, PLCγ or Ras-GAP often contain only a single SH3 domain, but the adaptor proteins Crk and Grb-2, which lack catalytic domains
contain two SH3 domains, while the adaptor protein Nck contains three SH3 domains (Figure 1.4).

SH3 domains have a low degree of sequence similarity at the amino acid level. Amino acid sequence alignments are generally limited to approximately 60 amino acid residues which constitute the core of the SH3 domain. The highest degree of conservation within the SH3 domain is localised at the amino- and carboxy-termini and in some parts of the central region. There is one amino acid motif, ALYD, that is characteristic of SH3 domains and which lies within residues in the amino-terminal part of the domain (Figure 1.7(D)).

1.2.5.1 Structure of SH3 domains

SH3 domains with known three dimensional structure all have the same overall topology, despite their limited sequence similarity, and other SH3 domains are predicted to have the same fold. The first three-dimensional structure of an SH3 domain determined was that of chicken α-spectrin, (Musacchio et al., 1992), which revealed a compact β-barrel of five anti-parallel β-strands. The five β strands, designated a to e form two orthogonal β-sheets of three strands each, with one β strand shared by both sheets (Figure 1.6A). The amino- and carboxy-termini of the domain are close together, as are those of several other protein modules, including SH2 domains. This allows the domain to be incorporated into any protein as an independently folding module, with little effect on the structure of the host protein.

The residue numbering adopted in this section is according to (Musacchio et al., 1992). Beta strand a in the SH3 domain of spectrin and Fyn is composed of four amino acids (residues 3-6), and is connected to strand b (residues 25-30) by a long loop (RT-loop), which resembles an irregular anti-parallel β-hairpin loop. Mutating residues R13 and T14 in the RT loop of the Src SH3 domain causes cell transformation (Kato et al., 1986). In the three-dimensional structures of the Src and PLC-γ SH3 domains (Kohda et al., 1993; Yu et al., 1992), the hairpin structure formed by strands a and b is extended beyond the β sheet. An extra β-strand is also observed around positions 17-20 in these SH3 domains.

Strand b in the SH3 domains of spectrin, Fyn, Src and PLCγ can be divided into two halves, designated b1 and b2, that each participate in one of the β-sheets that makes the SH3 domain structure. The neuronal variant of Src (n-Src) has a six-residue insert within the loop connecting strands b2 and c, and this loop has been named the n-Src loop. This insert increases the tyrosine kinase activity and alters the transforming potential of n-Src (Levy and Brugge, 1989; Levy et al., 1987; Martinez et al., 1987;
Pyper and Bolen, 1990) and it has been shown that a different set of proteins which binds to the n-Src SH3 domains (Cicchetti et al., 1992; Ren et al., 1993; Weng et al., 1993). A 15-residue insert in the n-Src loop of the SH3 domain of the p85α subunit of PI3K completely disrupts strand b2. Two different three-dimensional structures have been reported for this region of the p85α SH3 domain. In one NMR derived structure, the n-Src loop forms a large loop on the surface of the domain which is stabilised by hydrophobic interactions (Booker et al., 1993). In another NMR structure, this insert forms a short α helix and two 3_10 helices (Koyama et al., 1993). As the experimental conditions and numbers of nuclear Overhauser effect and hydrogen-bond restraints were very similar, the reason for this discrepancy remains unclear. The recently determined x-ray crystal structure of the p85α SH3 domain is in better agreement with the latter NMR structure (Liang et al., 1996).

Strands c and d (36-41 and 46-50) are connected by a tight turn in the spectrin SH3 domain but an insert of two amino acids is present in the same loop in the Fyn and Src SH3 domains. This loop was designated the distal loop (Noble et al., 1993) because it is on the opposite face of the SH3 domain to the putative ligand binding site. A turn of a 3_10 helix, starting at the conserved P51 (positions), connects strand d and strand e (residues 55-58).

In summary, the overall fold of an SH3 domain, therefore, is a β-sandwich, composed of two three-stranded β-sheets. The first sheet is composed of strands a, b1 and e, while the second comprises strands b2, c and d (Fig). In the p85α SH3 domain, strand b2 is completely absent. In the Src and PLCγ SH3 domains strand a is longer, and a short additional β strand at positions 17-20 seems to be present.

1.2.5.2 SH3 domain binding

Mutagenesis studies of two SH3 domain binding proteins, 3BP1 and 3BP2, mapped their Abl SH3 domain binding site to a 10 amino acid proline-rich region (Ren et al., 1993) with a potential consensus amino acid motif XPXXPPPZXP (where X is any amino acid and Z is a hydrophobic amino acid). A number of SH3 domain binding proteins subsequently have been identified, including SOS, the p85α subunit of PI3K, and p47phox, and the binding sites have also been mapped to proline-rich sequences (Finan et al., 1994; Musacchio et al., 1994) Proline-rich regions are a relatively common feature in proteins (Reviewed in Williamson, 1994), so it was important to establish whether specific rules exist which define proline-rich sequences constitute high-affinity SH3 domain binding sites, and the basis of ligand binding specificity among different SH3 domains.
The ligand binding pocket of SH3 domains comprises a hydrophobic patch that contains a cluster of aromatic residues, and is flanked by the RT and n-Src loops (Figure 1.7 (C)). The first study to attempt to locate the ligand binding site in an SH3 domain was a structural study of the Src SH3 domain using NMR (Yu et al., 1992). The amino acid residues comprising the binding site were inferred from changes in the NMR spectrum upon addition of proline-rich peptides derived from 3BP-1. Peptide-induced chemical shifts in an NMR spectrum used to map the binding site on the bovine p85αSH3 domain but, in this case, the peptide ligand was derived from dynamin, a GTP-hydrolysing, microtubule-associated enzyme, dynamin (Booker et al., 1993). The binding data indicated that ligand-SH3 domain interactions involved charged residues in the loops surrounding the hydrophobic face of the SH3 domain as well as the proposed hydrophobic peptide binding site. This gave the first clue to a potential mechanism for achieving SH3-ligand specificity.

The hydrophobic patch/charged loop ligand binding surface seem to be common to all SH3 domains for which structures have been determined, but whereas the hydrophobic patch is relatively conserved, there is significant variation in the charged amino acid residues in the flanking regions. The hydrophobic patch on the surface of the SH3 domain is composed of aromatic amino acids at positions 8, 36, 49 and 54 as well as proline 51 (Musacchio et al., 1992), which form a ladder on the surface, surrounded by the n-Src and RT-loops. Mutational analysis of conserved residues in this hydrophobic patch of the SH3 domain of SEM-5 has confirmed their importance in SH3 binding (Lim and Richards, 1994). However, all residues in this hydrophobic patch are well conserved between SH3 domains. For example residue 49 is a phenylalanine in the spectrin SH3 domain whereas the corresponding residue in the Src and Fyn SH3 domains is a tyrosine and in the p85α SH3 domain it is an aspartate. The variations in amino acid sequence within the hydrophobic pocket and surrounding loops between different SH3 domains may constitute the structural basis of their binding specificity for their putative ligands.

The first ligated SH3 domain three dimensional structure solved was that of the human p85α SH3 domain bound to the peptide RLP1 (sequence: RKLPPRPSK) (Yu et al., 1994) (Figure 1.6B). This peptide had been previously identified from screening combinatorial peptide libraries with the SH3 domains of p85α and Src (Chen et al., 1993). As with the SH2 domain, very little structural alteration in the SH3 domain is detected upon ligand binding. The peptide was shown to form a left-handed polyproline (PPII) helix that lies along the ligand-binding site of the SH3 domain, with two of the proline residues, Pro4 and Pro7, interacting with aromatic residues on the
hydrophobic face of the SH3 domain (Fig). Other residues in the peptide, Lys2, Pro5, Ser8 and Lys9, show little contact with the SH3 domain, and it was found that these residues could be altered to alanine with only minor effects on the binding affinity of the peptide for the SH3 domain. The remaining residues in the peptide, Arg1, Leu3 and Arg6 also interact with the SH3 domain surface. Arg1 and Arg6, most notably, form electrostatic interactions with Asp21 and Glu51 of the SH3 domain. These results were consistent with the observed specificity of ligand-binding of different SH3 domains. For example, the Abl SH3 domain has a threonine in the equivalent position to Asp21, and its ligand, 3BP-1, contains a methionine residue at the position equivalent to Arg1 in RLP1. This suggested that the general nature of the SH3-ligand interaction was the same for Abl-3BP1 and p85α-RLP1 complexes. With the growing number of SH3 domain complexes and mutagenic studies of known binding sites (Gout et al., 1993; Musacchio et al., 1994; Ren et al., 1993), an initial consensus SH3 binding sequence of Arg-X-X-Pro-X-X-Pro emerged, in which the unspecified amino acids were usually either proline or hydrophobic residues. These consensus sequences are now termed class I sites.

It was then demonstrated that the SH3 domain of Src could also bind other sequence motifs, termed class II sites, which contained the consensus sequence Pro-X-X-Pro-X-Arg, and that these peptides bound with opposite orientation to that seen for class I ligands (Feng et al., 1994). At the same time, the three dimensional structure of the adaptor protein Grb2 showed that its amino-terminal SH3 domain bound to a peptide derived from its natural ligand, Sos, in the class II orientation (Goudreau et al., 1994; Lim et al., 1994) The ability of the SH3 domain to bind ligands in both amino-to-carboxyl and carboxyl-to-amino orientations derives from the pseudosymmetry of the PPII helix. One can recast the ligand consensus sequences as Arg-X-q-Pro-X-q-Pro for class I sites and q-Pro-X-q-Pro-X-Arg for class II sites, to reflect this symmetry (Figure 1.7 (a)). In these sequences, the ‘q’ specifies any hydrophobic residue, but is often proline, leucine or valine. The ‘Pro’ is an essential proline residue. The PPII helix is roughly triangular in cross section. The ‘q-Pro’ sequences at the base of the triangle intercalate with the conserved hydrophobic residues of binding sites 1 and 2 on the surface of the SH3 domain. The terminal charged residue, usually an arginine, binds an acidic residue in site 3. The amino-acid side chains at the apex of the triangle extend away from the domain and are not well conserved, but proline is relatively common at these positions, probably because it stabilises the PPII helical conformation. The face of the PPII helix that packs against the binding surface is determined by the orientation of the arginine with respect to the Pro-X-X-Pro sequence. It has also been suggested that sites I and II select ‘q-Pro’ sequences over ‘Pro-q’ because they are more compact. Two bonds, rather than three, separate the hydrophobic side chains
when the proline is in the second position (Feng et al., 1994). In addition, the side chain of the non-prolyl residues in the leading position usually adopts an 'internal' packing conformation, in which the side chain extends into the binding site (Lim et al., 1994). In class I binding, the amino-terminal arginine residue occupies site 3, whereas in class II binding the orientation of the peptide is reversed and a carboxy-terminal arginine occupies this site. Interestingly, the two hydrogen bonds between the SH3 domain and the peptide backbone are conserved regardless of orientation (Wittekind et al., 1994). In both types of binding the 'q-Pro' sequences pack into sites 1 and 2, but the positions of the prolyl and non-prolyl residues are reversed within the sites (Figure 1.7(A) and (B)). Since the 'q-Pro' sequences are only roughly symmetrical, they are likely to fit better into the binding pockets in one particular orientation. Thus, the selection for class I or class II binding modes lies in both the amino- or carboxy-terminal position of the arginine residue and the subtle differences in van der Waal's contacts within binding sites. The capacity of SH3 domains to bind ligands in either orientation both expands the range of potential binding partners, and determines the spatial organisation of the resulting complex, which maybe critical for signalling.

The fact that different SH3 domains select different optimal binding sites and a particular proline-rich region of a protein can bind preferentially to a subset of SH3 domains (Gout et al., 1993), suggests specificity must exist for SH3 domain interactions. One determinant of specificity seems to be site 3, which interacts with the arginine residue in the ligand in SH3 domains from Src, p85α and Grb2. In contrast, the optimal high-affinity ligand for the SH3 domain of Abl contains another turn of the PPII helix, with a proline residue at position 5 relative to the Pro-X-X-Pro motif which bends inwards to occupy site 3 (Musacchio et al., 1994). As with SH2 domains, the specificity of SH3 domain binding is thought to be determined by the variable amino acids that surround the invariant residues. This specificity is evident in that the Abl SH3 domain does not bind well to Src SH3 domain ligands, while the Src SH3 domain binds poorly to Abl SH3 domain peptides (Rickles et al., 1994). However, SH3 domain specificity is not absolute. For example, the Fyn SH3 domain contains residues homologous to those in Src, but is able to bind a peptide derived from 3BP-2, a ligand for the Abl SH3 domain, whereas Src cannot (Musacchio et al., 1994; Rickles et al., 1995). Therefore, binding specificity could also be influenced by residues lying outside the PPII helix. Another determinant of specificity could be the small variations in the packing of hydrophobic side chains in the hydrophobic patch of the SH3 domain which may result in small differences in binding energy that are nevertheless sufficient to account for the specificity seen between SH3 domains (Mayer and Eck, 1995). For example, the peptide selected from a combinatorial library as optimal for binding to the Src SH3 domain binds with only a two-fold lower affinity to the p85α SH3 domain.
Conversely, the peptide that binds optimally to the p85αSH3 domain binds with a five-fold lower affinity to the Src SH3 domain (Yu et al., 1994). These subtleties in binding specificity are difficult to rationalise according to the primary structures of the SH3 domains and their ligands.

Another level of specificity that may be important in vivo is the additive effects of multiple potential SH3 domain ligands and, in some cases, multiple SH3 domains in a single protein. To date, most studies of the binding of SH3 domains have used peptide ligands, and the dissociation constants observed range between 5-100μM (Chen et al., 1993). However, many natural ligands for SH3 domains are embedded in long proline-rich regions of more than 100 amino acids that contain multiple potential binding sites. Higher affinity binding and greater specificity may therefore be achieved in vivo because the multiple potential binding sites for SH3 domains decrease the likelihood for the SH3 domain to diffuse away without re-binding than in the case of a peptide ligand. Small differences in the affinity of different SH3 domains for an individual site would be magnified when multiple copies of the binding site are present within a potential ligand. For example, the Abl-binding protein, 3BP-1, contains five potential SH3 domain binding sites in a 105 amino-acid region and, although it was possible to identify a minimal 10 amino-acid binding site, this binds the Abl SH3 domain more weakly than does the whole protein containing multiple sites (Ren et al., 1993). Similarly, the Sos protein, which binds the adaptor protein Grb2, contains four potential Grb2 SH3 domain binding sites in a 200 residue stretch (Yang et al., 1995). In this case, the affinity of binding may be increased still further by the presence of two SH3 domains in the intact Grb2 protein.

1.2.5.3 SH3 domain function

The known three-dimensional structures of liganded and unliganded SH3 domains display few ligand-dependent differences, ruling out major conformational changes within the domain. Upon ligand binding however, the binding event juxtaposes the SH3 domain containing protein and the proline-rich ligand containing protein which may have effects on the activity of one or both proteins. The physiological role of SH3-mediated interactions is still poorly understood, but recent studies suggest that SH3 domains mediate critical protein-protein interactions. These interactions are used to organise protein complexes within the cell, bring substrates to enzymes, and regulate enzymatic activities, as discussed in the following section.

Numerous oncogenes have been isolated from acutely transforming retroviruses including the adaptor protein, Crk, and the non-receptor tyrosine kinases, Abl and Src. Mutation of the SH3 domain of the viral form of Crk (v-Crk) mitigates its transforming
potential (Mayer and Hanafusa, 1990). Mutational analysis of the SH3 and SH2 domains of v-Src and c-Src suggest a role for these domains in the regulation of kinase activity and in the induction of morphological changes associated with transformation (Cross et al., 1984; Erpel et al., 1995; Hirai and Varmus, 1990) (section 1.3.3.1.4). Studies of c-Abl have shown that either deletion of or point mutations within the SH3 domain result in the constitutive tyrosine kinase activity and oncogenic activation (reviewed in Wang, 1993). Unlike c-Src protein, c-Abl is not regulated by binding of its SH2 domain to a carboxy terminal phosphorylated tyrosine (section 1.3.3.1). Instead, mutational analysis has implicated the SH3 domain as a potential regulator of c-Abl kinase activity (Franz et al., 1989; Jackson and Baltimore, 1989; Van Etten et al., 1995). The SH3 domain of c-Abl is thought to normally interact with a transregulator which blocks the kinase activity (Jiangyu and Shore, 1996). The HIV protein, Nef, was found to contain a PXXP motif that mediates its interaction with the SH3 domains of Hck and Lyn (Saksela et al., 1995). The PXXP motif is required for the higher replicative potential of Nef-bearing viruses, suggesting that the virus uses this SH3-domain mediated interaction to regulate a signalling pathway that facilitates viral growth.

Another type of regulatory mechanism is found in proteins involved in superoxide generation in neutrophils. Two proteins required for activation of a mitochondrial oxidase, p67phox and p47phox, contain two SH3 domains each. Genes encoding components of this pathway are often mutated in patients with hereditary chronic granulomatous disease (CGD), who have neutrophils lacking the capacity to synthesise superoxide. Activation of the superoxide-producing by treating neutrophils with arachidonic acid or sodium dodecyl sulphate causes the SH3 domain of p67phox, to bind to the proline-rich tail of p47phox, whose SH3 domains binds in turn to p22phox, a membrane-bound component of the oxidase complex (Finan et al., 1994; Sumimoto et al., 1994). Full activation requires both the SH3 domain and the small GTP-binding protein, Rac. (Abo et al., 1991; Clark et al., 1990). One mutation found in a CGD patient maps to the proline-rich region of p22phox and abolishes binding to p47phox. Presumably, the molecule is normally held in an inactive conformation by intramolecular interactions of SH3 domains with proline-rich sequences, and these must be disrupted before the active complex can be assembled.

Other examples of the regulation of enzymes by SH3 domain-containing proteins are the activation of PI3K by binding of SH3 domains to its 85 kDa subunit (Pleiman et al., 1994) (discussed in Chapter 5), and the increase in the GTPase activity of the endocytic protein dynamin that is observed upon binding of various SH3 domains (Gout et al., 1993). Dynamin belongs to a family of large GTPases which mediate vesicle trafficking.
Mammalian dynamins have approximately 80% homology to the *Drosophila melanogaster* *shibire* gene (van der Bliek and Meyerowitz, 1991). *ShiTS* flies were initially isolated as temperature-sensitive mutants (Grigliatti et al., 1973) that were paralysed as a result of depletion of neurotransmitter-containing vesicles at nerve terminals (Poodry et al., 1993). Similar mutations in the GTP-binding domain of mammalian dynamin inhibit receptor-mediated endocytosis (Herskovits et al., 1993; van der Bliek et al., 1993). The enzymatic activity of dynamin is markedly increased through association with microtubules (Herskovits et al., 1993; Shpetner and Vallee, 1992), acidic phospholipids, in particular PtdIns(4,5)P$_2$ (Salim et al., 1996; Tuma et al., 1993), and certain regulatory proteins that contain SH3 domains (Gout et al., 1993; Herskovits et al., 1993). A study using affinity chromatography showed that dynamin binds selectively to the SH3 domains of PLC$_\gamma$, Grb2 and p85$\alpha$, but not to several other SH3 domains. Dynamin has consensus SH3 domain binding sequences located at its carboxyl terminus. Furthermore, fusion proteins of Grb2, Src and p85$\alpha$ SH3 domains were able to stimulate the GTPase activity of brain dynamin (Gout et al., 1993). It is as yet unclear whether SH3 domain binding regulates dynamin GTPase activity in vivo. There is evidence that the activity of dynamin can also be modulated by phosphorylation. Protein kinase C (PKC) can phosphorylate dynamin, leading to stimulation of its GTPase activity, whilst the calcium-dependent phosphatase, calcineurin, can reverse this stimulation (Liu et al., 1994; Liu et al., 1994; Robinson et al., 1993). Interestingly, the PKC phosphorylation site is located within the proline-rich regions of dynamin, and phosphorylation and dephosphorylation of these sites correlates with the polarisation and depolarisation of synapsomes at the nerve terminals (Liu et al., 1994). Whether these phosphorylation events involve or regulate SH3 domain-binding has yet to be investigated.

Compartmentalisation of proteins within the cell plays an important role in the regulation of signal transduction processes. Many SH3 domain-containing proteins associate with the cytoskeleton or actin filaments, including $\alpha$-spectrin (Wasenius et al., 1989), myosin-1 (Jung et al., 1987) and cortactin (Wu and Parsons, 1993). Genetic analysis in yeast has demonstrated that SH3-domain containing proteins such as ABP-1, SLA1, BEM1 and BEM2 are required for the organisation and polarisation of the cytoskeleton (Kavanaugh et al., 1994). In *D.melanogaster*, mutations in the tumour suppressor gene, *discs large (dlg)*, leads to a loss of the tight septate junctions between epithelial cells, and aberrant proliferation of cells in the imaginal disk (Woods and Bryant, 1989). In mammalian fibroblasts, the SH3 domains of Grb2 and PLC$_\gamma$1 localise to membrane ruffles and actin stress fibres respectively, suggesting a role for SH3 domains in the control of protein distribution within the cell (Bar Sagi et al., 1993). Many ion-channel proteins contain proline-rich sequences (Rotin et al., 1994).
The interaction between the SH3 domain of α-spectrin and the proline-rich carboxy-terminal tail of the amiloride-sensitive Na⁺ channel dictates the localisation of α spectrin to the apical membrane of polarised epithelial cells (Rotin et al., 1994).

Thus, SH3 domains can fulfil a variety of roles in the regulation of enzyme activity, the assembly of multiprotein complexes or specification of protein localisation within the cell.

1.2.5.4 Co-operative regulation by SH3 and SH2 domains

While SH2 and SH3 domains are separate modules, capable of functioning independently, they are frequently found within the same protein on the same polypeptide chain, and there is increasing evidence to suggest that their functions may be co-ordinated. Crk contains a single SH2 domain and two SH3 domains, in the order SH2-SH3-SH3 (see Figure 1.4). A proline-rich insert in the Crk SH2 domain constitutes an SH3 domain-binding site (Anafi et al., 1996). This proline-rich sequence is predicted to lie within an extended loop of the SH2 domain which is dispensable for the binding of pTyr-containing proteins. Binding of one of the SH3 domains to this sequence is enhanced when the SH2 domain is bound to pTyr containing sequences.

RasGAP, which contains an SH3 domain flanked by two SH2 domains, forms an SH2-domain mediated complex with RhoGAP in tyrosine kinase transformed cells (Moran et al., 1991; Settleman et al., 1992). Two tyrosine-containing sequences that are close together in three dimensional space in RhoGAP are thought to bring the two SH2 domains of RasGAP into close proximity, resulting in a conformational change in RasGAP that causes a 100-fold increase in the accessibility of the ligand binding surface of its SH3 domain. This tandem arrangement of SH2 and SH3 domains can provide a conformational mechanism for regulating SH3 domain dependent interactions through tyrosine phosphorylation and regulation of SH2 domain interactions (Hu and Settleman, 1997).

The x-ray crystal structure of a fragment containing the SH2 and SH3 domains of lck, a Src tyrosine kinase family member, bound to a phosphorylated peptide derived from its sequence of its carboxyl tail (Eck et al., 1994). gives insight into the structural nature of SH2 and SH3 co-operativity. The fragment crystallised as a dimer with extensive intermolecular SH2-SH3 contacts. Interestingly, a pro residue within the SH2 domain lies in the binding pocket of the opposing SH3 domain, and is bound in an orientation similar to that seen in PXXP consensus SH3 domain ligands. While the pTyr of the bound phosphopeptide is in an orientation similar to that observed in SH2 domain phospho-peptide complexes, the peptide chain does not, lying in the crease of the
intermolecular SH2-SH3 interface. Although Src family kinases do not dimerise (Weijland et al., 1997), it has subsequently been shown that the SH3 domains of Src and Hck interact with pseudo PPII helices at the amino termini of these proteins (section 1.3.3.1). This structure illustrates how an SH3 domain might participate in intramolecular interactions involving distant regions of the protein, that would render it inaccessible to other proteins.

1.2.6 The WW domain

The WW domain is present in a number of signalling and regulatory proteins, and is often present in several copies (reviewed in Andre and Springael, 1994; Bork and Sudol, 1994; Hofmann and Bucher, 1995; Sudol, 1996). It consists of approximately thirty eight amino acids (Bork and Sudol, 1994) with a high content of hydrophobic, aromatic and proline residues, and has two highly conserved tryptophan residues (hence WW domain). Like SH3 domains, WW domains bind proline-rich sequences and have been implicated, directly or indirectly, in several human genetic disorders (Einbond and Sudol, 1996). The best characterised is Liddle’s syndrome, a hereditary form of hypertension in which there are lesions in the gene for the amiloride-sensitive epithelial sodium channel (Rossier, 1996). Patients with this syndrome have carboxy-terminal deletions in this protein that encompass the sequence PPPNY, which prevents the interaction with a WW domain in the regulatory protein Nedd4 (Bork and Sudol, 1994; Schild et al., 1996; Staub et al., 1996).

WW domains were first identified in the Yes-associated protein (YAP), and have since been found in dystrophin, ubiquitin-protein ligases, the transcriptional activator FE65 and proteins that bind to formins (Andre and Springael, 1994; Bork and Sudol, 1994; Chan et al., 1996; Hofmann and Bucher, 1995). Two putative ligands were identified for the YAP WW domain by screening of a cDNA expression library for YAP binding sequences. Both ligands have a proline-rich sequence followed by a tyrosine residue, PPPPY. Subsequent binding assays and site-specific mutagenesis have established a preliminary consensus sequence XPPXY that is thought to mediate WW domain binding. This consensus sequence is distinct from the SH3 domain-binding motif PXXP (Yu et al., 1994), and a recent three dimensional structure of the WW domain of human YAP in complex with its cognate peptide ligand confirmed that WW and SH3 domains are also structurally distinct (Macias et al., 1996).

In contrast, it has been suggested that WW domains may regulate the function of SH3 domains through direct competition for the same proline-rich sequences on target proteins (Sudol, 1996). This hypothesis emerged from a study of formins, which are
proteins involved in murine limb and kidney development (Woychik et al., 1990). Screening of cDNA expression libraries from the mouse limb bud for binding to the polyproline region of formin identified two types of formin binding proteins (FBPs). One set contained SH3 domains while the other contained WW modules (Chan et al., 1996). The WW domains of FBPs were shown to compete with the Abl SH3 domain for binding to the polyproline sequence in formin. If it is shown that these interactions can occur in vivo, it will be one of the first examples of degeneracy in the 'protein-protein interaction code'.

1.2.7 PH domains

Pleckstrin-homology (PH) domains are protein modules of approximately 120 amino acids. They were identified initially as repeat regions of weak amino acid sequence homology in pleckstrin, the major substrate of PKC in platelets (Haslam et al., 1993; Tyers et al., 1988). PH domains are found in many proteins involved in intracellular signalling pathways (Mayer et al., 1993; Shaw, 1996) and seem most likely to have a role in the signal-dependent membrane localisation of proteins (Shaw, 1996). It is striking that many proteins with PH domains also contain SH2 and SH3 domains.

PH domains were only recognised relatively recently due to their poor amino acid sequence conservation. The PH domain amino acid sequence alignment shows a series of rather poorly conserved regions interspersed with even less well conserved linker sequences, which may vary from a few amino acids to 100 or more and as is the case for PLCγ, may include other functional domains. The best conserved region of PH domains is the carboxy-terminal 15 amino acids, which contains the only nearly invariant residue, a single Trp residue (Downing et al., 1994; Fushman et al., 1995; Shaw, 1996). However, the secondary and tertiary structure of PH domains is strongly conserved.

Despite their lack of sequence similarity, all five PH domains for which three dimensional structures have been determined (reviewed in Lemmon et al., 1996) share the same basic fold, in which two nearly orthogonal β sheets of four and three strands form a β sandwich that is closed off at one corner by a carboxy-terminal amphipathic α helix which contains the relatively conserved Trp residue (Figure 1.8). Three loops of variable length and sequence separate the β strands and lie roughly on one face of the PH domain. This face usually contains several positively charged residues and is thought to be the site through which PH domains bind to negatively charged ligands.
The best characterised ligands for PH domains are phospholipids. A number of PH domains bind, albeit weakly, to membranes containing PtdIns(4,5)P2 (Harlan et al., 1994). Many of the reported ligands for PH domains bind only weakly and apparently with poor specificity, and thus have questionable physiological relevance. A notable exception is the PH domain of PLC-δ1, which binds specifically to PtdIns(4,5)P2 with a $K_d$ in the micromolar range (Lemmon et al., 1995) and to its soluble headgroup Ins(1,4,5)P3, with a $K_d$ of approximately 200nM. PLC-δ1 has been shown to bind both PtdIns(4,5)P2 containing lipid vesicles and the plasma membrane, and its PH domain is both necessary and sufficient for lipid association (Cifuentes et al., 1993). The role of the PH domain of PLC-δ1 may therefore be to bind to PtdIns(4,5)P2 in cellular membranes, thus co-localizing the enzyme with its substrate, membrane-bound PtdIns(4,5)P2, which is hydrolysed to produce Ins(1,4,5)P3. Free Ins(1,4,5)P3 could then negatively regulate PLC-δ1 by occupying the PtdIns(4,5)P2 binding site and preventing access of the enzyme to its substrate (Cifuentes et al., 1994; Kanematsu et al., 1992; Lomasney et al., 1996). A role for PH domains in signalling mediated by phosphoinositide lipids has also been reported for Btk. (Fukuda and Mikoshiba, 1996) and the serine/threonine kinase PKB also known as AKT and RAC, which has a PH domain at its amino-terminus. The latter protein is a downstream effector of PI3K, whose regulation by phosphoinositides will be described in detail in (section 1.4.7.9.1).

Some PH domains have also been reported to bind $\beta\gamma$-subunits of small G proteins in vivo. The PH domains of the $\beta$-adrenergic receptor kinase (β-ARK) and Bruton’s tyrosine kinase (BTK) have been shown to interact with G$\beta\gamma$ subunits (Touhara et al., 1994). G$\beta\gamma$ subunits, together with an unidentified membrane factor(s), which may be phospholipids, have been shown to activate immunoprecipitated Itk/Tsk, a cytoplasmic tyrosine kinase which is homologous to Btk (Langhans Rajasekaran et al., 1995). In contrast, it has recently been shown that the $G\alpha_q$ subunit directly stimulates the kinase activity of BTK and that G$\beta\gamma$ has little effect on the stimulation of BTK by $G\alpha_q$ (Bence et al., 1997). The reported affinities of PH domains for G$\beta\gamma$ subunits varies widely and thus the functional significance of these interactions remains unclear.

A third class of ligands that have been reported to bind PH domains are WD40 repeats (reviewed in Bence et al., 1997). WD40 repeats are approximately 40 amino acid repeat sequences that frequently contain a Trp-Asp motif. WD40 repeat-containing proteins are found in all eukaryotes, and their amino acid sequence is unusually highly conserved across species boundaries (Neer et al., 1994). The carboxy-terminal region of the G$\beta$ subunit contains five WD40 repeats. It has been suggested that the function of some WD40 repeats is to bind PH domains and that the differing affinities of various
PH domains for Gβγ subunits may be because some PH domains bind preferentially to other WD40 repeat containing proteins.

Thus, it appears that PH domains can bind both inositol lipids and proteins, but a different region of the PH domain is implicated for each binding event. It has been reported that Gβγ subunits and membrane lipids act synergistically to activate β-ARK (DebBurman et al., 1995; Pitcher et al., 1995). This raises questions about allosteric regulation of the PH domains, which have not been addressed to date.

Thus interaction of protein modules has emerged as a major theme in virtually all aspects of signalling. Given the multitude of interacting partners that gives rise to an even greater number of possible protein-protein complexes, unravelling the specificity and biological significance of these interactions is urgently required.

1.3 Signalling molecules downstream of RTK's

1.3.1 Substrates without catalytic activity

A number of proteins have been identified that link activated receptors to downstream enzymatic effectors. They possess no obvious enzymatic activity and often consist almost entirely of non-catalytic SH2 and SH3 domains. Such proteins serve as adapters. These adaptor proteins include Grb2, Shc, Crk, Nck, and the p85 subunit of PI3K. There are many other molecules that possess an adaptor function but only the best-characterised proteins are described below.

1.3.1.1 Grb2

Grb2, the mammalian homologue of the C.elegans sem-5 gene, is an approximately 25kDa protein with the structure SH3-SH2-SH3. The acronym Grb2 stands for growth factor receptor bound protein 2. Grb2 was originally cloned by screening bacterial cDNA expression libraries using the tyrosine phosphorylated carboxy-terminal tail of the EGF receptor as a high affinity probe (Lowenstein et al., 1992) Grb2 binds to activated EGF, PDGF and CSF-1 receptors but it does not become tyrosine phosphorylated itself (Lowenstein et al., 1992)

Microinjection of neutralising anti-Grb2 antibodies into mammalian cells blocked induction of their entry into S phase and the induction of membrane ruffling in response to PDGF and EGF (Matuoka et al., 1993). In C.elegans, Sem-5 is involved in vulval induction, and larval survival and has been demonstrated to be downstream of the EGF receptor homologue Let-23 and upstream of the Ras protein homologue Let-60 (Clark
et al., 1992; Sternberg and Horvitz, 1991) (see Figure 1.14). An analogous pathway exists in *D. melanogaster.* During the development of the photoreceptor R7 cell in the fruit fly retina, Sevenless, a receptor tyrosine kinase on the surface of the R7 precursor cell interacts with its ligand, Boss, on the apposed R cell. This interaction generates a signal leads to the differentiation of the precursor cell to an R7 phenotype. Ras1 was shown to be downstream of Sevenless. Then another component of this pathway downstream of Sevenless and upstream of Ras1 named son of Sevenless (Sos) (Bowtell et al., 1992; Fortini et al., 1992; Rogge et al., 1991; Simon et al., 1991) was shown to have homology to CDC25, a Ras guanine nucleotide exchange factor in *Saccharomyces cerevisiae.* (Bowtell et al., 1992; Fortini et al., 1992; Rogge et al., 1991; Simon et al., 1991) Subsequent studies identified mammalian homologues of SOS, (Bowtell et al., 1992) and demonstrated their ability to activate Ras by exchanging GTP for GDP (Chardin et al., 1993; Fantl et al., 1993; Gale et al., 1993). Grb2 was found to be in complex with SOS in mammalian cells (Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993; Li et al., 1993; Rozakis Adcock et al., 1993) In most cells types, the association of SOS and Grb2 was constitutive, and co-immunoprecipitation of both proteins with the EGFR was demonstrated to be EGF-dependent (Buday and Downward, 1993; Egan et al., 1993; Li et al., 1993; Rozakis Adcock et al., 1993) The SH3 domains of GRB2 bind at least two proline-rich sequences in the carboxy-terminal tail of SOS. (Li et al., 1993; Olivier et al., 1993; Rozakis Adcock et al., 1993; Stern et al., 1993). Point mutations in the SH3 domains of mammalian Grb2, corresponding to those found in *Sem5* loss-of-function mutations (Lowenstein et al., 1992) reduced or abolished binding of Grb2 to Sos (Egan et al., 1993; Rozakis Adcock et al., 1993), suggesting that a stable association between Grb2 and SOS requires interactions of SOS with both of the SH3 domains of Grb2. Short synthetic peptides, with the sequence PPPVPPRRR or sequences derived from the carboxy terminal tail of Sos block the binding of Grb2 to Sos or other proline-rich peptides, confirming the importance of SH3 domain interactions in Grb2 (Rozakis Adcock et al., 1993).

EGF binding to its receptor triggers tyrosine phosphorylation of the receptor cytoplasmic domain, creating a high affinity site for binding the Grb2 SH2 domain (Buday and Downward, 1993; Fantl et al., 1993; Rozakis Adcock et al., 1993) and recruiting the Grb2/SOS complex to the plasma membrane, where Ras, the substrate for Sos is located. In addition to the activated EGF receptor, Grb2 has also been shown to be capable of binding to the tyrosine phosphorylated insulin receptor substrate, IRS-1 (reviewed in Yenush and White, 1997), the adaptor protein SHC (Rozakis Adcock et al., 1992), the chimaeric oncogene product BCR-ABL (Pendergast et al., 1993) and the middle T antigen of polyoma virus (Dilworth et al., 1994).
1.3.1.2 Shc

The \textit{SHC} mRNA can be spliced to produce several variant proteins that become tyrosine phosphorylated when various receptors are activated. The Shc proteins comprise an amino-terminal SH2 domain and an adjacent glycine/proline-rich motif (known as a collagen like region, (Pelicci \textit{et al.}, 1992)). Shc binds to a tyrosine-phosphorylated receptor via its SH2 domain, then phosphorylated on tyrosine by the kinase activity of the receptor, creating a binding site on Shc for other SH2 domains. One of the principle binding partners for phosphorylated Shc is Grb2 with consequent activation of Sos which is constitutively bound to Grb2. (Rozakis Adcock \textit{et al.}, 1992; Skolnik \textit{et al.}, 1993). The importance of the SHC-Grb2-SOS pathway in \textit{ErbB2} receptor (also known as p185, or \textit{HER-2/neu}) signalling was demonstrated by the finding that a deletion mutant of Grb2 which lacked the amino-terminal SH3 domain was able to substantially decrease the oncogenic signal from activated \textit{ErbB2}. This deletion mutant of Grb2 was still able to bind Shc, but could no longer bind Sos (Xie \textit{et al.}, 1995). Shc can itself transform fibroblasts and promote differentiation of a neuroblastic cell line PC12 in a Ras-dependent fashion (Pelicci \textit{et al.}, 1992; Rozakis Adcock \textit{et al.}, 1992). Indeed in v-Src transformed rodent cells, Shc is constitutively phosphorylated on tyrosine and is bound to the Grb2-Sos complex through the SH2 domain of Grb2 (Rozakis Adcock \textit{et al.}, 1992). A complex of Shc, Grb2 and SOS is also assembled in cells stimulated by insulin, FGF and other growth factors (Pelicci \textit{et al.}, 1992; Pronk and Bos, 1994; Skolnik \textit{et al.}, 1993; Skolnik \textit{et al.}, 1993)). Moreover, in response to insulin and in the absence of IRS docking proteins, the insulin receptor stimulates the phosphorylation of Shc which then recruits the Grb2-Sos complex to the membrane. Therefore, one of the major roles is to link receptors with no Grb2 binding sites to the Grb2-Sos pathway. In these signalling pathways, two adaptor proteins are required to activate the enzymatic activity of Sos. Shc can also couple non RTK's to the Grb2-Sos complex and other signalling pathways. There is evidence that Shc associates with the T cell receptor and members of the cytokine receptor family (Ravichandran \textit{et al.}, 1993; Ruff Jamison \textit{et al.}, 1993). Thus Shc has many functions but it is not entirely understood yet how it is regulated. It's involvement in a number of different signalling pathways highlights it's complexity.

1.3.1.3 Crk and Nck

The adaptor proteins encoded by the \textit{Crk} and \textit{Nck} genes consist almost entirely of SH2 and SH3 domains, with no other motifs or domains of known function. The Crk gene was first isolated as a retroviral oncogene, \textit{v-Crk} (Mayer \textit{et al.}, 1988). Although the SH2 and SH3 domains in Crk proteins are non catalytic, transformation by \textit{v-Crk} is accompanied by tyrosine phosphorylation of a number of proteins that become
associated with the Crk SH2 domain (Birge et al., 1992; Mayer and Hanafusa, 1990). Binding of Crk to the EGF or PDGF receptor has been shown to result in significant protection against dephosphorylation of the receptor by a cellular phosphatase activity (Birge et al., 1992; Rotin et al., 1992). It is likely that c-Crk also lies in the Ras pathway, as microinjection of Crk was found to induce neuronal differentiation of the rat pheochromocytoma cell line, PC12, through activation of Ras (Tanaka et al., 1995).

Moreover, C3G, a ubiquitously expressed Ras guanine nucleotide exchange factor (GNEF), interacts with the SH3 domain of Crk (Tanaka et al., 1994), thereby providing further evidence that Crk may act as an adaptor in the Ras activation pathway, in a manner analogous to Grb2, linking phosphorylated receptors to RasGNEF.

Nck is an ubiquitously expressed protein comprising one SH2 and three SH3 domains (Lehmann et al., 1990). EGF or PDGF stimulation leads to the phosphorylation of the Nck protein on tyrosine, serine, and threonine residues. Nck binds to these activated receptors through its SH2 domain. Nck is thought to function as an adaptor protein which links cell surface receptor tyrosine kinases to downstream signalling pathways involved in the control of cell proliferation (Li et al., 1992; Meisenhelder and Hunter, 1992) however, the identities of other proteins in this pathway have yet to be fully defined.

Overexpression of human Nck in rat fibroblasts results in transformation as judged by alteration of their morphology, and their ability to form colonies in soft agar, or tumours in nude BALB/c mice (Chou et al., 1992; Li et al., 1992). Although overexpression of Nck does not induce a detectable elevation of the phosphotyrosine content of specific proteins, it can clearly bind tyrosine phosphorylated proteins in vitro. Moreover, Nck binds to both Src and to an as yet unidentified serine/threonine kinase activity. Thus, Nck may play a role in linking tyrosine and serine/threonine kinase pathways within the cell (Chou et al., 1992; Meisenhelder and Hunter, 1992).

It seems clear, therefore, that adaptor molecules primarily serve as molecular integrators, through which signal transduction networks can be co-regulated. However, most adaptor proteins appear to function within a number of pathways, and the details of the interactions of these multifunctional proteins remains to be elucidated.
1.3.2 Docking proteins

Receptor activation triggers the phosphorylation of a number of tyrosine residues within the cytoplasmic domain of the receptor, which can serve as binding sites for up to a dozen signal transducing proteins. Whether all of these molecules bind the receptor at once is not clear, but steric considerations suggest this is unlikely. In some instances, RTK's may employ an intermediate docking protein which is able to bind SH2 domain containing proteins, rather than binding directly to these proteins. Docking proteins become tyrosine phosphorylated at multiple sites after receptor stimulation, thus creating binding sites for many SH2 domain containing proteins. The best characterised docking proteins are insulin receptor substrate 1 (IRS-1) and insulin receptor substrate 2 (IRS-2) which are substrates for the tyrosine kinase activity of the insulin receptor (Sun et al., 1991; Sun et al., 1995). Like most RTK's, insulin-stimulated autophosphorylation of the β subunit of the insulin receptor activates its intrinsic tyrosine kinase. Recent structural work revealed that phosphorylation of three tyrosine residues in the regulatory loop of the insulin receptor stabilises a conformational change that exposes the catalytic pocket and activates the receptor kinase (Hubbard et al., 1994). However, autophosphorylation of the insulin receptor does not recruit SH2 containing proteins into a complex with the receptor. Instead, the activated receptor phosphorylates the docking proteins IRS-1, and IRS-2, Grb2-associated binder (Gab1) (Holgado Madruga et al., 1996) or p62 in mammalian cells (Richard et al., 1995) or daughter of sevenless DOS in D.melanogaster (Raabe et al., 1996). The carboxy-terminal tail of this family of docking proteins contain many tyrosine-phosphorylation sites in various amino acid sequence contexts that bind distinct SH2 domain containing proteins, including the p85 regulatory subunit of PI3K, Grb2, SHP2, Nck, Crk, Fyn and others, reviewed in (Yenush and White, 1997). Coupling of IRS-1 and IRS-2 to the insulin receptor is thought to occur via both a PTB (section 1.2.2) and a PH domain (section 1.2.7), which reside in the amino-terminal portion of these docking proteins. Use of a docking protein for receptor signalling has several implications. Firstly, the docking protein can amplify a receptor signal by eliminating the stoichiometric constraints encountered by receptors that directly recruit SH2 domain containing proteins to their autophosphorylation sites. Secondly docking proteins dissociate the intracellular signalling complex from the endocytic pathways which down regulates the activated receptor. Thirdly, docking proteins allow the signalling complex to move to other cellular compartments than the plasma membrane. In insulin signalling, some responses such as glucose uptake take place in cell compartments that are inaccessible to the insulin receptor (Heller Harrison et al., 1996). Fourthly, a single receptor may engage multiple IRS proteins to expand its repertoire of
signalling pathways, or a single IRS protein may integrate signals from multiple receptors. IRS-1 has been shown to be important in other growth factor and cytokine signalling systems, including those emanating from the GH receptor and several interleukin receptors (reviewed in Yenush and White, 1997). Analysis of mice which have been made nullizygous for IRS-1 confirm that it has an important physiological role in glucose metabolism and general cell growth.

The movement of glucose across the cell membrane is largely accomplished by a family of six glucose transporters, GLUTs. GLUT4 is constitutively expressed in adipose and muscle tissue where it is usually sequestered in an intracellular vesicular compartment. Considerable evidence now indicates that the activation of PI3K by its association with IRS-1 is essential for GLUT4 translocation (Hara et al., 1994; Okada et al., 1994; Yarden et al., 1987). In addition to glucose transport, the synthesis of glycogen in skeletal muscle is an important mechanism for glucose storage following a meal. Two signalling cascades are implicated as upstream mediators of glycogen synthase, including the MAP kinase cascade and the PI3K/AKT pathway (Cross et al., 1995; Hara et al., 1994; Okada et al., 1994; Yarden et al., 1987). In skeletal muscle, IRS-1 is the principal regulator of both pathways following insulin stimulation (Yamauchi et al., 1996).

Thus docking proteins are substrates that are specifically phosphorylated at multiple sites by an activated RPTK and then bind a selection of SH2 domain containing proteins, which in turn propagate a downstream signal. Each docking protein is not specific for a single RPTK but can act as substrates for a number of RPTKs thereby increasing a cell’s signalling potential.

1.3.3 Substrates with catalytic activity

1.3.3.1 Protein tyrosine kinases (The Src Family)

1.3.3.1.1 Introduction

The protein tyrosine kinases (PTKs) are a large and ever expanding class of proteins possessing a highly conserved catalytic domain that specifically phosphorylate tyrosine residues. These enzymes, first recognised among retroviral oncoproteins, have been found only in metazoan cells where they have roles in transducing growth and differentiation signals. PTKs are distinct from RTKs in that they do not contain a membrane spanning region and their activation by extracellular ligands is generally indirect. Like RTKs, PTKs can be regulated by phosphorylation, either by autophosphorylation or by phosphorylation by upstream protein kinases in a signal
transduction cascade. PTKs often contain, in addition to their kinase domains, non-catalytic modules for example SH3 and SH2 domains. It is beyond the scope of this review to describe all PTKs thus one PTK family, the Src family, will be discussed in detail for two reasons. Firstly, Src has been linked with PI3K in a number of signalling processes and its interaction with p85 will be examined in Chapter 5. Secondly, the recently determined x-ray crystal structure of a large fragment of Src has facilitated a greater understanding of the regulation of its activity, and may be a model that is generally applicable to other PTKs.

The cellular Src gene was the first molecularly-defined proto-oncogene, and its product, Src (also known as p60^"S"
 or c-src) is now known to be the source of the first detected tyrosine phosphorylation event (Courtneidge and Smith, 1983; Eckhart et al., 1979). The oncogenic properties of Rous sarcoma virus, an avian retrovirus that causes the rapid eruption of tumours in infected animals, led to the identification of the viral oncogene v-Src, and to the recognition that this is a subtly altered form of the normal cellular gene, c-Src (Stehelin et al., 1976). Although over expression of unadulterated c-Src has little effect on cellular behaviour, the substitution of a single amino acid can convert the protein to a form that induces profound changes in cell growth control, gene expression, metabolism and cytoskeletal architecture, which suggests that c-Src is normally involved in these cellular processes (Figure 1.9).

To date, there are nine members of the Src family, namely Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk, and Yrk (Figure 1.10) (Courtneidge, 1994). While the expression of most members is restricted, particularly to haemopoietic cells, at least three, Src, Fyn and Yes are more generally expressed. However, they are all structurally similar and are distinguished by a conserved arrangement of functional domains. From the amino to carboxy-terminus, each Src family member contains (i) a short amino-terminal membrane anchor or SH4 domain, (ii) a poorly conserved 'unique' region of 40-70 residues, (iii) an SH3 domain of 50 residues (iv) an SH2 domain of 100 residues (v) a catalytic (tyrosine kinase) domain of 250 residues and (vi) a short carboxy-terminal region containing a conserved tyrosine residue. (Figure 1.10) (Cooper, 1990; Resh, 1994; Superti Furga and Courtneidge, 1995)

1.3.3.1.2 Domain structure and regulation
Src family kinases are synthesised in the cytoplasm but are found associated with cell membranes (Courtneidge et al., 1980; Krueger et al., 1983). Membrane association is critical for transformation by activated v-Src (Kamps et al., 1986), and a recent study of mutants of lck showed that an activated Src-family kinase needs to remain at the membrane in order to signal (Spencer et al., 1995) The major determinant of membrane
localisation is the first 15 amino acids in the SH4 region of the protein. All Src members bear a signal for myristylation and most also have one for palmitoylation (reviewed in Brown and Cooper, 1996) which direct the association of these proteins with cellular membranes (Resh, 1994). It has also been reported that appreciable quantities of c-Src also exist in several intracellular membrane compartments and localise to cytoskeletal structures in the cell.

The lipid attachment sequences are followed by a unique domain which is of variable size. As its name implies, this portion is the least conserved between the different family members and may confer specificity of function. One known function of this region is to mediate the association of Lck with the tail of the transmembrane receptors CD4 and CD8 in T lymphocytes via two cysteine residues in the unique domain of Lck (Rudd et al., 1993; Weiss and Littman, 1994). The unique domain of many Src family members is also subject to serine/threonine phosphorylation. In Src, for example, Ser 17 represents a major, constitutive, phosphorylation site for cyclic AMP-dependent protein kinase C, and serine 12 is a site of phosphorylation by protein kinase C. The function of these phosphorylations, however, is not clear, since mutation of these residues has no detectable effect (Taylor and Shalloway, 1993).

Carboxy-terminal to the unique domain are the SH3 and SH2 domains, which can bind to proline-rich and phosphotyrosine-containing sequences respectively (section 1.2.1 and 1.2.5). The SH2 domains of several Src family kinases have the same binding specificity (Songyang et al., 1993), whereas the SH3 domains seem to have different binding specificites (Weng et al., 1994). In accordance with this observation, the SH2 domains are slightly more conserved among Src family members than the SH3 domains.

The majority of the carboxy-terminal amino acids (260-516) of c-Src comprise the catalytic domain, which has homology to the catalytic domain of all protein tyrosine kinases (Hanks et al., 1988). Contained within these sequences are the ATP binding site (in particular a lysine at position 295 (Kamps et al., 1984), and an autophosphorylation site Tyr 416 (Hanks et al., 1988) which is in the so-called 'activation loop'. The catalytic domain is followed by a stretch of 20 amino acids known as the tail and contains a conserved tyrosine residue, (Tyr 527 in chicken c-Src) (Smart et al., 1981), whose phosphorylation negatively regulates kinase activity.

A protein tyrosine kinase called Csk phosphorylates tyrosine 527 in the carboxy terminal tail of all members of the Src group that have been tested (Bergman et al., 1992; Okada et al., 1991; Superti Furga et al., 1993). In many cases, it has been shown
that this phosphorylation occurs in vivo and is associated with down-regulation of enzyme activity (Chow et al., 1993; Murphy et al., 1993; Okada et al., 1993; Sabe et al., 1992; Superti Furga et al., 1993). Mutation of Tyr527, to phenylalanine is sufficient to convert the proto-oncogene c-Src to an oncogenic form, and dephosphorylation or mutation of this residue is accompanied by an at least 10-fold increase in kinase activity (Courtneidge, et al., 1995). Phosphatases specifically acting on Tyr 527 of c-Src have not been identified but a number of candidate molecules have been implicated (reviewed in Brown and Cooper, 1996).

Activated forms of Src, such as v-Src or c-Src expressed in yeast in the absence of Csk, are autophosphorylated on Tyr 416 which is in a region known as the activation loop. Autophosphorylation of residues in the activation loop has been shown to regulate the activity of several serine/threonine and tyrosine kinases (reviewed in Morgan and De Bondt, 1994; Superti Furga and Courtneidge, 1995; Zhang and Stoltzfus, 1995). Mutation of Tyr 416 to phenylalanine impairs the ability of activated c-Src to transform cells, but has only very little effect on the transformation potential of v-Src (Hunter, 1987; Superti Furga and Courtneidge, 1995). This mutation does not abrogate kinase activity, although it is reduced 2-3 fold. Phosphorylation of Tyr 416 is therefore not strictly required for Src kinase activity, however it can upregulate kinase activity in certain forms of Src. The mechanism behind this observation and its physiological outcome is not yet fully understood (Ferracini and Brugge, 1990; Kmiecik and Shalloway, 1987; Piwnica Worms et al., 1987).

1.3.3.1.3 Three dimensional structure of Src tyrosine kinases
The three dimensional structure of an active form of Src in which Tyr527 is phosphorylated has elucidated the mechanism behind these observations and helped to explain many aspects of Src, and Src-family kinase, regulation (Xu et al., 1997) (Figure 1.11). This interaction forces conformational constraints on the Src kinase domain, such that the catalytic cleft is occluded. The interaction of Tyr527 and the SH2 domain is strengthened by a co-operative binding of the SH3 domain to the linker region between the SH2 and the kinase domains. This linker does not have the consensus sequence of an SH3 domain binding site, but nevertheless adopts a conformation almost identical to a type II polyproline helix. Both the SH2 domain and the linker region form contacts with the kinase domain. A recent study shows that mutations in the linker region give rise to Src mutants that are not only highly active but also transforming in murine fibroblasts (Gonfloni et al., 1997). The structure of the catalytic domain is similar to that of cyclin-dependent kinase (CDK) 2 (De Bondt et al., 1993) and the activity of both kinases is primarily regulated by the orientation of the 'αC helix', which sits near the active site cleft. The inactive orientation is stabilised by
interactions of this helix with the SH2-SH1 linker and the activation loop. The three dimensional structure of Hck, a Src family member, has also been determined and is similar to that of c-Src (Sicheri et al., 1997).

In both structures, the SH2 domains and catalytic domains are held together by a few charged interactions and more strongly, by the SH2-carboxy-tail association. The large separation between the SH2-carboxy-terminal tail and the active site implies that their influence on catalytic activity must be exerted at long range. Surprisingly, however, the SH3 domain does not interact directly with the catalytic domain (or does so only very poorly), but rather binds the segment that links the SH2 domain to the kinase domain, namely the SH2-SH1 linker. The linker itself adopts a very extended conformation, typical of polyproline type II helices, and interacts with the small lobe of the catalytic domain. The SH3 domain thus makes use of the linker as an adapter to bind the catalytic domain. In turn, the small lobe presents the ligand for the SH3 domain, a situation that resembles presentation of the polyproline type II helix by the rest of the Nef structure in the binding to the Hck SH3 (Lee et al., 1996).

The elucidated structures of the inactive c-Src and Hck account for the co-operative role of SH2 and SH3 domains in the full activation of Src family kinases. When Tyr 527 is phosphorylated, not only is the kinase domain repressed, but the SH2 and SH3 domains are locked up in intramolecular interactions, such that they cannot associate with exogenous ligands and disturb SH2/SH3-regulated signalling events. Although it is likely that dephosphorylation of Tyr 527 is the key regulatory switch, it is possible that the intramolecular interactions in which the SH3 and SH2 domains are engaged are probably very weak. As a consequence more potent exogenous ligands for either domain may compete for binding, thereby disrupting the inactive structure. Direct evidence for the displacement mechanism is provided by the demonstration accompanying the Hck crystal structure that the HIV Nef protein activates Hck by binding to its SH3 domain (Moarefi et al., 1997) This activation is as strong as by the SH2 domain binding a receptor phosphopeptide. Moreover, a combination of the SH3 and SH2 binders activates Hck more than the SH2 binder alone, suggesting that the SH3 domain may also be inhibiting when the carboxy-terminal phosphotyrosine does not bind the SH2 domain. Thus the regulation of Src activity by its own SH3 and SH2 domains is a fundamental feature of this family of kinases and ensures that Src is only active when on the appropriate cellular targets. (Figure 1.12).

1.3.3.1.4 Biology of the Src tyrosine kinase family

An increase in the catalytic activity of Src, was first reported in quiescent fibroblasts stimulated with PDGF receptor (Ralston and Bishop, 1985). The Src family kinases
associate with the activated PDGF receptor via their SH2 domains (Twamley et al., 1992); About 5% of the PDGF receptor and Src family PTK populations associate, thus PDGF treatment causes a phosphorylation-dependent shift of ~5% of Src molecules on one and two dimensional gels (Gould and Hunter, 1988). c-Src binds predominantly to Tyr 579/Tyr 581 in the juxtamembrane region of the PDGF receptor (Mori et al., 1993). Upon association, the Src kinases are phosphorylated by the receptor (Gould and Hunter, 1988; Mori et al., 1993; Ralston and Bishop, 1985; Twamley et al., 1993) on Tyr 136 and Tyr 142 in the SH3 domain (Broome and Hunter, 1997) and although phosphorylation at Tyr 136 interferes with binding of an SH3-containing Src fragment to an SH3 domain binding peptide, the functional importance of these phosphorylations remains to be elucidated. Src has been shown to act downstream of a number of receptors including those for EGF (Sato et al., 1995), CSF-1 (Courtneidge et al., 1993), FGF (Osherov and Levitzki, 1994) and members of the antigen receptor family (Mustelin, 1994). To date, the c-Src SH2 binding consensus YEEI is only found in the PDGF and CSF-1 receptors.

Src family kinases are also activated by GPLR’s (Jalink et al., 1993; Liebenhoff et al., 1993; Wong et al., 1992). Although they are not required for the mitogenic effect of the GPLR ligands LPA or bombesin (Roche et al., 1995), it seems likely that Src family kinases are involved in the cytoskeletal rearrangements that accompany stimulation of cells by LPA or thrombin. Many of the cytoskeletal proteins which are phosphorylated upon stimulation of cells with LPA (Saville et al., 1994; Seufferlein and Rozengurt, 1994) have also been described as Src tyrosine kinase substrates in transformed cells (Kanner et al., 1990). These proteins include focal adhesion kinase (FAK) (Guan and Shalloway, 1992; Schaller et al., 1992), paxillin (Glenney, 1989) actin filament associated protein p110 (AFAP110) and p130CAS (Sakai et al., 1994). Thrombin treatment of platelets results in a redistribution of Src from the plasma membrane to a detergent-insoluble cytoskeletal fraction, (Clark and Brugge, 1993; Horvath et al., 1992; Oda et al., 1993).and transforming versions of Src are often localised in focal adhesions (Schaller et al., 1992). Thus the involvement of Src in GPLR signalling correlates with the change in cytoskeletal organisation associated with transformed and growth factor stimulated cells.

A role for the c-Src in normal mitotic regulation was first suggested by the observation that Src PTK activity increases at the onset of mitosis (Chackalaparampil and Shalloway, 1988). Src activation is accompanied by phosphorylation of Src on serine and threonine residues by Cdc2 kinase in vitro (Morgan et al., 1989; Shenoy et al., 1989). These phosphorylations alone are not sufficient to cause the activation of the Src kinase, but have a role in displacing the Src SH2 from pTyr 527 or making pTyr

45
more accessible to phosphatases. In addition, the ability of the Src SH2 domain to bind exogenous phosphotyrosine-containing ligands is increased during mitosis (Bagrodia et al., 1993). Increased Src activity during mitosis results in the phosphorylation of an (Fumagalli et al., 1994; Taylor and Shalloway, 1994) abundant nuclear protein of 68 kDa, Sam68 (Src associated in mitosis, 68Kda), that binds to both the SH2 and SH3 domains of Src family kinases. The role of Src in mitosis may therefore be to regulate aspects of RNA processing in a cell-cycle dependent manner.

There is considerable functional redundancy within the Src family. Mice made homozygous null for a single Src family kinase have only modest abnormalities, for example Src-/- mice are superficially normal, apart from a deficiency in bone resorption which leads to osteopetrosis (Soriano et al., 1991) while Fyn -/- mice, are also essentially normal except for defects in T-cell receptor signalling in thymocytes, abnormal development of the hippocampus and impairment of long term potentiation (a measurement related to memory) (Appleby et al., 1992; Grant et al., 1992; Stein et al., 1992). Deletions of genes of multiple Src tyrosine kinase family members however are more severe, if not lethal (Lowell and Soriano, 1996; Roche et al., 1995).

1.3.3.2 Serine /Threonine kinases

1.3.3.2.1 Introduction
Like the PTK family, The serine threonine protein kinases include a large family of enzymes that are either transmembrane receptors or are cytosolic proteins. The PTKs share some degree of homology with protein serine/threonine kinases (Yarden and Ullrich, 1988). They are often part of protein cascades, the best known example is the Mitogen-activated protein kinase (MAPK) cascade.

1.3.3.2.2 MAPK cascade
MAPKs, also known as extracellular signal-regulated kinases (ERKs) are serine/threonine protein kinases thought to play an important role in signalling cell proliferation and differentiation. This cascade is found in all eukaryotic organisms and consist of a three-kinase module that includes a MAPK, which in turn is activated by a MAPK/ERK kinase (MEK) , which in turn is activated by a MEK kinase (MEKK) (Cobb and Goldsmith, 1995). The first and best characterised MAPK cascade consists of Raf isoforms, MEK1/2, and ERK1/2, and is regulated by Ras (section 1.3.4.1.3) (Morrison and Cutler, 1997). Each consecutive kinase step is regulated by phosphorylation and dephosphorylation. This multistep pathway provides the means for signal amplification. It provides numerous branch points that serve different sets of targets as the signal is relayed along the pathway In the MAP kinase cascade, the degree
of branching, as gauged by the number of substrates that have been identified for a given kinase, changes throughout the cascade. Raf and MAP kinase kinase (MAPKK), especially the latter, appear to be highly specific kinases (Seger et al., 1992). However MAP kinase and Rsk (a MAP kinase substrate) have numerous substrates. MAP kinase transmits signals to many targets, both nuclear and non-nuclear, including, in addition to ribosomal protein S6 kinase II, Rsk (Sturgill et al., 1988). MAPK rapidly translocates to the nucleus upon activation (Chen et al., 1992), and recent findings have demonstrated that a substantial proportion of active MAPK is associated with the cytoskeleton (Reszka et al., 1995). Consequently, MAP kinase signalling is effectively disseminated throughout the cell, a factor that is critical with respect to its activation by MAPKK and its ability to phosphorylate numerous substrates at diverse locations within the cell. Another benefit that derives from a multistep cascade system is that such a scheme provides an opportunity for the exertion of independent control over some (but not necessarily all) functions regulated by the cascade. The impact a given signal will have depends on its site of action in the cascade. For example, an agent that acts on an early step in the cascade would be expected to affect multiple functions, whereas agents acting at the end of the cascade would affect only a few functions. Finally another feature of such cascades is that they contain feedback loops, which can act in both a negative and positive direction.

Growth factors, phorbol esters, G-protein-coupled receptor agonists, and hormones regulate MAPK through a series of phosphorylation events (Pelech and Sanghera, 1992). Translocation of activated MAPK to the nucleus (Chen et al., 1992; Lenormand et al., 1993) and subsequent phosphorylation of a variety of transcription factors including c-Myc, Elk-1, and ATF-2 (Davis, 1993) support the involvement of MAPK in transducing cytoplasmic signals to nuclear responses (Rodriguez Viciana et al., 1994). Experiments using inhibitors, antisense, or dominant-negative MAPK constructs to decrease MAPK activity have further demonstrated the involvement of MAPK activation in specific cellular responses including neuronal differentiation in PC12 cells and thrombin-induced mitogenesis in fibroblasts (Freshney et al., 1994; Pages et al., 1993; Qui and Green, 1992). Clearly, MAPK activation is important for many growth responses and represents a point of integration of signals emanating from both RTK's and GPLR's.

1.3.3.3 Protein phosphatases (PTPs)
The study of protein kinases has clearly demonstrated phosphorylation is a key mechanism of regulation of many critical proteins and enzymes. However, the protein phosphatases, which catalyse the reverse reaction, are equally important in the
regulation of protein function by phosphorylation. Thus, the levels of phosphorylation of a protein after agonist stimulation is determined by the opposing actions of protein kinases and protein phosphatases, which subsequently dictate whether a cell will grow and divide, change shape, move, differentiate, or die.

The tyrosine phosphatase superfamily, contains at least 3 classes of protein with tyrosine phosphatase activity. There is little amino acid sequence similarity amongst members of the three groups, but they have similar tertiary structures and share the same mechanism of catalysis, which is characterised by the formation of a thiophosphate intermediate involving a conserved catalytic cysteine residue which is essential for phosphatase activity (reviewed in Denu et al., 1996). The first class of protein tyrosine phosphatases (PTPs) are the 'Classical' PTPs which exist in transmembrane forms (receptor-like PTPs or RPTPs) and non-transmembrane (non-TM) forms. These enzymes have at least one catalytic domain of approximately 240 residues (the PTP domain), containing the conserved 'signature motif', [I/V]HCxAGxxR[S/T]G (where X represents any amino acid).

The three dimensional structures of several classical non-TM PTPs and RPTPs have been determined (Barford et al., 1994; Bilwes et al., 1996; Jia et al., 1995; Su et al., 1994). Non-TM PTPs have a similar overall architecture, comprising of a central twisted, mixed β sheet flanked by α helices. The signature motif residues are found within a single loop, at the base of the catalytic cleft. The essential cysteinyl residue is positioned in this loop so that it is accessible to the phosphotyrosyl residue. The topology of the RPTPα catalytic domain is similar to that of the non-TM PTPs, except that RPTPα crystallises as a dimer in which the amino-terminal helix-turn-helix motif of each monomer inserts, into the catalytic domain of the other, making specific contacts with residues at the mouth of the catalytic cleft (Bilwes et al., 1996). In dimeric form, RPTPα is presumably inactive, as helix-turn-helix structure blocks access of the substrate to the active site, suggesting that dimerization-multimerisation may be a general mechanism for RPTP regulation. RPTP ligands, which would be predicted to be promote RPTP oligomerization, would thus inactivate RPTPs, which is in contrast to the activation of RPTK's by oligomerization. However little direct experimental evidence exists for RPTP dimer formation in vivo.

Several SH2 domain containing non-TM PTPs (SHPs) have been identified in mammals, Xenopus and Drosophila (reviewed in Frearson and Alexander, 1997). These enzymes are normally catalytically inactive due to an intramolecular inhibition by their two SH2 domains (Pei et al., 1993; Townley et al., 1993). The mechanism of this autoinhibition is not understood, but is thought not to be phosphotyrosine-dependent,
as in the case of the Src family tyrosine kinases (section 1.3.3.1). Studies using phosphorylated peptides as substrates suggest that while the amino terminal-SH2 domain has both a regulatory and a recruitment role, the carboxy-terminal SH2 domain is mainly involved in correct localisation of the enzyme (Pei et al., 1996) but binding of both SH2 domains is required for maximal activation of the catalytic domain.

SHP-1 has been shown to both up regulate and down regulate signal transduction pathways mediated by a variety of haemopoietic receptors (reviewed in Frearson and Alexander, 1997). In addition to its phosphatase activity, SHP-2 may also act as an adaptor molecule coupling receptors to downstream signalling pathways. Binding of SHP-2 to pTyr 1009 of the PDGF-R via its amino terminal SH2 domain results in phosphorylation of SHP-2 on a carboxy terminal tyrosine residue, creating a binding site for the SH2 domain of Grb2 and thus recruiting the Grb2/Sos complex to the cell membrane and activating the Ras-MAP kinase pathway (Bennett et al., 1994; Li et al., 1994). In both insulin and EGF-responsive cell lines over expression of an inactive form of SHP-2 results in a dominant negative effect on endogenous SHP-2 and inhibition of growth factor-induced mitogenesis (Milarski and Saltiel, 1994; Tauchi et al., 1994). Thus SHP-2 has many functions; not only is it able to switch off RTK signals but in certain situations it can act as an adaptor molecule. In contrast to its negative regulatory role, PTP2 can therefore also act as a positive signal transducer. How this is achieved and how RTK signals are negatively regulated is not yet known.

The third class of PTPs are the dual specificity phosphatases that dephosphorylate tyrosine, and serine or threonine residues. Most dual specificity PTPs act on a limited range of substrates. For example, PAC1, a PTP expressed only in haemopoietic cells, is only known to dephosphorylate and down regulate MAP kinase (Ward et al., 1994). There may be large numbers of dual specificity PTPs, each specific for a particular substrate but few are known and they are poorly characterised.

1.3.3.4 Lipid modifying enzymes
Although these enzymes are signalling molecules downstream of RTKs, they will be discussed in a separate section as PI3K is the subject of this thesis (section 1.4).

1.3.4 Ras and Ras-related GTP-binding proteins
The Ras-related GTPases superfamily is made up of several subfamilies such including Ras, Rho/Rac, Rab, Arf and Ran subfamilies reviewed in (Boguski and McCormick, 1993). All members of this superfamily are proteins with a molecular mass between 20-25 kDa with amino acid homology of approximately 30% over the entire
superfamily. All Ras related GTPases bind and hydrolyse GTP and thus modulate the activity of a great diversity of effector molecules. These GTPases function as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state. (Figure 1.13) illustrates the cycling of Ras between these two forms. This cycle is regulated by the activities of guanine nucleotide exchange factors (GNEFs), which accelerate GTP loading of GTPases and GTPase-activating proteins (GAPs) which increase the rate of GTP hydrolysis by GTPases. The activities of GAPs and GEFs are modulated by signals emanating from cell surface receptors, a process that is conserved in eukaryote evolution (Feig and Cooper, 1988) (Figure 1.14).

The Ras subfamily is mainly involved in the regulation of cell growth and differentiation, while the Rab subfamily is involved in the regulation of secretion and vesicular trafficking, and the Rho subfamily, is primarily involved in the regulation of cell growth and apoptosis (section 1.3.4.2.2). In addition the Ras and Rho subfamilies affect cell morphology (section 1.3.4.2.1). The ARF subfamily are ADP-ribosylation factors which are involved in the activation of cholera toxin, regulation of PLD and membrane trafficking (Boman and Kahn, 1995). The Ran family are involved in nuclear import pathways and the proper regulation of entry into mitosis. It is beyond the scope of this review to describe all the subfamilies, therefore emphasis will be on the Ras and Rho subfamilies, as they have been implicated in PI3K function.

Ras is a membrane-localised guanine nucleotide-binding protein with intrinsic GTPase activity. An alignment of the amino acid sequences of Ras proteins from various species reveals two regions of homology which have different functions (Milburn et al., 1990). The amino-terminal region (residues 1-165 of human Ras) is highly conserved and necessary for guanine nucleotide binding and GTPase activity. The carboxy-terminal portion (residues 166-189 of human Ras) is more variable except for the four carboxy-terminal amino acids CAAX which specify membrane localisation.

The Ras genes were first identified as the transforming agents in the Harvey and Kirsten murine sarcoma viruses (Lowy and Willumsen, 1993). Identification of the cellular Ras homologues H-Ras, K-Ras, and N-Ras (Hall et al., 1983) and the presence of mutated alleles in human tumours emphasised the importance of the Ras genes in the control of cellular proliferation. Oncogenic activation of the Ras proteins is a consequence of genetic mutations that result in either impaired GTPase activity or an increased rate of guanine nucleotide exchange. Both types of mutation increase the active GTP-bound Ras in the cell.
Point mutations in Ras genes are found in almost one-third of human tumours, one reason for the strong interest in this G-protein. These in vivo point mutations are usually found at either Gly 12/Gly 13 or Gln 61. Unlike wild type Ras, the oncogenic protein is unable to hydrolyse GTP in the absence or presence of GTP resulting in a persistently activated state of the protein. Activated Ras initiates some of the intracellular signals normally seen in response to mitogenesis that signal via RTKs and therefore can mimic growth factor stimulation of cells. Consistent with this, inhibition of cellular Ras blocks activation of the same signals by ligands that activate RTKs. The initial discovery that certain point mutations convert Ras genes into oncogenes in mammalian cells immediately implicated Ras in the control of cell growth. Subsequently microinjection of activated Ras into cultured fibroblasts induced DNA synthesis and morphological transformation, (Stacey and Kung, 1984) whereas Ras-neutralising monoclonal antibodies inhibited serum-stimulated DNA synthesis and morphological transformation (Mulcahy et al., 1985; Smith et al., 1986).

1.3.4.1.1 Structure of Ras
The first three dimensional structure of a small GTP-binding protein in the Ras superfamily to be solved was that of Ras itself. It was solved in both the active trisphosphate-bound conformation and the inactive diphosphate conformation (Kraulis et al., 1994; Milburn et al., 1990; Pai et al., 1990). The structure consisting of six β-strands and five α helices, led to the prediction that all GTP-binding domains have a common topology, known as the 'G domain'. By comparison of the two states, two highly flexible regions surrounding the γ-phosphate have been established: the switch I region, from residues 30-37 within loop L2 and β2 (the 'effector region'). Point mutations in this region render Ras biologically inactive and unable to interact with downstream effector molecules such as Raf (Nassar et al., 1995) and the switch II region, from residues 60-76 within loop L4 and helix α2. The switch II region is highly mobile, existing in multiple conformations. Mutations in this region can compromise Ras function and lead to selective disruption of binding to PI3K or neurofibromin, implying that this region may contribute to the selectivity of binding (Moodie et al., 1995).

The mechanism of the GAP-mediated GTP hydrolysis on Ras has been a matter of considerable debate in recent years and has centred around the issue of whether Ras itself is an efficient GTPase device and GAP acts catalytically to push it into the active state or whether GAP actively participates in the reaction to make it efficient (Geyer et al., 1996; Maegley et al., 1996). Evidence for the latter option came from experiments with aluminium fluoride, which binds in the γ-phosphate binding site of most ATP/GTP-utilising phosphoryl transfer enzymes where it mimics the transitions state of
the reaction, as has been most clearly shown for the α subunits of heterotrimeric G proteins (Coleman et al., 1994; Sondek et al., 1994). Contrary to results on Gα proteins, Ras itself does not bind aluminium fluoride but rather needs stoichiometric amounts of RasGAPs to do so (Mittal et al., 1996), implying that the catalytic centre of Ras is incomplete unless GAP is present. This was revealed by the three-dimensional structure of the complex between RasGDP and the catalytic domain of p120GAP crystallised in the presence of aluminium fluoride. The structure shows that GAP supplies an arginine finger to the active site of Ras, which apparently stabilises negative charges in the transition state of the GTPase reaction via its guanidinium sidechain believed to proceed by a mostly associative mechanism (Scheffzek and et al, 1997). The structure also shows that Gln 61 of Ras, which in uncomplexed Ras is very mobile, becomes fixed by the presence of GAP and also participates in the stabilisation of the transition state and also explains a structural requirement for a glycine in position 12 for GAP catalysis to work.

1.3.4.1.2 Biochemistry and function of Ras

Many signal transduction pathways involving Ras were initially dissected in lower eukaryotes. Ras has been implicated in various developmental processes in amphibians, flies and nematodes, all of which are normally controlled by receptor tyrosine kinases. For example, insulin-induced maturation of Xenopus laevis oocytes is mimicked by activated Ras and abrogated by the inhibition of cellular Ras (Grigorescu et al., 1994). In the nematode C. elegans, the induction of vulval cell differentiation is dependent on the activity of the let-23 gene product, a receptor tyrosine kinase, and the let-60 gene product, a Ras homologue (Han and Sternberg, 1990). Similarly, in the eye of the D. melanogaster the induction of the R7 photoreceptor requires the activity of the Sevenless receptor tyrosine kinase and the Drosophila Ras1 homologue (reviewed in Yamamoto, 1994). Both fission yeast and budding yeast also contain Ras homologues, although these organisms lack receptor tyrosine kinases and the signalling pathways upstream and downstream of Ras are quite different to those in multicellular organisms (Tokiwa et al., 1994). However, yeast GAPs and GEFs have catalytic domains that are highly homologous to those found in higher eukaryotes (Figure 1.14).

In contrast to the mitogenic effects observed in fibroblasts, activated Ras induces growth arrest and differentiation in other cell types. For example, in the rat pheochromocytoma-derived PC12 cell line which differentiates into neurones upon stimulation with NGF, activated Ras mimics the action of NGF and induces growth arrest and the extension of neurite-like processes (Bar Sagi and Feramisco, 1985), whereas the inhibition of cellular Ras inhibits NGF-stimulated neurite extension.
(Szeberenyi et al., 1990). The way in which Ras induces opposing effects in different cell types is not fully understood, although the initial state of the cell and differences in the duration of downstream signalling events are possible explanations (Szeberenyi et al., 1990). The availability of groups of effector proteins in different cell types would also the Ras pathway.

The immunoprecipitation of cellular Ras from 32P-labelled cells, followed by the resolution of Ras-bound guanine nucleotides using thin-layer chromatography, has been widely employed to demonstrate that numerous extracellular signals stimulate RasGTP accumulation. In addition to ligands known to bind to RTK’s or to activate non-receptor tyrosine kinases, GPLR agonists and direct activators of PKC also activate Ras in certain cell types, although in most cases Ras activation is sensitive to tyrosine kinase inhibitors. (Downward, 1990; Szeberenyi et al., 1990; van Corven et al., 1993). Ligation of integrins, which are receptors for components of the extracellular matrix has also been reported to activate Ras, possibly via the activation of the focal adhesion-associated tyrosine kinase, p125FAK (Schlaepfer et al., 1994).

In principle, the total amount of Ras-GTP in a cell may be raised either by stimulation of guanine nucleotide exchange by a GEF or inhibition of a GAP molecule (Gaul et al., 1992; Rogge et al., 1992). Two examples where the stimulation of Ras has been linked to a decrease in GAP activity are the phorbol ester or T-cell receptor agonist stimulation of T cells (Downward, 1990; Downward et al., 1990) and erythropoietin stimulation of human erythroleukaemic cells (Torti et al., 1992). In other cell types, an increase in nucleotide exchange activity has been associated with the activation of Ras, for example in PC12 cells stimulated with NGF (Li et al., 1992), and rat-1 fibroblasts overexpressing wild type Ras stimulated with EGF (Buday and Downward, 1993; Medema and Bos, 1993) or insulin (Medema and Bos, 1993).

### 1.3.4.1.3. Ras Effectors

Many proteins, including protein kinases, lipid kinases and GEFs have been shown to interact with the active GTP-bound form of Ras in order to transmit signals for proliferation, differentiation and oncogenesis. Transformation by Ras oncoproteins requires the activation of multiple signal transduction pathways, including those which transmit signals via members of the Rho subfamily of GTPases (section 1.3.4.2).

The best characterised effector of Ras is the serine/threonine kinase c-Raf-1. c-Raf-1 consists of an amino-terminal regulatory region and a carboxy-terminal kinase domain. The amino-terminal regions of c-Raf-1 interact with Ras (Chuang et al., 1994). The Ras binding domain (RBD) encompassing residues 51-131 binds with high affinity
to Ras (Herrmann et al., 1996) and with somewhat lower affinity to Rap1A, a Ras-like protein. The structure of Raf-RBD alone has been solved using NMR spectroscopy (Emerson et al., 1995), and the complex of Rap1A with a GTP analogue has been solved using x-ray crystallography (Nassar et al., 1995). The latter structure shows the main interactions between Rap and Raf are mediated by an interprotein β-sheet, which is formed by two anti-parallel strands from the two proteins. The Raf-RBD binds to residues within the core effector region of Rap1A which encompasses amino acids 32-40 of Rap1A. The cysteine-rich domain of c-Raf-1, residues 139-184 has been proposed as a second Ras-binding site (Ghosh and Bell, 1994). Its structure determination has shown it to have a double Zn^2+ binding motif (Mott et al., 1996). The structure is slightly different from that of two related cysteine-rich domains from PKC which correlates with its inability to bind diacylglycerol and phorbol esters. These amino terminal regions are missing in the v-Raf oncoprotein, which leads to the constitutive activation of the v-Raf kinase domain (Daum et al., 1994).

Biochemical and genetic studies initially identified Raf as a kinase involved in Ras-dependent signalling in mammalian cells, C.Elegans and D.melanogaster (reviewed in Dickson and Hafen, 1994). Subsequently, Raf has been shown to be a direct target of Ras by a number of methods (reviewed in Marshall, 1995). Activation of Raf appears to be critical for transactivation and transformation by Ras (Bruder et al., 1992). Raf-1 is normally a cytosolic protein but in Ras-transformed cells, Raf-1, is translocated to the plasma membrane and is concentrated in membrane ruffles where it is co-localised with Ras (Traverse et al., 1993). In NIH-3T3 cells, the essential role of c-Raf 1 in the transduction of proliferative signals initiated by receptor PTKs and transmitted through c-Ras, is demonstrated by the ability of dominant negative c-Raf-1 mutants (Bruder et al., 1992; Kolch et al., 1991) to block DNA synthesis and cell proliferation stimulated by serum, the phorbol ester TPA or by Ras oncogene proteins. Further evidence that Raf-1 is a downstream effector of Ras is the ability of v-Raf expression to overcome the growth arrest produced by the microinjection of inhibitory anti-Ras antibodies (Huleihel et al., 1986; Smith et al., 1986). GTP-bound Ras binds cytoplasmic Raf-1 and causes it to be translocated to the plasma membrane (Leevers et al., 1994) where it becomes activated by a mechanism that is still poorly understood. Activation of Raf-1 initiates the MAPK cascade (described in section 1.3.3.2.2).

Numerous other proteins have been found that bind to Ras in both a GTP-dependent and an effector residue-mediated manner and therefore qualify as potential effectors of Ras (Wittinghofer and Herrmann, 1995). These additional proteins include the p110 subunit of PI3K (section 1.4.7), and RalGEF, the guanine nucleotide-exchange factor.
for Ral (Kikuchi et al., 1994). Other putative effectors include PKCζ (Diaz Meco et al., 1994), rin (for Ras-interacting) and Rsb (for Ras-binding) (Marshall, 1996).

**1.3.4.2 Rho family GTPases**

The Rho GTPases form a subgroup of the Ras superfamily and have been shown to regulate a wide spectrum of cellular functions. These proteins are ubiquitously expressed across the species, from yeast to man (Table 1.2). The mammalian Rho-like GTPases comprise at least 10 distinct proteins: RhoA, B, C, D, E and G; Rac1 and 2; Cdc42Hs and TC10. A comparison of the amino acid sequences of the Rho proteins from various species has revealed that they are conserved in primary structure and are 70% or more homologous to each other. As with Ras, Rho family members cycle between an inactive GDP-bound state and an active GTP-bound state and this cycle is regulated by Rho-specific GAPs and GEFs (Figure 1.15) reviewed in (Van Aelst and D'Souza Schorey, 1997). In addition, nucleotide dissociation is regulated by guanine nucleotide dissociation inhibitors (GDIs). RhoGDI binds equally well to both the GDP-and GTP-bound forms of Cdc42, inhibiting both GDP dissociation and GTP hydrolysis. It inhibits the interaction of the Rac, Rho and Cdc42 proteins with GAP and stimulates the release of these GTPases from cell membranes, perhaps allowing them to translocate to another cell compartment (reviewed in Ridley, 1995).

Rho GTPases were originally thought to be primarily involved in the organisation of the actin cytoskeleton, but it has become apparent that they regulate a wide array of cellular responses including endocytosis, secretion, transcriptional regulation and growth control (Ridley, 1996; Symons, 1996; Tapon and Hall, 1997).

**1.3.4.2.1 Rho family GTPase function**

The actin cytoskeleton of animal cells maintains cellular shape and plays a pivotal role in cell motility, cytokinesis, phagocytosis, and intracellular transport processes. Actin stress fibers are long bundles of filaments that traverse the cell and are linked to the extracellular matrix through integrins and focal adhesion complexes, while a highly compact meshwork of actin filaments can be found at the leading edge of motile cells in lamellipodia and ruffles (Burridge et al., 1988; Small, 1981). In addition, short bundles of actin filaments are often found protruding from the cell surface, particularly in motile cells and at the ends of growth cones in neurones, to produce microspikes and filopodia (Bentley and O'Connor, 1994). At mitosis these structures disappear, and a contractile ring made of actin filaments is assembled at the cleavage furrow during cytokinesis (Cao and Wang, 1990).
It is now generally accepted that three members of the Rho family, namely Rho, Rac and Cdc42 are involved in regulating the organisation of the actin cytoskeleton. In growth factor stimulated fibroblasts, it has been shown that Rho regulates the formation of actin stress fibres, whereas Rac regulates lamellipodium formation and membrane ruffling, and Cdc42 regulates filopodium formation (reviewed in Hall, 1994; Machesky and Hall, 1996). Microinjection studies using Swiss 3T3 fibroblasts showed that Ras-induced lamellipodium and stress fibre formation depend on Rac and Rho respectively, indicating that they act downstream of Ras (Ridley and Hall, 1992; Ridley et al., 1992). Regulation of the cytoskeleton is thought to be controlled by direct coupling of these multiple GTPase switches in a cascade. In Swiss 3T3 fibroblasts, Cdc42 activates Rac, which in turn activates Rho (Nobes and Hall, 1995). The molecular links between these GTPases in mammalian cells are unknown. A similar cascade is believed to control bud formation and morphogenesis in the yeast *S. cerevisiae* and *Schizosaccharomyces pombe.* In the latter organisms, a role for GEFs in linking these GTPases has been demonstrated (section 1.3.4.3); however no such connections have yet emerged in animal cells. A number of proteins that bind Rho, Rac and Cdc42 in a GTP-dependent manner have been identified. It is beyond the scope of this chapter to discuss them all but their characterisation has provided major insights into the molecular mechanisms by which the Rho GTPases affect the cytoskeleton (Van Aelst and D'Souza Schorey, 1997).

Several lines of evidence have implicated the involvement of PI3K in PDGF- and insulin-induced cytoskeletal rearrangements. Treatments of fibroblasts with wortmannin, inhibits membrane ruffling induced by PDGF, EGF, and insulin, although not by microinjected Rac protein (Kotani et al., 1994; Nobes et al., 1995; Wennstrom et al., 1994). Furthermore, PDGF could stimulate the level of RacGTP by increasing GEF activity in a PI3K-dependent manner (Hawkins et al., 1995). Hence PI3K appears to function upstream of Rac for the induction of membrane ruffling in response to extracellular growth factors. Moreover, a constitutively active PI3K kinase mutant has been shown to trigger membrane ruffles and stress fibres in a Rac and Rho-dependent manner (Reif et al., 1996). However, this mutant failed to stimulate Rac/Rho signalling pathways that regulate transcription (Reif et al., 1996). A plausible explanation given for this observation is that the Rho GTPases are linked to different upstream regulatory proteins, which may determine the interaction with different GTPase effector pathways leading to the diverse biological activities. The mechanism by which PI3K activates Rac is unknown but may involve GEFs, GAPs, or GDIs. Recently, the putative PI3K homologue TOR2 has been shown to be required for organisation of the actin cytoskeleton in *S. cerevisiae* (Schmidt et al., 1997).
and activates the GTPases RHO1 and RHO2 via their exchange factor ROM2 (Schmidt et al., 1997).

As wortmannin did not inhibit the RasV12-induced membrane ruffling in Swiss fibroblast cells, it suggested that PI3K was not involved in Ras-mediated membrane ruffling (Nobes et al., 1995). In contrast, another study has shown that wortmannin partially blocked RasV12-induced membrane ruffling in PAE cells and that the inhibition was complete when the RasV12, C40 mutant was used (Rodriguez Viciana et al., 1997). RasV12, C40 is a Ras mutant that fails to bind the serine/threonine kinase Raf and RalGDS but can still bind PI3K (Joneson et al., 1996; Khosravi Far et al., 1996; Rodriguez Viciana et al., 1997; Van Aelst et al., 1994). Furthermore, the same study showed that a dominant-negative form of PI3K completely blocked RasV12-induced membrane ruffling. As mentioned before, it is possible that the pathways leading to the activation of PI3K may vary in different cell types.

Mechanism whereby Rho, Rac and Cdc42 induce actin re-organisation is unclear, but one possibility is that Rho has been shown to associate with a PtdIns P-5-kinase (Ren et al., 1996) and activate it (Chong et al., 1994). PtdIns(4,5)P₂, the lipid product of PtdIns P-5-kinase has been shown to bind and modulate the activities of a number of actin-binding proteins, thus the pathway via which Rho induces actin polymerisation may involve an increase in cellular PtdIns(4,5)P₂ concentration. A similar link has been demonstrated between Rac and PtdIns P-5-kinase reviewed in (Zigmond, 1996).

A number of studies have implicated the members of the Rho family in various membrane-trafficking processes including phagocytosis, endocytosis and the regulation of secretory vesicle transport (reviewed in Van Aelst and D'Souza Schorey, 1997). It is possible that the effects of Rho GTPases on phospholipid metabolism may provide a point of intersection between the co-ordinated control of membrane trafficking and cytoskeletal organisation.

1.3.4.2.1 Rho family members and growth control
A number of oncogenes encode exchange factors for Rho family GTPases, suggesting that Rho GTPases may have a role in cell growth control themselves (Ridley, 1995). Rac and Rho can transform some cell types and they have been shown to be essential for cell transformation by Ras (reviewed in Symons, 1995). Rac and Rho therefore appear to act downstream of Ras (Ridley and Hall, 1992; Ridley et al., 1992). A dominant-negative mutant of Rac inhibits transformation induced by oncogenic Ras, but not by a constitutively active form of Raf suggesting that Ras dependent MAPK and Rac/Rho pathways bifurcate at the level of Ras. Constitutively active Raf and
constitutively active Rac or Rho are synergistic in their ability to transform cells (Khosravi Far et al., 1996; Qiu et al., 1995) and the finding that ERK activation and membrane ruffling are mediated by distinct Ras effectors suggests that both these activities are required for transformation (Joneson et al., 1996).

The signalling pathways via which Rac and Rho influence cell proliferation remain to be identified. The p21-associated kinase (PAK) family of serine/threonine kinases may be downstream effectors of Rac, as they bind to and are activated by GTP-bound Rac or CDC42. (Brown et al., 1996). Other signal transduction proteins linked with Rac/Cdc42, and PAKs activation are the 'stress-activated' protein kinases (SAPKs), which are also known as Jun N-terminal kinases (JNKs) and p38 MAP kinase (Kyriakis and Avruch, 1996). However, JNKs and p38 are most potently activated by various cellular stresses rather than by growth factors. In cells a transcription factor known as SRF binds to the serum response element (SRE) found in the promoters of a number of 'early' genes induced by growth factors (Hill et al., 1995). Over-expression of Rho, Rac or Cdc42 stimulated the activity of SRF, as well as other transcription factors such as Jun (Minden et al., 1995; Perona et al., 1997).

Rac and Cdc42 have also been reported to interact with and activate pp70S6K both in vitro and in vivo although the mechanism by which this occurs is unclear (Chou and Blenis, 1996). PI3K may link these molecules as it interacts with both Rac and Cdc42 (Chou and Blenis, 1996; Tolias et al., 1995; Zheng et al., 1994) (section 1.3.4.4) and is an upstream regulator of pp70S6K (Proud, 1996) (section 1.4.7.6.2).

Several Rho family GTPases also modulate apoptotic pathways. RhoA, RhoC, and Rac1, enhance apoptosis in murine fibroblasts and the human K562 erythroleukaemia cells after serum deprivation by a p53-independent mechanism (Jimenez et al., 1995). Studies have shown apoptosis by Rho proteins is related to the generation of ceramides (Esteve et al., 1995) but can be blocked by Bcl2 expression both in vitro and in vivo (Esteve, submitted, 1997). Thus Ras and Rho pathways seem to be involved in signalling pathways culminating in both proliferation and apoptosis.

1.3.4.3 GEFs for the Rho family GTPases: The DH domain

GEFs for Rho-like GTPases belong to a rapidly growing family of proteins that share a common motif, designated the Dbl-homology (DH) domain for which the Dbl oncogene product is the prototype (Cerione and Zheng, 1996). Dbl was first identified as an oncogene which catalysed nucleotide exchange on Cdc42 (Eva and Aaronson, 1985) and is homologous to a yeast protein that fulfils the same function for the yeast homologue of Cdc42 (Ron et al., 1991). Deletion analysis of the Dbl protein
demonstrated that the DH domain was essential and sufficient for this activity and that this domain was also necessary to induce oncogenicity (Hart et al., 1991; Hart et al., 1994; Ron et al., 1991). In addition to the DH domain, Dbl and the yeast Cdc24 share a PH domain which is essential for the cellular localisation of the Dbl proteins.

Several oncogene products and growth regulatory molecules have now been recognised as having a DH domain in tandem with a PH domain. The signalling cascade coupling Dbl-like GEFs to the upstream components remains elusive. The specific biological functions of many of the Dbl family members have yet to be determined. Among the currently known members of the Dbl-like GEF family, Cdc24, Tiam-1 and Lbc are the only proteins that have been shown to act as specific regulators for Rho family GTP binding proteins, namely for Cdc42, Rac, and Rho, respectively (Cerione and Zheng, 1996). Both Dbl and Vav have been shown to trigger the formation of filopodia, lamellipodia, and stress fibers and stimulate the SAPK/JNK signal transduction pathway (Olson, 1996). In addition to the PH and DH domains, many of the exchange factors have other domains such as SH3 and SH2 domains, suggesting they may bind other proteins and have additional functions (Cerione and Zheng, 1996). Indeed it has recently been shown that tyrosine phosphorylation of Vav by Lck activates members of the Rho family both in vitro and in vivo. (Crespo et al., 1997; Han et al., 1997).

1.3.4.4 GAPS for the Rho/Rac-related GTPases

1.3.4.4.1 BCR protein

BCR is a protein that was first identified as part of the BCR-ABL fusion oncogene, which is generated by the translocation of sequences encoding the ABL tyrosine kinase on chromosome 9 to BCR sequences on chromosome 22. It is present in human leukaemias positive for the Philadelphia chromosome (Heisterkamp et al., 1985). This translocation results in the expression of two chimaeric oncogenic proteins, p210BCR-ABL and p185BCR-ABL, which are strongly implicated in the etiology of chronic myelogenous leukaemia (CML) and acute lymphocytic leukaemia (ALL), respectively.

The non oncogenic form of BCR is a 143 kDa cytosolic phosphoprotein. It contains and amino terminal serine/threonine protein kinase (Maru and Witte, 1991), and a carboxy terminal GAP domain that stimulates the GTPase activity of Rac and cdc42 but not of Rho (Diekmann et al., 1991). The central region of this protein has some homology with a GEF called DbL. Interestingly, both the chimaeric oncogenes, p210BCR-ABL and p185BCR-ABL, lack this GAP domain, and p185BCR-ABL also lacks the DbL-related domain. Both oncogenic proteins localise to the cytoplasm and possess deregulated Abl tyrosine kinase activity (Konopka et al., 1984).
Approximately 70% of CML patients express the reciprocal chimaeric transcript ABL-BCR but the biological significance of this observation has yet to be determined (Melo et al., 1993) It could be suggested that deregulation of GAP activity may have an important biological role in leukaemic cells.

1.3.4.4.2 BH domain containing proteins

A family of proteins with homology to the carboxy terminus of the BCR protein has now emerged and comprises GAPs that hydrolyse GTP bound to the Rho subfamily of small G proteins. To date, these include seventeen mammalian genes, the *rotund* locus in *Drosophila melanogaster*, the *BEM2* and *BEM3* genes from *Saccharomyces cerevisiae*, and a *Caenorhabditis elegans* gene called *CeGap*. Table 1.3 summarises the substrate specificity for this family of GAPs. Each of these proteins contains a single domain that has between 20-40% amino acid identity to the GAP domain of BCR. This putative domain has become known as the BCR homology (BH) domain. The remainder of these BH domain containing proteins are otherwise unrelated and multifunctional (Figure 1.16) The BH domain may occupy any position with the linear sequence of these proteins, suggesting that it acts as a functionally independent module.

An amino acid sequence alignment of a subset of BH domains was used to evaluate the degree of conservation within this domain. Although the overall sequence identity of BH domains from various proteins is weak (20-40%), the alignment reveals 3 blocks of conserved amino acid residues (Figure 1.17 and Figure 1.18).

*N*-chimaerin (α1-chimaerin) is expressed specifically in brain neurons (Hall et al., 1990; Lim et al., 1992)). The carboxy-terminal GAP domain of N-chimaerin stimulates the GTPase activity of Rac1 in vitro (Diekmann et al., 1991) as well as of Cdc42Hs to a lesser extent, but not that of Rho A (Ahmed et al., 1994; Manser et al., 1992)). Mutational analysis of this BH domain gave the first indication that amino acid residues responsible for binding to Rac were different from those that conferred Rac-GAP activity (Ahmed et al., 1994). In this study, N-chimaerin proteins containing a variety of point mutations within each of the three blocks of conservation of the BH domain were expressed as GST fusion proteins and tested for GAP activity on Rac1. and their ability to bind Rac-1. Of the seven deletion mutants, four possessed GAP activity, and three ΔEIE, ΔYRV, and ΔLKLY were inactive yet able to bind Rac1. This suggested that BH domain containing proteins may have the ability to bind the Rho subfamily proteins but not necessarily have concomitant GAP activity. A similar study was used to delineate the limits of a functional GAP domain of the *S.cerevisiae* proteins BEM2 and BEM3, which act on the yeast homologue of Cdc42 (Zheng et al., 1993).
suggested that no single one of the three conserved regions of the BH domain was sufficient either to bind to GTP-bound Cdc42 species or to catalyse its GTPase activity, but that all three homology regions combine to confer a specific tertiary conformation on the BH domain that forms the proper binding and catalytic interfaces.

1.3.4.4.3 Functions of BH domain containing proteins

Microinjection experiments of isolated BH domains from BCR, p50RhoGAP and p190, into Swiss 3T3 cells interfere with Rho and/or Rac signalling pathways (Ridley et al., 1993). The BCR BH domain has been shown to have a greater affinity and/or avidity for Rac
vitro, and when microinjected specifically blocked Rac-induced membrane ruffling but not the Rho-induced formation of stress fibres. p50RhoGAP is a ubiquitously expressed protein which has been reported to have GAP activity for the Rho subfamily of GTPases (Diekmann et al., 1991). Indeed, p50RhoGAP acts as a GAP on all known members of Rho-related GTPases in vitro, however when it is microinjected, it inhibits the activation of Rho biological responses but not those of the Rac. p190 was first identified as a tyrosine-phosphorylated, p120-rasGAP-associated protein in cells stimulated by certain growth factors or transformed by tyrosine kinases (Ellis et al., 1990). p190 has GAP activity for Rho, Rac and cdc42Hs in vitro. This strongly suggests that the interaction of p120rasGAP with p190 could serve as a regulatory link between the Rho and Ras signalling pathways (Settleman et al., 1992). Despite having GAP activity for Rho, Rac and Cdc42 in vitro, microinjected p190 inhibits the Rho mediated formation of stress fibres but is unable to inhibit Rac mediated formation of membrane ruffles. 3BP-1 was isolated as an Abl-SH3 binding protein in the mouse (Cicchetti et al., 1992). In vitro, 3BP-1 showed GAP activity towards both Rac and Cdc42Hs, however microinjection of the isolated BH domain of 3BP-1 in to serum-starve Swiss 3T3 fibroblasts inhibits only Rac-mediated PDGF-induced membrane ruffling (Cicchetti et al., 1995).

In addition to GAP activity towards Rho subfamily GTPases, BH domains may be involved but not directly in membrane ruffling. The BH domain from n-chimaerin and full length n-chimaerin were microinjected into Swiss 3T3 fibroblasts and NIE-115 neuroblastoma cells (Kozma et al., 1996). The n-chimaerin BH domain was found to act as a specific downregulator of the Rac1 pathway, and also eliminated growth factor-mediated membrane ruffling and lamellipodium formation. In contrast, microinjection of full length n-chimaerin induced the formation of lamellipodia, filopodia and pinocytosis which implies it is involved in the activation of both the Rac1 and Cdc42Hs pathways.

With the exception of the chimaerins, all these BH domain containing proteins appear to be ubiquitously expressed. Clearly, they must be highly regulated in vivo so that the
Rho-like GTPases are not always GDP bound and therefore down regulated. The multidomain and nature of BH domain-containing proteins suggests other domains with the protein are involved in regulation of GAP activity or recruitment to its site of action at the plasma membrane. For example, the chimaerins contain cysteine-rich sequences similar to those found in the regulatory domain of PKC (Ahmed et al., 1990). It has been reported that phosphatidylserine (PS) and phosphatidic acid (PC) activate Rac-GAP activity of n-chimaerin, and that phorbol esters can synergise with these phospholipids. In contrast, LPA, phosphatidylinositol lipids (PtdIns, PtdIns(4)P, PtdIns(4,5)P2) and arachidonic acid (aa) inhibit the GAP activity of n-chimaerin (Ahmed et al., 1993). The modulation of the GAP activity of n-chimaerin by phospholipid/phorbol esters requires the PKC-like cysteine-rich domain, but whether this regulation is physiologically significant is unclear.

1.3.4.3 Three-dimensional structure of members of the Rho family and their GAPs

The structure of human Rac1, has been solved in its active conformation using X-ray crystallography (Hirshberg et al., 1997) Rac1, with an overall sequence identity of 30% to Ras, has a 13-residue insertion between β5 and α4 (residues 123-135) which forms an additional α helix. The switch II region, which is usually one of the most flexible elements in GTP-binding proteins differs between Ras and Rac1 in that the latter has two short 310 helices after the DTAGQ γ-phosphate binding motif. The switch II region is different in the structures of two other members of the Rho family, Cdc42GDP (Feltham et al., 1997; Hirshberg et al., 1997) and RhoA GDP (Wei et al., 1997). In Cdc42, no evidence has been found for an α helix in the switch II region, whereas RhoA has a regular α helix.

The structures of two members of the RhoGAP family, the BH domain of the p85α regulatory subunit and from p50RhoGAP (Barrett et al., 1997; Feltham et al., 1997; Hirshberg et al., 1997; Musacchio et al., 1996) have also been determined recently. The crystal structure of the GAP domain from human p50RhoGAP is an unusual arrangement of nine α-helices, the core of which includes a four helix bundle (Figure 1.19). Residues conserved across the RhoGAP family are largely confined to one face of this bundle, and is the proposed interaction site for Rho proteins (Rittinger et al., 1997). The structure of the p85αBH domain is very similar (discussed in Chapter 4).

The three dimensional structure of the complex between Cdc42 in the GTP-bound state and the catalytic domain of p50RhoGAP has also been solved recently (Rittinger et al., 1997). As in the Ras.RasGAP complex, the switch regions of Cdc42 are mostly
responsible for the interaction. As the Cdc42-RhoGAP complex represents the ground state of the GTPase reaction, it was interesting to find that the arginine which is also believed to be involved in the GTPase reaction in RhoGAP, is not contacting the γ-phosphate, which implies that it may do so only in the transition state of the reaction, as has been found for Gia1 (Tesmer et al., 1997).

These studies have revealed that Ras related GTPases are versatile signalling molecules that regulate a diverse set of cellular functions and are capable of interacting with a large number of proteins. The signal transduction pathways mediating these cellular functions appear to be complex and interwoven. Very recently, a number of structures have been solved that allow us to take a first look at the regulation of these proteins and gain more insight into the function of these molecular switches, and their modes of regulation.

1.4 Lipid metabolism

1.4.1 Introduction
The plasma membrane of most eukaryotic cells contains a variety of phospholipids which include phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE) and sphingomyelin. Together these four phospholipids constitute more than half the mass of lipid in most membranes. With headgroups that differ in size, charge and shape, these phospholipids provide the correct environment for membrane proteins to function optimally. Another class of phospholipid known as phosphoinositides (PI) are functionally important but are only found as a minor component of many subcellular membranes, however they have a crucial role in cell signalling.

It was first demonstrated in 1953 that stimulation of cholinergic receptors in pancreas slices leads to turnover of the phosphate groups in PtdIns (Hokin and Hokin, 1953). This effect was later attributed to a net breakdown and subsequent resynthesis of PtdIns which forms the basis of a signal transduction system, resulting in mobilisation of Ca$^{2+}$ from intracellular storage sites (Berridge and Irvine, 1984). The occupancy by agonists of many different cell surface receptors stimulates the activity of phospholipase C and the rapid breakdown of plasma membrane phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P$_2$) leading to the generation of two products, diacylglycerol and inositol 1,4,5-trisphosphate (IP$_3$) (Berridge, 1984). These molecules then act as signalling messengers in a bifurcating pathway (Berridge and Irvine, 1989). IP$_3$ mobilises Ca$^{2+}$ from intracellular stores to elevate cytoplasmic Ca$^{2+}$ concentration, and diacylglycerol activates protein kinase C (PKC), which in turn phosphorylates many cellular protein.
substrates and is known to regulate a multitude of cellular functions (Takai et al., 1979). Alternatively, IP₃ can be metabolised to generate a vast number of inositol polyphosphates most of which have no known biological functions. DAG can be phosphorylated to form phosphatidic acid (PA), thereby terminating signalling through PKC. Inositol is produced by the action of various phosphoinositol phosphatases, and this is used together with phosphatidic acid to regenerate PtdIns in the endoplasmic reticulum. This pathway may be considered to form a cycle (Figure 1.13) within which key intermediates are exploited for their biological signalling ability. There is some evidence that the cycle defines a closed pool or compartment of metabolites, termed the agonist-sensitive pool, which is somehow distinguished in cells from the agonist-insensitive compartment. However, the physical nature of these compartments is not understood and in other studies their existence was not apparent (reviewed Monaco and Gershengorn, 1992).

A large number of enzymes are involved in the biosynthesis and breakdown of PI and the lipid products generated have been implicated in many cellular processes. However, these will be discussed briefly and this review will focus on the structure and function of the phosphoinositide 3-kinase (PI3K) family. Studies with this enzyme led to the discovery of a set of lipids phosphorylated at the D-3 position of the inositol ring. In the 3-phosphoinositide pathway PtdIns, PtdIns(4)P and PtdIns(4,5)P₂ are phosphorylated at the D-3 position of the inositol ring, resulting in the production of PtdIns(3)P, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ respectively. (Figure 1.20).

1.4.2 Phospholipase C Family

Phosphoinositide-specific phospholipase C (PLC) isoforms hydrolyse PtdIns, PtdIns(4)P or PtdIns(4,5)P₂ in vitro to produce DAG and inositol 1-phosphate, inositol 1,4-bisphosphate or IP₃, respectively (Lee and Rhee, 1995). The hydrolysis of PtdIns(4)P and PtdIns(4,5)P₂ is optimal when micromolar Ca²⁺ concentrations are present, while higher Ca²⁺ concentrations are required for PtdIns hydrolysis (Ryu et al., 1987). Phosphoinositide 3-phosphates are refractory to all PLC activity. There are three known classes of PLC isoforms, termed β, γ and δ (Murthy and Pande, 1993). All PLC isoforms are single polypeptides containing a catalytic domain composed of two separate regions, designated X and Y, which are both required for hydrolytic activity (Lee and Rhee, 1995). Sequence comparisons suggests that a PH domain exists at the amino-terminus of all three classes of PLC isoforms, and that a putative split PH domain lies between the X and Y domains of PLCγ. In addition, each isoform contains a putative Ca²⁺ and phospholipid binding region known as a C2 domain (Ponting and Parker, 1996) (section 1.4.7.4). Within each class of isoforms, there are
several family members allowing for very subtle regulation. The roles of all the isoforms are not yet fully understood.

For example, PLCβ activity is regulated by a wide variety of GPLRs, but different isoforms are differentially stimulated by Gαi/o βγ subunits, which they may bind via their amino terminal PH domain. The two known PLCγ isoforms, types 1 and 2, are differentially expressed in a wide range of cell types. In addition to two putative PH domains these enzymes also possess an SH3 and two SH2 domains. PLCγ1 is found predominantly in the cytosol of quiescent cells, but is translocated to the plasma membrane by receptor binding after receptor activation (Margolis et al., 1989; Margolis et al., 1990; Todderud et al., 1990). PLCγ1 binds to a number of activated receptors (Rotin et al., 1992; Vega et al., 1992) and becomes phosphorylated following stimulation (Kim et al., 1991; Meisenhelder et al., 1989), however, it has been shown that tyrosine phosphorylation is necessary but not sufficient for the activation of PLCγ in vivo (Li et al., 1991; Vega et al., 1992). This is consistent with a model in which, following association with and phosphorylation by the EGFR, PLCγ is released from the receptor and associates with membrane or cytoskeletal elements via its SH3 domain (Bar Sagi et al., 1993; Lee and Rhee, 1995). The PH domain of PLCγ1 is both necessary and sufficient for binding of the enzyme to the plasma membrane (Paterson et al., 1995) It is connected to the rest of the enzyme by a flexible linker (Essen et al., 1996), and tethers intact PLCγ1 to membranes containing its substrate PtdIns(4,5)P2, thus allowing processive PtdIns(4,5)P2 hydrolysis (Cifuentes et al., 1994; Lemmon et al., 1996). By competing with PtdIns(4,5)P2 for binding to the PH domain, the PLCγ1 product IP3 removes this tether and reduces catalytic efficiency (Cifuentes et al., 1994; Kanematsu et al., 1992).

The biological importance of PtdIns(4,5)P2 has been emphasised by the realisation that it is the preferred in vivo substrate of PI3Ks, and that it possesses a signalling ability of its own. It has been reported recently that activation of PI3K via receptor stimulation or a non-receptor tyrosine kinase causes PLCγPH domain-mediated membrane targeting and PLCγ1 activation (Falasca et al., 1998). These experiments provide the first evidence for the mutual regulation of activity between two enzymes that participate in the control of phosphoinositide metabolism. PtdIns(4,5)P2 has been shown to regulate the function of several actin-binding proteins including gelsolin and profilin (Goldschmidt Clermont et al., 1990) but virtually all of these observations have been made using in vitro experiments with micellar PtdIns(4,5)P2. Nevertheless, it has been proposed that PtdIns(4,5)P2 may be an intermediate in the regulation of actin polymerisation by extracellular stimuli, possibly by binding and releasing gelsolin and other actin-capping proteins from actin filament ends reviewed in (Zigmond, 1996)
1.4.3 Protein kinase C

A large number of isoforms of PKC have been described and are divided into three groups: conventional PKCs (cPKCs α, β1, β2 and γ), novel PKCs (nPKCs δ, ε, σ and υ) and atypical PKCs (aPKCs ζ and λ) (Nishizuka, 1992). All PKC's require phospholipids for activity, and are the best defined target for DAG produced by PLC's. DAG, or its analogue phorbol esters, activates cPKCs and most nPKC isoforms but not aPKCs. Calcium is required as a cofactor which increases the affinities of cPKCs for acidic phospholipids. The novel PKCs are activated by DAG in a Ca^{2+} independent manner. Elucidating the precise functions of the protein kinase Cs has been confounded by their broad substrate specificity \textit{in vitro}, the abundant effects of phorbol esters \textit{in vivo}, and the existence of multiple isoforms within single cell types.

PKC's are made up of two regions, the regulatory and the catalytic domains, which are joined by a hinge region. The hinge region, mediates the lipid and protein interactions that regulate PKC localisation and contains a nuclear localisation sequence that is masked by the regulatory domain when PKCa is inactive. The regulatory domain contains several PS-binding sites including the pseudosubstrate autoinhibitory sequence, a cysteine rich region, a C1 domain which is involved in phorbol ester binding and α C2 domain which is involved in calcium binding (Jaken, 1996) (section 1.4.7.4). The novel PKCs also have a C2 like domain, but this is unable to bind Ca^{2+}. The amino acid sequence of the catalytic domain of PKC is highly homologous to that of PKA (Hanks and Hunter, 1995). Modelling of the kinase core of PKC suggests that it is maintained in an inactive state by interaction with the pseudosubstrate domain.(Orr and Newton, 1994).

Most of the PKC in unstimulated cells is phosphorylated suggesting that phosphorylation is involved in the processing of the enzyme, rather than in modulating the function of the enzyme in response to specific signals (Newton, 1997). The activation loop in the kinase core is the first site to become phosphorylated when PKC is activated. From modelling studies, this activation loop appears to be buried when the pseudosubstrate region occupies the active site, suggesting that the pseudosubstrate region must be removed from the active site before phosphorylation can occur (Keranen, 1995). Following phosphorylation at the activation loop, PKC becomes phosphorylated at Thr 641 (for PKC βII) at the carboxy-terminus of the active site which appears to lock PKC in a catalytically active conformation, perhaps by anchoring the carboxy-terminus away from the active site. Phosphorylation of Thr 641 regulates enzyme activity (Cazaubon et al., 1994; Zhang et al., 1994). The third phosphorylation occurs at Ser 660 and is an autophosphorylation reaction (Keranen, 1995) which
correlates with release of PKC into the cytosol, suggesting that its role is to direct the 
subcellular location of PKC.

Immunocytochemical analysis has clearly established that different PKC isozymes are 
localised to different subcellular compartments and a number of proteins have been 
identified that bind PKC (reviewed in Jaken, 1996) More recently, it has been 
demonstrated that PKC is a downstream target of PI3K (section 1.4.7.6.3).

1.4.4 Phosphoinositide kinases
A comparison of the amino acid sequences of the PI kinases shows there are regions of 
homology which have functional significance either as conserved motifs in the PI 
kinase family, in protein kinases or in other proteins (Zvelebil et al., 1996) Homology 
region 1 encompasses the kinase domain. Homology region 2, previously referred to 
as the PIK domain, is found in all PI3Ks and PtdIns4Ks. Homology region 3 appears 
to be specific to PI3Ks while homology region 4 is restricted to the PI3Ks of subgroup 
I (section 1.4.7.6). Sequence alignment of the putative kinase domain showed that 
PtdIns4Ks fall into two subgroups. The first includes the Saccharomyces cerevisiae 
enzyme, which is encoded by the gene STT4, (Yoshida et al., 1994) and a human PI4-
kinase (Wong and Cantley, 1994). The second subgroup of PtdIns4Ks includes the 
Saccharomyces cerevisiae PIK1 125 kDa gene product (Flanagan et al., 1993; Garcia 
Bustos et al., 1994; Wong and Cantley, 1994) and three putative PtdIns4K from 
Dictyostelium discoideum whose enzymatic function has yet to be confirmed (Zhou and 
Cantley, 1995) The alignment of the putative catalytic domains of PI3K, and PtdIns4K 
show considerable sequence variation carboxy terminal of the conserved DFG pattern 
that forms part of the substrate binding site. This variation maybe the result of 
differences in the substrate specificity of distinct PI kinases.

Several groups have reported the purification of Type II and Type III PtdIns4Ks from 
various tissues and cell types to apparent homogeneity (Hsuan and Tan, 1997). 
PtdIns4K activity in mammalian cells can be detected in the Golgi, lysosomal, 
mitochondrial, vesicular, nuclear and membrane compartments. The membrane-bound, 
55 kDa, type II PtdIns4K and a PtdIns5K activity have been found in EGFR 
immunoprecipitates of the EGFR from EGF-stimulated A431 cells (Cochet et al., 1991; 
Kauffmann Zeh et al., 1994). The site of interaction of PtdIns4K and an unknown 
PtdIns5K with the EGFR has been mapped to the receptor juxtamembrane region using 
a series of carboxy-terminally truncated receptor mutants and synthetic peptides (Cochet 
et al., 1991).
Little is known about the mechanisms by which PtdIns4Ks are regulated, although there is some evidence that GPLR activation maybe involved. A pool of PtdIns(4)P may exist that is sensitive to the Gsa subunit inhibitor cholera toxin and this toxin was able to specifically inhibit an EGF-dependent increase in PtdIns(4)P in A431 cells (Pike and Eakes, 1987) Furthermore, it has been shown that a synthetic peptide corresponding to the reported interaction site for PtdIns4K on the EGFR can differentially regulate G-proteins in vitro (Stryjek Kaminska et al., 1996; Sun et al., 1995).

The association of type II PtdIns4K with the EGFR is likely to be dependent on receptor autophosphorylation, however, tyrosine phosphorylation of PtdIns4K itself inhibits its activity (Payrastre et al., 1990) The EGF receptor tyrosine kinase is probably not responsible for PtdIns 4-kinase phosphorylation since partially purified PtdIns4K cannot be phosphorylated by receptor in vitro (Kauffmann Zeh et al., 1994). However since the 55 kDa isoform of PtdIns4K has not been cloned, little is understood about the regulation of its activity.

Two isoforms of PtdInsP5K have been characterised from brain, erythrocytes and platelets and denoted type I and type II (Bazenet et al., 1990; Jenkins et al., 1994). A third PtdIns5K activity has also been partially purified from brain microsomes (Schmidt et al., 1993). These isoforms can be distinguished immunologically by differences in stimulation by phosphatidic acid, and differences in activity towards native membranes. PtdInsP5K activity has been shown to be stimulated by G-protein agonists in brain, neutrophils and HL60 cells (Cunningham et al., 1995; Smith and Chang, 1989; Stephens et al., 1993) and by the small G-protein Rho during thrombin or PDGF-stimulated phosphoinositide turnover in Swiss 3T3 cells (Chong et al., 1994) (section 1.3.4.2.1). Type I enzymes are distinguished by their ability to be activated by phosphatidic acid (PA). The PtdInsP5K isoforms have distinct subcellular distributions, which may be important for their functions. For example, type I PtdInsP5K activity was identified in one of three cytosolic fractions which enhanced ATP-dependent priming of Ca^{2+}-regulated noradrenaline secretion from rat neuroendocrine PC12 cells (Hay et al., 1995), whilst a purified type II enzyme did not exhibit similar activity.

1.4.5 Phosphatidylinositol transfer protein (PtdInsTP)

Exactly how activated cells control the supply of PI signalling precursors is still poorly understood and many questions remain to be answered. As most phospholipids are synthesised in the endoplasmic reticulum in mammalian cells and are not freely diffusable in the cytosol, specific transport mechanisms must exist to redistribute
phospholipids from their site of synthesis to other metabolic and structural compartments within cells. PtdInsTP belongs to a super family of lipid transfer proteins that are characterised by their ability to transport lipids between membranes in vitro. Members of the PtdInsTP family include the mammalian isoforms, PITPα and PITPβ, the rdgB protein from D.melanogaster, and sec14 protein from S.Cerevisiae (Trotter and Voelker, 1994; Wirtz, 1991). The rdg B gene is required for visual and olfactory signalling in Drosophila, both of which involve a PtdIns(4,5)P2-specific PLC (Vihtelic et al., 1993). Unlike most PITPs, rdg B is a membrane bound protein, however the significance of this is unclear.

The amino acid sequence of PtdInsTP is strongly conserved between species. Known PITP isoforms all possess a marked preference for PtdIns but are also able to transfer PC and, to a lesser extent, phosphatidylglycerol (Wirtz, 1991). A role for the cytosolic PITP was demonstrated by its ability to reconstitute PLC signalling in response to G-protein agonists in permeabilised HL60 cells (Cunningham et al., 1995; Thomas et al., 1993) and EGF signalling in permeabilised A431 cells (Kauffmann Zeh et al., 1995) Initial experiments with PITP (Thomas et al., 1993), suggested that its role is to replenish PtdIns in the plasma membrane by transporting it from its site of synthesis in the ER (Downes and Batty, 1993). However, recent evidence from receptor signalling and secretion studies suggest that PITP may play a more subtle role to play (reviewed in (Hsuan and Tan, 1997; Liscovitch and Cantley, 1995; Thomas et al., 1993). Briefly, permeabilised and cytosol-depleted HL60 cells are capable of producing an InsP3 response, albeit over an extended time course, presumably by using the PtdIns which is normally present in the plasma membranes of all cells, but addition of exogenous PITP to these cells greatly enhances the initial rate of IP3 production (Cunningham et al., 1995) These data suggest that PITP promotes the synthesis of PtdIns(4,5)P2 and its subsequent hydrolysis by PLC. Furthermore PITP was found to co-precipitate with the EGF receptor, type II PtdIns4K and PLCγ in an agonist-dependent manner. As a consequence of these and other data (reviewed by Hsuan and Tan, 1997), PITP, type II PtdIns4K, type II PtdIns5K and either PLCβ or PLCγ are likely to form a multienzyme signalling complexes during GPLR and receptor tyrosine kinase signalling. According to this model PITP acts as a carrier by retaining and sequentially presenting PtdIns, PtdIns(4)P and PtdIns(4,5)P2 to the enzymes of the signalling complex (Cunningham et al., 1995; Liscovitch and Cantley, 1995). A major challenge is to investigate whether agonist-dependent complexes of PITP with PtdIns(4)P and PtdIns(4,5)P2 exist in vivo.

1.4.6 Lipid Phosphatases

The inositol polyphosphate 5-phosphatases (5-phosphatases) are a growing family of signal-terminating enzymes that regulate the levels of different inositol phosphate and
phospholipid messengers essential for proper cell function (Drayer et al., 1996; Mitchell et al., 1996). This class of enzymes remove the phosphate in the 5' position of the inositol ring of polyphosphoinositides and polyninositolophosphates. Several isoforms of the 5-phosphatases have been characterised in mammalian cells and each display substrate specificity. Type-I 5-phosphatases are relatively small (43-65 kDa) and terminate the Ca\(^{2+}\) dependent signalling of Ins(1,4,5)P\(_3\) and Ins(1,3,4,5)P\(_4\) but have no activity towards membrane-bound polyphosphoinositides. The only Type-I 5'-phosphatase for which the cDNA has been isolated is a 43 kDa protein which can be cytosolic or membrane bound. Stable cell lines with an 80% reduction in the levels of expressed 43 kDa 5'-phosphatase have a transformed phenotype (Speed et al., 1996).

Certain human acute and chronic leukaemias are associated with a decrease in cellular 5'-phosphatase activity, and it has been suggested that the resulting increase in intracellular Ca\(^{2+}\) may play a role in the increased proliferation of leukaemic cells (Mengubas et al., 1996).

By contrast, the type II phosphatases have activity towards one or more phosphoinositides and in most cases inositol phosphates. Based on protein sequence conservation, they can be divided into distinctive subgroups. The first subgroup (known as GAP domain-containing inositol 5-phosphatases or GIPs) is characterised by a BCR-GAP domain. The importance of this class of activities is exemplified by the Lowe's syndrome protein OCRL (Attree et al., 1992), as mutations in this gene lead to the juvenile onset of tissue dysfunction and morbidity. In Lowe's syndrome, PtdIns(4,5)P\(_2\) 5-phosphatase activity is specifically decreased in this cellular compartment. It has been postulated that in affected individuals, abnormal PtdIns (4,5)P\(_2\) levels in the Golgi may lead to aberrant protein trafficking (Attree et al., 1992; Olivos Glander et al., 1995).

The SHIP-subgroup owes its name to a particular member, the SH2 domain-containing inositol 5-phosphatase, which contains an N-terminal SH2 domain, while the other member of this subgroup INPPL1, resembles the N-terminal (i.e. SH2)-deleted splice variants of SHIP that have been described (Kavanaugh et al., 1996). This class of phosphatases associates with the tyrosine-phosphorylated receptors of the haemopoietic family. SHIP has a carboxy-terminal proline-rich domain and consensus binding sites for PTB domains which allows it to associate with adaptor molecules such as Shc or Grb2 after stimulation of haemopoietic receptors (Damen et al., 1996; Lioubin et al., 1996), linking growth factor signalling to 5-phosphatase activity. Overexpression of SHIP has been shown to inhibit cytokine-induced cell growth (Lioubin et al., 1996). This provides strong evidence for recruitment-dependent control and suggest that either the substrates or products play key positive or negative role respectively.
The third subgroup are the Sac domain-containing inositol 5-phosphatases or SCIPs. They are characterised by the presence of an amino-terminal SAC1 domain, homologous to the amino-terminus of the *Saccharomyces cerevisiae* Sac1p protein (Kearns et al., 1997). Members of this family include synaptoganin, a neural synapse-specific 5-phosphatase. Synaptoganin is thought to play a role in phosphoinositide-mediated regulation of membrane trafficking in synapses. Synaptoganin co-localises with dynamin, a GTPase involved in neurite outgrowth, and is associated with endocytic vesicles (McPherson et al., 1996; McPherson et al., 1994). Whether the 5-phosphatase of synaptoganin activity is involved in synaptic vesicle recycling has yet to be determined.

Several other 5'-phosphatases have been described, but are yet to be fully characterised. A novel PtdIns(3,4,5)P₃ 5-phosphatase was identified in the cytosolic fraction of human platelets which binds to and co-purifies with the p85/p110 form of the PI3K (Jackson et al., 1995). This enzyme is specific for PtdIns(3,4,5)P₂ and does not remove the 5' phosphate from PtdIns(4,5)P₂, InsP₄ or InsP₃. Antibodies directed against the p85 subunit of the PI3K co-immunoprecipitated the PtdIns(3,4,5)P₃ 5-phosphatase activity. The physical association of the PI3K with a PtdIns(3,4,5)P₃ 5-phosphatase may be a mechanism for generation of the second messenger PtdIns(3,4)P₂. Alternatively, an association of this phosphatase with this kinase may be required to maintain the low basal levels of PtdIns(3,4,5)P₃ observed in unstimulated cells. Another major PtdIns(3,4,5)P₃ 5-phosphatase activity has been purified to near-homogeneity from the cytosolic fraction of rat brain (Woscholski et al., 1995). This 145 kDa Mg²⁺-dependent 5-phosphatase hydrolyses both PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃, but the latter is the preferred substrate. Finally, an 85kDa novel membrane-bound polyphosphoinositide phosphatase was partially purified from rat brain (Rath-Hope and Pike, 1995) which could hydrolyse the phosphate at the 3' 4' or 5' position of the inositol ring.

1.4.7 Phosphoinositide 3-kinase (PI3K)

1.4.7.1 Characterisation of the enzyme

Phosphoinositide 3-kinase (PI3K) was originally identified as an 85kDa phosphoprotein which was associated with a PI3K activity in immunoprecipitates of v-Src and middle T/c-Src complexes from transformed cells (Courtneidge and Heber, 1987; Whitman et al., 1985). This kinase phosphorylated the hydroxyl group at position 3 on the inositol ring to produce PtdIns3P, a lipid that had not been previously described (Whitman et al., 1988). A similar lipid kinase activity, which phosphorylated
the 3’position on PtdIns(4)P, PtdIns(4,5)P₂ and PtdIns, was subsequently found to be associated with the activated PDGF receptor tyrosine kinase (Auger et al., 1989).

PI3Ks convert PtdIns, PtdIns(4)P, and PtdIns(4,5)P₂ to PtdIns(3)P, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ respectively (Figure 1.21). In quiescent cells only PtdIns(3)P is present, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ are virtually absent (Auger et al., 1989). A rapid increase in the levels of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ upon growth factor stimulation suggested that these lipids may act as second messengers (Stephens et al., 1993; Varticovski et al., 1994). Such 3-phosphorylated lipids are not hydrolysed by any known PLC and are not components of the canonical PI turnover pathway. Instead, their catabolism is mediated by phosphatases that remove the phosphate group at position 3 or 5 of the inositol ring (De Camilli et al., 1996; Stephens et al., 1993).

The PI3K was purified to homogeneity from rat liver, bovine brain, bovine thymus mouse, fibroblasts and Balb/c 3T3 (Carpenter et al., 1990; Escobedo et al., 1991; Fry et al., 1992; Morgan et al., 1990; Shibasaki et al., 1991). and was shown to be a heterodimer of two subunits with apparent molecular masses of 110kDa and 85kDa. The cDNAs encoding the p85 protein was subsequently isolated using three different approaches. Firstly, the amino acid sequence of tryptic peptides obtained from purified bovine brain PI3K was used to design oligonucleotides which were then used to probe bovine brain cDNA libraries (Otsu et al., 1991). Secondly, a phosphorylated, immobilised PDGF receptor was used as an affinity reagent to purify PI3-kinase from BALB/c 3T3 cell lysates. Partial amino acid sequence was then used in a similar manner to isolate a cDNA from a BALB/c 3T3 cDNA library (Escobedo et al., 1991). Thirdly, an 85 kDa protein was identified from a screen of a human λgt11 expression library using the phosphorylated carboxy terminus of the EGF receptor as a probe (Skolnik et al., 1991). Two isoforms of the p85 protein were isolated, both of which contained SH2 and SH3 domains but no catalytic activity. Cloning of the 110 kDa catalytic subunit of PI3K (Hiles et al., 1992) revealed a sequence similarity within its catalytic domain to yeast Vps34p, a protein involved in vesicular trafficking which was later shown also to possess PI3K activity (Schu et al., 1993). This sequence homology within the lipid kinase domain allowed the use of polymerase chain reaction (PCR) based screens to isolate and characterise novel PI3K family members.

1.4.7.2 Classes of PI3Ks
To date, the classification of PI3K family members is based on their lipid substrate specificity in vitro, then structure and their likely mode of regulation. In mammals, 7 forms of the catalytic subunit and 5 forms of the adaptor subunit are known and can be divided into three main classes (Figure 1.22).
1.4.7.3 Class I PI3Ks

Class I PI3K's form heterodimeric complexes with adaptor proteins which link them to upstream signal transduction pathways. All mammalian class I PI3Ks interact with active, GTP-bound Ras (Kodaki et al., 1994; Marte et al., 1997; Rodriguez Viciana et al., 1994; Rodriguez Viciana et al., 1996; Rubio et al., 1997; Vanhaesebroeck et al., 1997) and can utilise PI, PtdIns(4)P and PtdIns(4,5)P2 as substrates in vitro but they appear to preferentially phosphorylate PtdIns(4,5)P2 in vivo (Irvine and Divecha, 1992). The reason for this disparity is unclear, although it may be a consequence of substrate presentation. Class I PI3Ks can be divided into two subclasses (A and B), according to the type of adaptor subunit with which the catalytic subunit associates.

1.4.7.3.1 Class IA PI3Ks

Class IA PI3K's are comprised of a 110-130kDa catalytic subunit which interacts with an adaptor protein containing two SH2 domains (described in section 1.2.1). Mammalian Class IA catalytic subunits include p110α, p110β and p110δ (Hiles et al., 1992; Hu et al., 1993; Vanhaesebroeck et al., 1997). Each isoform contains a domain which binds p85, a region which binds ras (Rodriguez Viciana et al., 1996) a PI kinase (PIK) domain and a carboxy-terminal catalytic domain. The PIK domain is a region of amino acid sequence homology in PI3Ks and PI4Ks and, although its function has not yet been determined, it has been suggested that it is involved in substrate presentation (Flanagan et al., 1993). Both p110α and p110β have a wide tissue distribution, the expression of p110δ is restricted to leukocytes, which may imply that it has a specific function in these cells (Vanhaesebroeck et al., 1997). All members of this subclass bind the p85 adaptor subunit, through which they can be recruited to receptor signalling complexes upon activation of RTK activity (Domin et al., 1996).

The first adaptors to be identified for the Class IA catalytic subunits were p85α and p85β (Otsu et al., 1991) (Figure 1.23). Both proteins contain two SH2 domains separated by an inter SH2 domain, through which the adaptor interacts with p110 subunit (Dhand et al., 1994). At the amino-terminus there is a SH3 domain (Musacchio et al., 1992) and a breakpoint cluster region homology (BH) domain, which has homology with the GAP domain of the BCR gene product (Fry and Waterfield, 1992). In addition, two proline rich regions flank the BH domain. The roles of the amino domains and regions of p85 are investigated in this thesis and are introduced in Chapter 1 and described in detail in chapters 4, 5, and 6. A number of smaller adaptor proteins have been identified which also bind the Class IA catalytic subunits (Figure 1.17). These are described in chapter 4. Most recently a mutant of p85α, p65-PI3K has been cloned and characterised (Jimenez et al., 1998). This mutant includes the initial 571
residues of the wild type p85α-protein linked to a region conserved in the eph tyrosine kinase receptor family, and provides the first evidence linking the PI3K enzyme to mammalian tumour development. It is proposed that p65-PI3K induces transformation due to its ability to constitutively activate PI3K. To date, there has been no report to date of a preferential coupling between any of the class IA adaptors and catalytic subunits, although it is possible that tissue-specific differences in function or regulation may exist. In addition to their lipid kinase activity, class IA PI3Ks also possess an intrinsic ser/thr protein kinase activity (Carpenter et al., 1993; Dhand et al., 1994), which phosphorylates the associated adaptor on Ser608 within the inter-SH2 domain in the case of p110α and p110β (Dhand et al., 1994), or autophosphorylation as in the case of p110δ (Vanhaesebroeck et al., 1997). IRS-1 is the only reported exogenous substrate for the protein kinase activity of p110α (Lam et al., 1994). Phosphorylation of Ser608 of p85α has been proposed to downregulate the lipid kinase activity of the catalytic subunit (Dhand et al., 1994).

1.4.7.3.2 Class IB PI3Ks

Studies in platelets and neutrophils, have identified a form of PI3K which acts downstream of GPLRs (Kucera and Rittenhouse, 1990; Stephens et al., 1993) and was chromatographically distinct from the class IA enzymes, (Stephens et al., 1994). This PI3K was also heterodimeric, and activated by βγ subunits. It possessed a native molecular mass of 220 kDa but did not contain a p85-like adaptor subunit. cDNAs for two forms of the G-protein activated PI3K catalytic subunit have been isolated. The first was obtained from a human bone marrow cDNA library and was designated p110γ (Stoyanov et al., 1995), while the second encodes a 120 kDa, protein that is highly related to p110γ and was co-purified from a porcine neutrophil cytosol together with a 97 kDa adaptor termed p101 (Stephens et al., 1994). Binding of p110γ to the p101 adaptor subunit lipid kinase rendered it over 100-fold more sensitive to activation by βγ subunits. Although the activity of the p101/p110γ complex cannot be stimulated by tyrosine phosphorylated peptides, and is therefore not thought to function downstream of RTKs, p110γ does contain a functional Ras binding domain (Rubio et al., 1997).

1.4.7.4 Class II PI3Ks

Class II PI3Ks are the largest (170-220 kDa) and the most recently identified form of PI3K (MacDougall et al., 1995). Family members primarily phosphorylate PtdIns and PtdIns4P in vitro. Distinctively, class II PI3Ks all contain a carboxy-terminal C2 domain (Figure 1.16) (Domin et al., 1997). C2 domains bind to phospholipids and inositol phosphates either in a Ca^{2+}-dependent or independent manner (Rodriguez
Viciana et al., 1997). They have also been implicated in mediating Ca\(^{2+}\)-triggered dimerisation of C2 domain-containing proteins (Chapman et al., 1996). The C2 domains of class II PI3Ks lack an Asp residue that has been shown to be an essential residue in the first C2 domain of synaptotagmin for binding Ca\(^{2+}\) and Class II PI3Ks have not been shown to be regulated by Ca\(^{2+}\) (MacDougall et al., 1995) No distinct functional domains have been identified in the amino acid residues which lie amino-terminal to the PIK domain in class II PI3Ks. The only possible motifs in this region are one or more Pro-rich sequences, suggesting they may bind SH3 domain-containing proteins. At present, it is unknown whether class II PI3K activity is regulated by extracellular stimuli or whether it exists in a heterodimeric complex.

1.4.7.5 Class III PI3Ks

The yeast protein Vps34p and its human homologue, PtdIns 3-kinase, can only phosphorylate PtdIns but not PI4P or PtdIns(4,5)P\(_2\) (Volinia et al., 1995). Since Vps34p is the only PI3K isoform present in yeast, members of this class are considered to be a primordial form of PI3K. Members of this class of PI3Ks also form heterodimers. Vps34p binds the ser/thr kinase Vps15p which recruits it to membranes and activates its lipid kinase activity. Similarly, PtdIns 3-kinase binds p150, the human homologue of Vps15p (Panaretou et al., 1997). Mutational analysis of Vps34p has shown it is involved in vesicular trafficking, osmoregulation and endocytosis (De Camilli et al., 1996; Herman, 1996; Shepherd, 1996). Vps15p appears to regulate the sorting of proteins to the vacuole from the Golgi complex by selectively recruiting Vps34p to the appropriate membrane site, where vacuolar hydrolases are packaged into vesicular carriers (Herman, 1996). One possibility is that the localized production of PtdIns3P by Vps34 may either recruit or activate effector molecules, that catalyze the transport reaction (Stack et al., 1995). Inactivation of the Vps34p PtdIns-3K has also been shown to alter a late stage of the endocytic pathway (Munn and Riezman, 1994). As yet, it is not clear if this effect is direct. Using yeast Vps34p/Vps15p as a paradigm, it is likely that the human homologues will fulfill an equivalent biological role in mediating vesicular trafficking (De Camilli et al., 1996; Herman, 1996; Shepherd, 1996).

1.4.7.6 PIK-related kinases

PIK-related kinases are a group of proteins that display that show sequence similarities to the kinase domain of the PI3Ks. Despite the homology, they have not yet been shown to function as lipid kinases. In addition, they contain a unique, conserved carboxy-terminal extension, which has been used to distinguish this family of enzymes from PI3Ks and PI4Ks (Keith and Schreiber, 1995). PIK-related kinases are generally high molecular weight proteins (>270kDa) and have been implicated in a diverse range
of biological functions such as DNA repair, DNA recombination and cell cycle control (Keith and Schreiber, 1995; Zakian, 1995). Family members include the ataxia telangiectesia mutated (ATM) gene product (Zakian, 1995), the catalytic subunit of DNA-dependent protein kinase (DNA-PK)(Jackson and Jeggo, 1995) and the targets of rapamycin TOR (Kunz et al., 1993) and FRAP (Brown et al., 1994).

1.4.7.7 Inhibitors of PI3K
Wortmanin, a fungal metabolite, and LY294002, a bioflavonoid derived from quercitin, are low molecular weight, cell permeable inhibitors of PI3K activity (Vanhaesebroeck et al., 1997). Wortmanin binds irreversibly to Lys608 on the p110 subunit of PI3K by forming a covalent bond, and inhibiting this PI3K with an IC$_{50}$ in the low nanomolar range (1-5nM) (Arcaro and Wymann, 1993; Thelen et al., 1994; Woscholski et al., 1994; Yano et al., 1993). Wortmannin has been used to identify downstream PI3K signalling pathways which are dependent upon PI3K catalytic activity (Okada et al., 1994; Thelen et al., 1994; Yano et al., 1993). Wortmannin does not however inhibit all classes of PI3K to the same extent. Although wortmannin inhibits all known class I PI3Ks and human Vps34 with a similar IC$_{50}$ (Stephens et al., 1994; Stephens et al., 1994) yeast Vps34p, a bovine homologue and PI3KC2α have lower sensitivity in the PI3K catalytic activity (Yano et al., 1994; Thelen et al., 1994; Yano et al., 1993). Wortmannin has been used to identify downstream PI3K signalling pathways which are dependent upon PI3K catalytic activity (Okada et al., 1994; Thelen et al., 1994; Yano et al., 1993). Wortmannin does not however inhibit all classes of PI3K to the same extent. Although wortmannin inhibits all known class I PI3Ks and human Vps34 with a similar IC$_{50}$ (Stephens et al., 1994; Stephens et al., 1994) yeast Vps34p, a bovine homologue and PI3KC2α have lower sensitivity

The main limitation to the use of PI3K inhibitors in the investigation of cellular physiology is their lack of specificity. They fail to distinguish between different isoforms of PI3K and are known to also inhibit some isoforms of PtdIns 4-Ks, TOR kinases and other signalling molecules such as cytosolic phospholipase A$_2$ (Hartley et al., 1995; Yano et al., 1995). Since the concentration of the inhibitor inside the cell cannot be determined with confidence, the specificity of PI3K inhibitor effects should be treated with caution.

1.4.7.8 Upstream regulation of PI3K
Class I PI3K have been demonstrated to play a role in a large range of receptor mediated signalling events (Figure 1.24) (Cantley et al., 1991; Stephens et al., 1993; Varticovski et al., 1994). When receptor stimulation by a ligand results in phosphorylation upon a tyrosine residue within a YXXM motif, phosphotyrosine residues serve as a docking site for the SH2 domains of the adaptor subunit of class I PI3Ks (Kapeller and Cantley, 1994). Such adaptor-mediated translocation brings the catalytic subunit into close proximity with its lipid substrates in the cell membrane. Binding of a phosphopeptide which mimics the p85 SH2 domain binding site on activated receptors has also been reported to directly increase the activity of the catalytic subunit by two to four fold (Rordorf Nikolic et al., 1995).
The paradigm for receptor mediated activation of PI3K is the PDGFβ-R. PI3K was first shown to be important in PDGF stimulated mitogenesis in studies through the use of PDGFβ-R mutants in which tyrosine residues that were targets of the p85 SH2 domains were mutated to phenylalanine. When transfected into human Hep G2 cells, these mutant receptors were found to be defective in their ability both to recruit PI3-kinase and to respond to PDGF as a mitogen (Valius and Kazlauskas, 1993). Subsequently, microinjection of neutralising antibodies specific for the p110α subunit has been shown to block the growth stimulatory effect of PDGF and EGF in quiescent fibroblasts (Roche et al., 1994). PDGFβR mutants lacking the PI3K binding site have also been used to demonstrate the importance of PI3K activity for receptor internalization and degradation (Joly et al., 1995).

An alternative mechanism by which cytosolic PTKs may recruit and activate PI3K involves SH3 domain interactions. SH3 domains from the src family have been shown to associate with the proline-rich motifs of p85α which results in the activation of PI3K activity (discussed in Chapter 5). The small GTP-binding protein Cdc42, has been shown to interact with the BH domain and activate the lipid kinase (discussed in Chapter 4), but the physiological significance of both these results is yet to be established.

Class I PI3Ks have also been shown to interact with Ras proteins in a GTP-dependent manner. This interaction has only been studied in detail for the p110α/p85α complex (Marte et al., 1997; Rodriguez Viciana et al., 1994; Rodriguez Viciana et al., 1996). The Ras-related proteins Rac, which has been implicated in signalling downstream of PI3Ks (Hawkins et al., 1995), and Rho, do not bind p110α/p85α (Rodriguez Viciana et al., 1994). RasGTP bound an amino terminal region of the p110 subunit and activated PI3K invitro. Transfection of activated PI3K and an activated Ras mutant, Val 12 Ras into PC12 cells resulted in elevated levels of PtdIns 3,4-P2 and PtdIns(3,4,5)P3. Dominant-negative ras (type N17, in which Ser17 is mutated to Asn) blocked the increase in levels of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 in response to EGF and nerve growth factor (NGF) stimulation. Accumulation of GTP-bound Ras has been shown to be required for full activation of classIA PI3Ks by PDGF (Klinghoffer et al., 1996). It therefore appears that PI3K activation is downstream of Ras activation, however, it is not yet known whether PI3K activation by RTK’s also occurs independently of Ras.

PI3Ks may thus be another class of Ras effector molecules, together with proteins such as Raf Ser/Thr kinases (section 1.3.4.1.3). The interaction of Ras with a PI3K may
also result in allosteric activation of PI3K and/or contribute to PI3K recruitment to the plasma membrane. Interestingly, Ras effector mutants have been identified that interact with p110α but not with Raf-1 and vice versa (Kauffmann Zeh et al., 1997; Rodriguez Viciana et al., 1997), suggesting that PI3K is part of a Raf-independent signalling pathway downstream of Ras. Conversely, it has been reported that expression of a constitutively active form of PI3K results in induction of Fos transcription, which is blocked by both N17 dominant-negative Ras and a dominant-negative form of Raf (Hu et al., 1995). Furthermore, GTP-bound Ras is elevated in cells transfected with a constitutively active form of PI3K, which suggests that Ras is downstream of PI3K. This hypothesis is supported by experiments in which microinjection of the amino-terminal SH2 domain of p85α as a dominant-negative inhibitor of PI3K blocks induction of Fos transcription in response to insulin stimulation (Jhun et al., 1994). This block was rescued by expression of an activated form of Ras. Inhibition of Fos induction by a neutralising antibody raised to the amino terminal SH2 domain of p85α was also rescued by expression of activated Ras.

At present it is unclear how heterotrimeric G-proteins activate the class IB PI3K. Whilst Gβγ can directly stimulate p110γ lipid kinase activity this stimulation is considerably enhanced in the presence of the p101 adaptor (Stephens et al., 1997). The regulation of the class II PI3K activity remains unknown. Similarly for the class III PI3Ks, it can be hypothesised that the class II PI3Ks may be constitutively active (based on the housekeeping role of the yeast Vps34 in vesicular trafficking).

### 1.4.7.9 Downstream effectors of PI3K

The products of the lipid kinase activity of PI3K, PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ are thought to transmit PI3K signals by binding to other cellular proteins and modulating their activity or cellular location (Figure 1.25). Two modules present in many proteins, the PH domain (section 1.2.7) and the C2 domain (Newton, 1995) have been shown to specifically bind phosphoinositides, therefore PH and C2 domain containing proteins are the prime targets for effectors of PI3K transduced signals. It is unclear whether the protein ser/thr kinase activity of PI3K also results in signal propagation. Phosphorylation of p85 and p110 by PI3K results in a decrease in lipid kinase activity, and this may be part of a negative feedback loop. There is one report that IRS1 can serve as a substrate for the ser/thr kinase activity of PI3K, but the significance of this phosphorylation event has yet to be determined.

#### 1.4.7.9.1 PDK1-PKB/Akt

The Akt/PKB/RAC ser/thr kinase is the cellular homologue of the retroviral oncogene v-akt and is activated upon ligand stimulation of receptor tyrosine kinases (Bellacosa et
A large number of studies which suggest that Akt is downstream of PI3K. Treatment of cells with the PI3K inhibitors Wortmannin or LY294002, or over-expression of dominant negative forms of PI3Ks inhibit Akt activation downstream of RTK stimulation (Burgering and Coffer, 1995; Franke et al., 1995; Kohn et al., 1996). In addition, overexpression of PDGFβ receptor mutants that lack the PI3K binding site also fail to activate Akt (Burgering and Coffer, 1995; Franke et al., 1995). Elevation of levels of PI3K lipid products in cells by overexpression of constitutively active mutants of p110α results in activation of Akt (Didichenko et al., 1996; Klippel et al., 1996; Marte et al., 1997). Akt is a ser/thr kinase which contains an amino-terminal PH domain that has been shown to bind PI(3,4,5)P3 and PI(3,4)P2 with relatively high affinity and specificity (Franke et al., 1997; Frech et al., 1997; James et al., 1996; Klippel et al., 1997). Binding of PtdIns(3,4)P2, but not PtdIns(3,4,5)P3, to the PH domain of AKT increases its kinase activity (Franke et al., 1997; Frech et al., 1997; Klippel et al., 1997). PtdIns(3,4)P2 is mainly produced by the action of 5-phosphatases on PtdIns(3,4,5)P3 in vivo (Woscholski and Parker, 1997), suggesting that 5-phosphatases act downstream of PI3K in signalling via Akt. Binding of PtdIns(3,4)P2 also induces the dimerization of Akt in vitro (Franke et al., 1997). It is not clear whether dimerization directly affects kinase activity, although it has been shown that binding of a recombinant Akt kinase domain to Akt in vitro increases its kinase activity (Datta et al., 1995). Full activation of Akt requires two phosphorylation events as well as PtdIns(3,4)P2 binding. In insulin or IGF-1 stimulated cells, Akt becomes phosphorylated on thr 308, which is in the kinase domain, and ser 473, which is in the carboxy-terminal tail, in a wortmannin sensitive manner (Alessi et al., 1996). In the presence of PtdIns(3,4,5)P3, 3-phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates Thr 308 and PDK2 phosphorylates Ser 473 (Alessi et al., 1997; Stephens et al., 1998; Stokoe et al., 1997) Akt therefore integrates signals from several pathways, at least two of which involve the lipid products of PI3K. Akt can also be activated by stresses such as heat shock or hyper osmolarity in a PI3K independent manner (Konishi et al., 1996).

Activation of Akt mediates the insulin-induced inhibition of glycogen synthase kinase 3 (GSK3) which phosphorylates and down regulates glycogen synthase (Cross et al., 1995), as well as eIF2B, an initiation factor important in the control of mRNA translation and protein synthesis (Welsh et al., 1996). A number of other regulatory proteins are also thought to lie downstream of GSK3.

p70S6 kinase (p70S6k)

p70S6 kinase (p70S6k) phosphorylates the 40S ribosomal protein S6 in response to mitogenic stimuli and plays a key role in the progression of cells from the G1 to S
phase of the cell cycle (Brown and Schreiber, 1996; Kozma and Thomas, 1994; Proud, 1996). Activation of p70s6k is regulated by multiple independent phosphorylations on serine and threonine residues. Activation of p70s6k, is not dependent on the Raf/MAPK pathway, but does involve PI3K and the PIK-related kinase, mTOR, as well as PKC and an unidentified proline-kinase (Brown and Schreiber, 1996; Kozma and Thomas, 1994; Proud, 1996).

Overexpression of receptor mutants which do not bind the p85 adaptor and PI3 kinase inhibitors both block ligand induced activation of p70S6 kinase (Chung et al., 1994). Furthermore, constitutive PI3K activity results in the phosphorylation of p70S6 kinase and antisera which target p70S6 kinase and block the mitogenic activity of PI3-kinase (Weng et al., 1995).

Mammalian TOR (mTOR) is inhibited by the immunosuppressant drug, rapamycin, which is an analogue of another immunosuppressant, FK506. Rapamycin binds a 12kDa FK506 binding protein (FKBP12) which then binds to and inhibits mTOR. Rapamycin blocks the activation of p70s6k by all known agonists, however it is also known that mTOR does not phosphorylate p70s6k directly. mTOR protein kinase activity is also inhibited by Wortmannin, but PI3K is insensitive to rapamycin, thus the differential effects of these inhibitors have been used to the pathway involved in the regulation of p70s6k. Inhibitors of PI3K such as overexpressed receptor mutants lacking the PI3K binding site or wortmanin block p70S6K phosphorylation (Cheatham et al., 1994; Chung et al., 1994) and expression of constitutively active forms of PI3K leads to increased phosphorylation of p70s6k (Weng et al., 1995). In addition, neutralizing antibodies raised against p70s6k block PI3K mediated mitogenesis (Pullen and Thomas, 1997). PI3K therefore seems to lie upstream of p70s6k, but PI3K does not directly phosphorylate p70s6k. Mutant forms of mTOR that do not bind the rapamycin/FKBP12 complex overcome the inhibition of p70s6k by rapamycin, suggesting that mTOR is also upstream of p70s6k in vivo. It is not yet clear whether PI3K is upstream or downstream of mTOR, or whether the two act independently to activate p70s6k.

1.4.7.9.3 PKC and related kinases
Calcium independent isoforms of protein kinase C and protein kinase C related kinases have been found to be activated by 3' phosphoinositides in vitro (Derman et al., 1997; Palmer et al., 1995; Toker et al., 1995). Although one study has shown that both PI(4,5)P2 and PI(3,4,5)P3 activate these kinases to the same extent, other studies report conflicting data which might be attributed to differences in lipid presentation procedures. It was recently demonstrated that PI(3,4,5)P3 initiated cell motility of
NIH3T3 cells and that this effect was mediated by PKC (Derman et al., 1997). Although the study was not able to distinguish which PKC family member or members were directly involved, data suggested that the PKCe was strongly regulated by these lipids. More recently it has been demonstrated that binding of the PH domain of PLCγ to PtdIns(3,4,5)P3 in the membrane generated by PI3K in response to growth factor stimulation activates PLCγ1 (Falasca et al., 1998).

1.4.7.9.4 Rab GTPases
The Rab proteins are members of the Ras superfamily of GTPases (Boguski and McCormick, 1993). There are more than 30 Rab isoforms present in mammalian cells, but they are specifically localised to vesicles at different stages of the exocytic or endocytic pathways (Simons and Zerial, 1993). There is some evidence that at least one Rab protein, Rab5, may be downstream of PI3K (Li et al., 1995). In its GTP-bound, active form, Rab5 stimulates fusion of early endosomes and thus increases the rate of endocytosis of various proteins (Simons and Zerial, 1993). Wortmannin inhibits this process and has also been shown to inhibit endosome fusion in vitro. In contrast, wortmanin does not inhibit endosome fusion downstream of constitutively active forms of Rab5, suggesting that PI3K must lie upstream of Rab5. It has been suggested that PtdIns(3,4,5)P3 is able, either directly or indirectly to stimulate nucleotide exchange on Rab5 (Li et al., 1995).

1.4.7.10 PI3K and apoptosis
Recent studies have demonstrated that the activation of PI3K and its downstream target Akt are involved in a pathway that conveys survival signals from various cell surface receptors (Franke and Cantley, 1997; Franke et al., 1997). It has been previously demonstrated that inhibition of PI3K blocks the ability of survival factors to protect various cell types from programmed cell death (Yao and Cooper, 1995), (Yao and Cooper, 1996). Ras is capable of inhibiting apoptosis in murine fibroblasts and epithelial cells by activating PI3K independently of theRaf/MAP pathway (Kauffmann Zeh et al., 1997; Khwaja et al., 1997; Rodriguez Viciana et al., 1997) The inositide products of the PI3K can trigger survival signals through activation of the serine/threonine kinase PKB/AKT (Kauffmann Zeh et al., 1997; Khwaja et al., 1997; Rodriguez Viciana et al., 1997) but other pathways cannot be ruled out. Akt has been shown to be necessary and sufficient for neuronal survival. Overexpression of Akt prevents apoptosis in primary cultures of cerebellar neurons that are induced by survival factor withdrawal or inhibition of PI3K (Dudek et al., 1997). The overexpression of dominant negative forms of Akt interfered with IGF-1 mediated survival in these cells. Another study indicated that activated Akt/PKB prevented apoptosis, induced by the detachment of MDCK cells from their extracellular matrix (Khwaja et al., 1997).
Mutants of V12 Ras that selectively stimulate PI3K and Akt but not the Raf/MAPK pathway also prevent c-myc-induced cell death in Rat-1 cells (Kauffmann Zeh et al., 1997). In this system, activated forms of PI3K and Akt were sufficient to prevent apoptosis that was induced by c-myc. p70s6k activity was not necessary for the prevention of apoptosis caused by deregulated c-myc (Kauffmann Zeh et al., 1997), nor was it necessary for Akt dependent neuronal survival (Dudek et al., 1997). Therefore IGF-1 and certain other growth factors stimulate a cell survival pathway that involves Ras-dependent stimulation of PI3K, leading to activation of Akt. This pathway appears to be independent of MAPK and p70s6k and is able to prevent apoptosis induced by a variety of cellular agonists.

One mechanism by which Akt may facilitate cell survival is through the inhibition of a component which promotes apoptosis (Datta et al., 1997). Akt has been shown to phosphorylate a critical serine residue on Bad, a protein which promotes cell death by binding and inhibiting a cell survival factor termed Bcl-x1. Phosphorylation of this serine residue is required for the interaction of BAD with the phosphoserine binding protein 14-3-3 (Zha et al., 1996) and its dissociation from Bcl-x1. The released Bcl-x1 then promotes cell survival by blocking the caspase protease cascade (Reed, 1997). Furthermore, it has been shown that PI3K activity is necessary for the phosphorylation of BAD after interleukin treatment for its interaction with 14-3-3 (del Peso et al., 1997). These studies demonstrate a mechanism by which a growth factor-regulated kinase cascade mediates cell survival by inhibiting the intrinsic cell death machinery. However not all cell survival signals require PI3K activity and Akt (Philpott et al., 1997) and furthermore, PI3K and AKT are involved in other signalling pathways unrelated to cell survival. Since PtdIns products of PI3K also mediate responses unrelated to activation of Akt (section 1.4.7.9.1). Branch points must exist at points in the signalling cascade with each step having multiple upstream regulators.

It has also recently been demonstrated that p85 is involved in the cellular apoptotic response to oxidative damage. p85 is upregulated by p53 and involved in p53-mediated apoptosis by following H2O2 administration (Yin et al., 1998). Interestingly, the lipid kinase activity of PI3K was unaffected by oxidative stress, indicating that p85 may function independently of p110. As previously described, p110 is involved in inhibiting apoptosis and promoting cell survival. However it is possible that in response to oxidative stress, p85 may recruit different effectors to mediate cell death.

Clearly PI3K and its lipid products are involved in a variety of biological responses including cell growth, receptor internalisation, vesicular trafficking, glucose transport, chemotaxis, transformation (discussed in chapter 5), membrane ruffling (section
1.3.4.2.1) and cell survival. The structural diversity of family members facilitates multiple mechanisms of activation. Numerous downstream protein and lipid binding domains allow the enzyme and its lipid products to interact with separate pathways depending upon cell type and the nature of the stimulus.
Figure 1.1 Schematic structure of a G protein linked and a tyrosine kinase receptor.

The model at the far left illustrates an example of a receptor with intrinsic tyrosine kinase activity. The multimeric cytokine receptor and the T cell receptor complex are examples of receptors that lack intrinsic enzymatic activity but associate with intracellular tyrosine kinases. The model at the far right represents a seven transmembrane receptor which is coupled to a heterotrimeric G protein (see text).
Figure 1.2 Classification of the superfamily of receptor tyrosine kinases.

In subfamilies according to the structural domains present in their extracellular domains and the presence or absence of a kinase insert in their kinase domains (after A.D. Reith and G. Panayotou, Introduction to the Cellular and Molecular Biology of Cancer, pg 233, eds. I.M. Franks and N.M. Teich, Oxford University Press). Known ligands for each subclass of receptors are listed above each diagram while known receptors in each subclass are listed below each diagram. See abbreviations list and text for abbreviations used.
Figure 1.3 Initiation of Signalling by Receptor tyrosine kinases

(a) Ligand binding
(b) Receptor activation by ligand induced dimerization and subsequent autotransphosphorylation of cytoplasmic tyrosine residues.
(c) Formation of signalling complexes by binding of SH2 and PTB domain containing proteins to phosphorylated tyrosine residues.
PROTEINS WITH SH2, SH3 AND PH DOMAINS

A. Enzymes

Src
GAP
PLCγ
Vav
SYP

B. Adaptor molecules

p85
Nck
SHC
c-Crk
GRB2
p91

Figure 1.4 Schematic illustration of the SH2, SH3 and PH domain family of signalling proteins. Domains are defined as follows: PTPase (phosphotyrosine phosphatase domain), PLC (PLCy kinase domain), RasGAP (RasGAP kinase domain), Dbl (GDP/GTP exchange factor domain), Gly/PR (glycine/proline rich region), PR (proline-rich region), P, phosphotyrosine-containing SH2 binding site, BH (region of homology to the BCR protein), PTB (phosphotyrosine binding domain), see text.
Figure 1.5 Ribbon Representation of the three dimensional structures of the amino terminal (green) and carboxy terminal (blue) SH2 domains of p85α bound to a diphosphateptide derived from the p85α binding sites in the PDGFβ receptor (red, spacefilled model). The phosphotyrosine (pY) and the amino acid residue at position three carboxyl-terminal to pY (pY+3) are the principle residues in the phosphopeptide that make contact with both SH2 domains. (1) and (2) indicate the two phosphotyrosine binding pockets.

(personal communication Dr Marketa Zvelebil)
Figure 1.6 Three dimensional structure of the p85α SH3 domain

(A) Ribbon representation of the p85α SH3 domain indicating α helical (pink), β sheet (yellow) and loop (white) regions (see text) and (B) A three dimensional structure of the p85α SH3 domain illustrating the Van der Waals interaction surface and a space filled model of a bound proline-rich peptide derived from (Yu et al., 1994, Cell, 76, 933-945). The orientation of the proline rich peptide from its amino (N) to carboxy (C) terminus is indicated.
Figure 1.7 (A and B) The structures of class I and class II proline-rich peptide ligands for SH3 domains are depicted schematically. P and R indicate conserved proline and arginine residues; q and X indicate variable amino acids (see text). The ability of the SH3 domain to bind its ligands in both amino-to-carboxyl (class II) and carboxyl-to-amino (class I) derives from the pseudosymmetry of the PPII helix. (C) The three-dimensional structure of the SH3 domain of the tyrosine kinase, fyn, is shown diagrammatically (after D.E Noble et al., 1993, EMBO-J, 12, 2617-24). The position of the three variable loops, n-Src loop, distal loop and RT loop and the three sites on the surface of the SH3 domain that make contact with its ligand are indicated. The side chains of residues involved in ligand binding are depicted in colour, and correspond to the coloured boxes on the sequence alignment shown in (D). (D) Alignment of the amino-acid sequences of several representative SH3 domains. Coloured boxes indicate conserved residues involved in ligand binding. CrkN and GrbN are the amino-terminal SH3 domains of Crk and Grb2; n-Src is the alternatively spliced neural form of cellular Src.
Figure 1.8 Ribbon diagram of the three dimensional structure of the dynamin PH domain

The dynamin PH domain contains two nearly orthogonal anti-parallel β-sheets of three and four β strands (blue) and a single amphipathic carboxy-terminal α-helix (pink) positioned at one end of the interface between the β-sheets. The β strands are separated by three loops (white) of variable length. (After D Timm, et al., (1994), Nat.Struct.Biol, 11, 782-8).
Figure 1.9 Upstream agonists and downstream effectors of the Src tyrosine kinase family
<table>
<thead>
<tr>
<th>GENE</th>
<th>Expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>src</td>
<td>Ubiquitous; expression highest in neurons, platelets and osteoclasts</td>
</tr>
<tr>
<td>yes</td>
<td>Widespread</td>
</tr>
<tr>
<td>lyn</td>
<td>Widespread; expression highest in neurons</td>
</tr>
<tr>
<td>lyn</td>
<td>Alternative splice form (FynT) restricted to T and B cells</td>
</tr>
<tr>
<td>lck</td>
<td>T cells, natural killer cells</td>
</tr>
<tr>
<td>hck</td>
<td>B cells, myeloid cells; expression highest in granulocytes</td>
</tr>
<tr>
<td>lyn</td>
<td>B cells, myeloid cells</td>
</tr>
<tr>
<td>fgr</td>
<td>B cells, myeloid cells</td>
</tr>
<tr>
<td>blk</td>
<td>B cells</td>
</tr>
<tr>
<td>yrk</td>
<td>Widespread; high expression in cerebellum, spleen</td>
</tr>
</tbody>
</table>

---

**Figure 1.10** Below, primary structure of src illustrating domains, regulatory regions, their functions and physiological phosphorylation sites (by known kinases). PDGF-R, platelet-derived growth factor receptor.
Figure 1.11 A ribbon diagram of c-src in the inactive form (phosphorylated at Tyr527). Domains and other key sequences are annotated. The disordered activation loop and the unique region, which was not included in the crystal structure (indicated by '?'), are indicated by dashed lines (after W.Xu et al., (1997), 385, 595-602).
Figure 1.12 Routes by which Src may be activated. The SH3 domain is shown in blue, the SH2 domain in green and the catalytic domain in red (see text for details).
Figure 1.13 Cycling of Ras between GTP and GDP bound states
Known GTPase activating proteins (GAPs) are listed in purple while Guanine nucleotide exchange factors (GNEFs) are listed in red.
**Fig 1.14 Components of the Ras pathway are conserved through evolution**

The diverse signalling events controlled by Ras proteins are shown with components represented sequentially. These components are in an *S.pombe* Ras1 pathway distinct from byr2.
Table 1.2 Members of the Rho family, identified in the species shown, are listed in groups according to their closest homology with the mammalian proteins.

<table>
<thead>
<tr>
<th>Rho family members</th>
<th>Mammalian</th>
<th>S. Cerevisiae</th>
<th>S. pombe</th>
<th>Drosophila</th>
<th>Dictyostelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho-like</td>
<td>Rho (A,B,C)</td>
<td>Rho1</td>
<td></td>
<td>Rho1</td>
<td></td>
</tr>
<tr>
<td>Rac-like</td>
<td>Rac (1,2)</td>
<td></td>
<td></td>
<td>DRac (1,2)</td>
<td>Rac (1,2,3,B)</td>
</tr>
<tr>
<td>Cdc42-like</td>
<td>Cdc42, G25K</td>
<td>Cdc42</td>
<td>Cdc42</td>
<td>DCdc42</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>RhoD</td>
<td>Rho2</td>
<td></td>
<td></td>
<td>RacA</td>
</tr>
<tr>
<td></td>
<td>RhoE</td>
<td>Rho3</td>
<td></td>
<td></td>
<td>RacC</td>
</tr>
<tr>
<td></td>
<td>RhoG</td>
<td>Rho4</td>
<td></td>
<td></td>
<td>RacD</td>
</tr>
<tr>
<td></td>
<td>TC10</td>
<td></td>
<td></td>
<td></td>
<td>RacE</td>
</tr>
<tr>
<td></td>
<td>TTF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig 1.15 Cycling of the Rho family GTPases between the GTP and GDP bound states. Known GTPase activating proteins (GAPs) are listed in purple while Guanine nucleotide exchange factors (GNEFs) are listed in red. The pathway where guanine dissociation inhibitors (GDIs) act is also indicated.

<table>
<thead>
<tr>
<th>RhoGAP-containing proteins</th>
<th>Organism</th>
<th>Predicted Molecular Mass (kDa)</th>
<th>Rho</th>
<th>Rac</th>
<th>Cdc42</th>
</tr>
</thead>
<tbody>
<tr>
<td>p50RhoGAP</td>
<td>Mammalian</td>
<td>50</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Bcr</td>
<td>Mammalian</td>
<td>143</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Abr</td>
<td>Mammalian</td>
<td>92</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-chimaerin</td>
<td>Mammalian</td>
<td>34</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>β-chimaerin</td>
<td>Mammalian</td>
<td>34</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>p190</td>
<td>Mammalian</td>
<td>170</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p85α</td>
<td>Mammalian</td>
<td>83</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>p85β</td>
<td>Mammalian</td>
<td>81</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3BP-1</td>
<td>Mammalian</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rotund</td>
<td><em>Drosophila melanogaster</em></td>
<td>41</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BEM2</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>BEM3</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>125</td>
<td>+</td>
<td>ND</td>
<td>++</td>
</tr>
<tr>
<td>CeGAP</td>
<td><em>Caenorhabditis elegans</em></td>
<td>155</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Ability (+) or inability (-) of the RhoGAP-containing proteins to stimulate GTPase activity of the corresponding specific protein. ND, not determined.
Fig. 1.16 The multifunctional character of GAPs for the rho subfamily of GTPases. The homologous domains are identified at the bottom of the figure.
Figure 1.17 The dendrogram shows the degree of amino acid sequence similarity of the known BH domains.
Figure 1.18 Amino acid sequence alignment of the BH domain. The three regions of conservation in this domain are represented as blocks 1, 2 and 3 (see text). A consensus sequence, based on four or more occurrences of an amino acid residue at a single position, is derived from these seven proteins and compared with p85. Invariant residues in the consensus sequence are in red.
Figure 1.19 Ribbon representation of the GAP domain of p50RhoGAP

The helices are labelled from amino-to-carboxy-terminus as A0 to G. There are nine helical segments and two short helical turns (labelled E' and G'). Two short regions of $\beta$ helix between helices A1 and B, and helices C and D are indicated. Arg85 and Asn194 are involved in G protein binding and enhancing GTPase activity.
Figure 1.20 Cycle of phosphoinositide biosynthesis
Fig 1.21 The lipid products generated by the 3-phosphoinositide pathway are depicted in red to differentiate from the phosphoinositides involved in the canonical PI turnover pathway. The enzymes thought to catalyze the different reactions are shown. The solid arrows indicate pathways known to occur in vivo. The dashed arrows indicate activities that can be detected in cell lysates or with purified enzymes.
### Lipid substrates and structural features of catalytic subunits

<table>
<thead>
<tr>
<th>Class</th>
<th>Subunits</th>
<th>Catalytic</th>
<th>Adaptor</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I</strong></td>
<td>PtdIns, PtdIns(4)P, PtdIns(4,5)P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>p110γ, δ (m) Dp110 (Dm) AGE-1 (Ca) PIK1, PIK2 (Dd)</td>
<td>p85γ, δ (m) p55γ (m) p50γ (m) p60 (Dm)</td>
<td>Tyr kinases &amp; Ras</td>
</tr>
<tr>
<td>A</td>
<td>PtdIns(4)P</td>
<td>p110γ (m) PIK3 (Dd)</td>
<td>p101 (m)</td>
<td>Heterotrimeric G proteins &amp; Ras</td>
</tr>
<tr>
<td>B</td>
<td>PtdIns, PtdIns(4)P</td>
<td>PI3K-C2γ (m)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td><strong>II</strong></td>
<td>PtdIns</td>
<td>Vps34p&lt;sup&gt;**&lt;/sup&gt;</td>
<td>Vps15p (Sc) p150 (m)</td>
<td>Constitutive?</td>
</tr>
</tbody>
</table>

**Fig 1.22 The classification of PI3K family members.** The assignment of catalytic subunits to a particular class is based on sequence homology within the catalytic domain.
Fig 1.23 Structural organization of the class IA adaptor subunits for class IA PI3Ks

(P, proline-rich region; BH, BCR homology region). p50α and p55α (also known as p85/AS53) are splice variants of p85α, whereas p85β and p55γ (also known as p55PIK) are encoded by different genes. Triangles indicate further splice insertions in p85α and p55α (here named p85αi and p55αi).
Figure 1.24 A schematic representation of stimuli that activate one or more members of the PI3K family, and some biological responses that are elicited by the lipid products of the enzyme.
Figure 1.25 Signaling pathways linked to mammalian class IA PI3Ks
(see text for details)
Chapter 2: MATERIALS AND METHODS

2.1 DNA Cloning Techniques

2.1.1 Preparation of DNA fragments for ligation

2.1.1.1 DNA digestion with restriction enzymes
To generate appropriate amounts of DNA fragments for ligation, 1-5μg plasmid DNA was typically digested in a volume of 20-100μl. Restriction enzymes were obtained from Boehringer Mannheim Ltd, so digests were performed in the required restriction enzyme digestion buffer as directed. The restriction enzyme (0.5-1U/μl) was added to initiate digestion, and reactions were incubated at 37°C for 2-16h. Restriction enzymes were supplied in 50% (v/v) glycerol and were therefore restricted to less than 10% of the digestion reaction volume, to prevent inhibition of non-specific digestion of DNA.

2.1.1.2 DNA amplification by the polymerase chain reaction (PCR)
PCR reactions were performed in 50μl volumes buffered with 15mM Tris HCl pH8.8, and containing 60 mM KCl, 2.25mM deoxynucleotide triphosphates (dNTPs) (Pharmacia), 0.2 pmol/μl of each primer and 0.05U/μl Vent DNA polymerase (New England Biolabs). The template for synthesis was 1-10ng of plasmid DNA. Samples were overlaid with mineral oil (Sigma Chemical Co. Ltd), and amplification was performed using a Cetus thermal cycler (Perkin Elmer). Samples were denatured (94°C, 30 s), annealed (50°C, 30s) and extended (72°C, 60 s) for 30 cycles followed by 5 min at 72°C to permit a final extension of the PCR product. The PCR products were extracted with chloroform to remove mineral oil, resolved by TAE/agarose gel electrophoresis (see 2.2.1.1.5) and purified using a Quiex gel extraction kit (Quiagen) according to the manufacturer’s instructions.

2.1.1.3 End-filling of PCR products
To add 5' phosphate groups to PCR products, the purified DNA was eluted into 13μl of water and increased to a final volume of 19μl with 70mM Tris HCl pH7.5, 10mM MgCl₂, 10mM DTT and 1mM ATP. T4 Polynucleotide kinase (New England Biolabs) (1μl 5U/μl) was added and the reaction was incubated at 37°C for 30min. To remove the protruding 3' nucleotide that is often present at both ends of a PCR product, 1μl 5U/μl E.coli DNA polymerase I Klenow fragment [New England Biolabs (NEB)], 6μl water and 3 μl 0.5mM dNTPs (to inhibit 3'-5' exonuclease activity of the Klenow fragment) were added and the reaction mixture was incubated at 25°C for 15min. Both
enzymes were heat inactivated at 70°C for 15min then the PCR product was ligated into an alkaline phosphatase-treated vector (see 2.1.1.4).

2.1.1.4 Phosphatase treatment of digested plasmids
To prevent recircularization of plasmids bearing blunt or compatible cohesive ends, the 5' phosphate group was removed from both termini. Calf intestinal alkaline phosphatase (Boehringer Mannheim Ltd.) (0.075 U/μl) was added to a digested plasmid and incubated at 37°C for 20min. To remove alkaline phosphatase, the DNA was purified by agarose gel electrophoresis as described in section 2.1.1.5.

2.1.1.5 Identification and purification of digested DNA fragments
DNA was fractionated according to size by electrophoresis in agarose gels. Fragments between 0.3-7kb were resolved on 2%-0.8% (w/v) agarose gels, fragments less than 500bp in size were resolved on 2%(w/v) agarose gels and DNA larger than 4kb was resolved on 0.8% agarose gels. Gels were buffered with 40mM Tris-acetate pH 7.8, 1mM EDTA, containing 1μg/ml ethidium bromide. Excised DNA fragments were purified using a Quiaex gel extraction kit (Quiagen) according to the manufacturer's instructions.

2.1.1.6 Synthesis and Purification of oligonucleotides
Oligonucleotides were synthesised on small scale Controlled Pore Glass (CPG) columns using an Applied Biosystems 381A DNA synthesiser. Oligonucleotides up to 45 bases long were eluted from the column in 1 ml concentrated ammonia solution, and de-protected by incubation at 55°C for 8-16hrs. Butan-1-ol (10ml) was added to the cooled ammonia solution and the mixture was vortexed extensively. The oligonucleotide was pelleted (8,000g, 10min), then the pellet was washed in 100% (v/v) ethanol, dried, resuspended in water and stored at -20°C. Commercially synthesised oligonucleotides (Genosys) were dissolved in water and stored at -20°C. The oligonucleotide was then precipitated by the addition of 10mls butan-1-ol and centrifugation at 8000g for 10min.

2.1.2 DNA ligation and transformation

2.1.2.1 DNA Ligation
In order to minimise self-ligation of plasmids lacking inserts, the molar ratio of insert:vector DNA in most ligation reactions was between 3:1 and 5:1. Normally, ligations were performed in a total volume of 10-15μl in ligation buffer (Gibco BRL) supplemented with 1mM ATP 0.1U/μl DNA ligase (Gibco BRL) was added and the reaction mixture was incubated for 2-6 hrs at room temperature or for 16hrs at 16°C.
2.1.2.2 Preparation of competent *E.coli*

Competent XL-1 Blue or BL21 *E.coli* were prepared by a procedure modified from (Hanahan, 1983). A single colony from an L-agar plate (10mg/ml bacto tryptone, 5mg/ml NaCl, 5mg/ml yeast extract, 15mg/ml agar) was inoculated into 20ml TYM broth [2%(w/v) bactotryptone, 0.5%(w/v) yeast extract, 0.1M NaCl, 10mM MgSO₄] and shaken at 37°C in a 250 ml flask until the optical density at 600nm was 0.2-0.8 absorbance units greater than that of TYM broth alone. The culture was transferred to a 2l flask containing 100ml TYM broth and agitated at 37°C until the absorbance at 600nm reached 0.5-0.9. At this time, a further 500ml TYM broth was added and the incubation continued until the absorbance at 600nm was 0.6. The culture was then cooled rapidly by swirling the flask in an ice bath, the bacteria were pelleted (5000g, 15min) and resuspended in 100ml ice-cold TfBI [30 mM potassium acetate, 50 mM MgCl₂, 100mM KCl, 10mM CaCl₂, 15% (v/v) glycerol]. The bacteria were centrifuged once more (5000g, 8min) and resuspended in 20ml TfBI [[10mM NaMOPS pH7, 0.75mM CaCl₂, 10mM KCl, 15% (v/v) glycerol], then 0.6ml aliquots were snap-frozen in liquid nitrogen and stored at -70°C.

2.1.2.3 Transformation of *E.coli*

Frozen competent XL-1 Blue or BL21 *E.coli*, prepared as described in section 2.1.2.2, were thawed on ice and added to either approximately 10ng of circular DNA or 10μl of a ligation reaction mix. The transformation mix was then incubated on ice for 10min, heat shocked at 42°C for 2min, then returned to an ice bath for 5min. LB broth (1ml) (10g Bactotryptone, 5g Bacto yeast extract and 10g NaCl per litre) was added and the cells were agitated at 37°C for 45min. The transformed cells were pelleted, resuspended in 100μl L-Broth and 10μl and 100μl aliquots of the cell suspension were spread onto the pre-warmed L-agar plates containing 50μg/ml ampicillin and incubated at 37°C for 16hrs.

To analyse the bacterial colonies for recombinants, 5ml cultures were grown, the plasmid DNA isolated (section 2.1.3.1) and analysed by restriction enzyme digestion (section 2.1.1.1)

2.1.3 DNA preparation

2.1.3.1 Small scale double-stranded plasmid DNA preparation

Plasmid DNA preparations yielding 5-20μg plasmid DNA were performed by a (modified Alkali Lysis) method derived from Sambrook *et al.* (p1.25-1.28, 1989). A 5ml culture in LB broth supplemented with 50μg/ml ampicillin was shaken at 37°C for
6-16hrs. Cells were pelleted (5000g, 10min) then resuspended in 200μl GTE (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA) and transferred to 1.5ml microcentrifuge tubes. Freshly prepared 0.2M NaOH/1% SDS (300μl) was added, the tube inverted several times, incubated on ice for 5min and neutralized by the addition of 300μl of 3.0M potassium acetate pH4.8. The tube was mixed gently, incubated on ice for 5min and centrifuged at 1000g for 10min at room temperature to remove the cellular debris. The supernatant was treated with 20μg RNase A at 37°C for 20min, then extracted twice with 400μl of chloroform. DNA was then precipitated by adding an equal volume of isopropanol and centrifugating at 10000g for 10min at room temperature. The DNA pellet was washed with 70% ethanol, dried under vacuum, then dissolved in 32μl water. DNA was further purified by PEG precipitation by adding 8.0μl of 4M NaCl and 40μl of sterile 13% PEG8000. After thorough mixing, the sample was incubated on ice for 30min and centrifuged at 10,000g for 15min at 4°C. The pellet was washed with 70% ethanol, dried under vacuum, resuspended in 20μl of water and stored at -20°C. The quality of DNA prepared by this method was found to be suitable for DNA sequence analysis.

2.1.3.2 Large scale double-stranded plasmid DNA preparation

Large scale double stranded plasmid DNA preparations or 'maxi-preps', to provide 0.1-2mg plasmid DNA for transfection or digestion and use in the construction of other plasmids, were performed using the Wizard Maxi prep kit (Promega) according to the manufacturer's instructions. The concentration of plasmid was assessed by agarose gel electrophoresis and comparison of intensity of ethidium bromide staining. DNA was to be used for transfection of mammalian cells, was purified using an additional extraction with phenol, followed by another chloroform extraction.

2.1.4 DNA Sequencing

DNA sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977). DNA prepared as in section 2.1.3.1 was sequenced using the ABI PRISM™ Ready Reaction DyeDeoxyTM Terminator Cycle Sequencing Kit according to the manufacturer's instructions. This method uses four dideoxy nucleotides, G, A, T, and C, labelled with different fluorescent dyes. These dideoxy-nucleotides terminate PCR extension of template DNA, which is carried out using AmpliTaq DNA polymerase (Perkin-Elmer). PCR amplification is carried out for 25 cycles of denaturation (96°C, 30s), annealing (50°C, 30s) and extension (60°C, 4min). PCR products were resolved by electrophoresis on a 6% acrylamide gel (Sequagel-6, National Diagnostic) under denaturing conditions. The gel was electrophoresed for 12hrs at 30 watts and 1280 volts on a model 373 automated sequencer (Applied
Biosystems). DNA sequence assembly and analysis was performed using the AUTOASSEMBLER SEQUENCE NAVIGATOR software (ABI).

2.2 Cell Culture Methodology

2.2.1 General cell culture techniques and seeding densities for insect cells.

Spodoptera frugiperda (Sf9) cells were maintained in culture as previously described in (Smith and Johnson, 1988). All culture media were warmed to 27°C prior to use and the cells were routinely incubated at 27°C. The culture medium used throughout was IPL41 (Gibco.BRL) supplemented with 10% (v/v) FCS, 2% yeastolate and 1% lipid supplement (Gibco). Routine culture was performed in the absence of antibiotics although 1% (v/v) fungizone and 50µg gentamycin (Gibco.BRL) were added during amplification of virus stocks. When growing monolayer cultures, 80-90% confluent cells were routinely subcultured in a 175cm² flask (Sf9 cells have a doubling time of 18-24hrs. Cells were grown to a density of 3-4x10⁶/ml and diluted 1/10). Cells were detached by gentle agitation, then diluted 1:2 to 1:40 into a new flask containing fresh IPL41 media in a final volume of 20mls. The flask was rocked gently to distribute the cells evenly, then incubated at 27°C. For large scale protein production, Sf9 cells were grown in suspension. Suspension cultures were started at an initial density of 5x10⁵ cells/ml. They were incubated at 27°C with constant stirring at 50-60 rpm and required subculturing every 72hrs when the cell density reached 3-4x10⁶ cells/ml.

2.2.1.2 Transfection of Sf9 cells.

The insertion of a gene of interest into the baculovirus genome is usually achieved by homologous recombination between a transfer plasmid and the viral DNA. This occurs when the plasmid and viral DNAs are simultaneously introduced (co-transfected) into host insect cells. A liposome-mediated transfection was employed (O'Reilly, 1994).

Cells were seeded (3x10⁶/5ml) into 5ml IPL41 medium in 25 cm² flasks and allowed to attach for at least 1hr. The lipofectin solution (GibcoBRL) was diluted 2:1 with sterile water and then mixed with an equal volume of a 1:6 mixture of BaculoGold DNA (Pharmingen) together with the recombinant plasmid in a polystyrene tube. The mixture was incubated at room temperature for 15min to allow the liposomes and DNA to fuse. Meanwhile, the cells were washed twice in serum and lipid free IPL41, and the transfection mixture was then added to the cells in 1.5 mls of serum free IPL41 and incubated overnight, after which time the transfection medium was replaced with complete IPL41. Medium containing recombinant virus was harvested after 4-5 days.
2.2.1.3 Baculovirus production and amplification

The virus produced as described in section 2.2.1.3 was harvested by centrifugation at 3000g and 1ml aliquots were used to infect $3 \times 10^6$ Sf9 cells 75 cm$^2$ flasks. Four days post-infection, virus was harvested and 1ml of this virus stock was used to infect $2 \times 10^9$ cells in a 175cm$^2$ flask. This amplified virus was then used to make a passage 4 virus stock which was titred to determine the amount of virus required for optimum protein production. A range of dilutions of the virus stock, generally between 1/5 and 1/10,000 were used to infect $1 \times 10^6$ cells in 25cm$^2$ flasks. Cells were harvested at 2-3 days post-infection and washed once in PBS prior to lysis and analysis of protein production as described in section 2.3.

2.2.1.4 Identification and purification of recombinant baculovirus

The passage 4 virus stock contains the required recombinant virus mixed with non-recombinant and single crossover viruses (O'Reilly, 1994) which affect the maximum levels of protein expression. If protein expression was low, recombinant viruses were plaque purified (O'Reilly, 1994) 149-165) to obtain clonal isolates of a potential recombinant virus, which was then used to make a high titre virus stock.

2.2.1.5 Protein expression in Sf9 cells

Sf9 cells were infected with different amounts of the final amplified virus and harvested at different times between days 1-3 post infection. The levels of protein expression were monitored by analysing the whole cell lysates (section 2.3.1) at each time point using SDS-PAGE (2.3.4) and coomassie blue staining. In this way, the time required for maximum expression of the protein was determined.

2.2.2 Mammalian cell culture techniques

2.2.2.1 Cell lines and culture

All cells were grown at 37°C, 10% (v/v) CO$_2$, 99% relative humidity (RH) in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM). HL60 and Jurkat cells were grown in RPMI containing 10% (w/v) FCS and 60 IU/ml penicillin and 100µg/ml streptomycin. 3T3L1 fibroblasts and COS7 cells were grown in DMEM containing 10% (w/v) foetal calf serum, 60IU/ml penicillin and 100µg/ml streptomycin. HL60 and Jurkat cells were passaged every 2-3 days Cells were harvested by centrifugation at 900rpm for 5min, then washed in PBS, re-centrifuged and stored at -20°C. COS7 cells were passaged when 80-90% confluent by washing adherent cells twice with PBS and incubating with 1ml of trypsin-EDTA (Gibco) for 4-5 min at 37°C. 5mls
of fresh media was then added to inactivate the trypsin and the cells were then diluted 1 in 10. Cells were passaged every 4-5 days.

2.2.2.2 In vivo labelling with $^{35}$S methionine.

To prepare $^{35}$S methionine labelled cell lysates, sub-confluent Jurkat cells were incubated for 16 hours at 37°C in 90% (v/v) methionine-free DMEM, 10% (v/v) DMEM, 1% (v/v) FCS and 150-200μCi/ml $[^{35}$S] methionine (<1000 μCi/mmol methionine, NEN Research Products), then washed twice in cold PBS and lysed as described in section 2.3.2.

2.2.2.3 Transfection of COS cells

COS7 cells were transfected with SV40-based expression plasmids (for example pcDNA3 (Invitrogen), pEFBOS (Mizushima and Nagat, 1990)) using a modified DEAE-dextran method (Vaheri and Pagano, 1965). Cells were passaged the previous day and transfected when 50% confluent. Cells were transfected with 15μg plasmid DNA per 15cm tissue culture dish (10^6 cells). The DNA was first added to RPMI containing 10% heat-inactivated NuSerum, 2mM glutamine and 50μg/ml gentamycin. This was followed by the addition of 100μm chloroquine and 200μg/ml DEAE-Dextran to the transfection medium. 10mls of transfection medium were added per dish and incubated at 37°C for 4 hours. (Timing here is important and the transfection must not be allowed to exceed 4 hours as chloroquine and DEAE-Dextran are toxic to the cells). The transfection medium is removed and replaced with 6mls of PBS containing 10%DMSO and incubated for a further 2min. The DMSO solution was removed and the cells were washed in TBS before the addition of 25mls of complete RPMI medium and further incubation. 48 hours post transfection the cells were detached with HBSS containing 0.05% Trypsin and 0.53mM EDTA.

2.2.2.4 Cell culture and scrape loading with recombinant proteins

Swiss 3T3 cells were maintained at 37°C in DMEM containing 10% (w/v) foetal calf serum and 10% (v/v) CO₂. In preparation for scrape loading, 8x10^5 cells were seeded into 100mm dishes, grown to confluence then washed and incubated in serum-free DMEM for 16 hours. The cells were washed twice with phosphate buffered saline (PBS), recombinant protein was added in 160ul of scrape-loading (SL) buffer (10mM Tris-HCl pH7.0, 114mM KCl, 15mM NaCl, 5.5mM MgCl₂ (Leevers and Marshall, 1992) and cells were scrape loaded according to a previously described method (Morris et al., 1989).
2.2.2.5 Immunofluorescence and microscopy
Sterile glass coverslips were placed in multiwell cell culture dishes, washed with distilled water and incubated with 13.3μg/ml poly-L-lysine (PLL) for 10min at room temperature. Scrape loaded cells were washed with serum free medium and added to PLL coated coverslips at a density of 1x10^5 cells per ml. After incubation at 37°C, and 10% CO_2 for between 10 min and 1 hour, cells were fixed with 3% paraformaldehyde and permeabilised with 0.2% Triton X-100. Actin filaments were detected as previously described (Ridley and Hall, 1992) using tetramethylrhodamine isothiocyanate (TRITC)-labelled phalloidin. Cells were viewed using a Zeiss Axiophot microscope and photographed using Kodak TNY-400 film.

2.3 The Analysis Of Cellular Proteins

2.3.1 Preparation of whole cell lysates
Cells were washed twice with PBS and resuspended in between 20-500μl of SDS-PAGE sample buffer (2% (w/v) SDS, 62.5mM Tris-HCl pH7.5, 10% (v/v) glycerol, 1mM DTT, 0.01% (w/v) bromophenol blue). The sample was heated at 95°C for 5 min and analysed by SDS-PAGE.

2.3.2 Triton X-100 lysis of cells
Cells were washed twice with PBS and resuspended in 0.5 ml of ice-cold extraction buffer ( 50 mM Tris.HCl pH 7.4, 150mM NaCl, 50mM NaF, 5mM EDTA, 1% (w/v) Triton X-100, 500μM sodium orthovanadate, 2mM PMSF, and 100 kallikrein inhibitor units of aprotinin). The lysate was incubated on ice for 20min, followed by centrifugation at 10,000g for 20min at 4°C to remove cell debris. The lysate was then subjected to further biochemical analyses. For rat brain and liver extracts the tissue homogenate was first centrifuged for 10min at 10,000rpm then lysed in ice-cold extraction buffer. Following incubation on ice, the lysate was recentrifuged for 45min at 50,000 rpm at 4°C.

2.3.3 Immunoprecipitations of proteins
Cell lysates were prepared as described in section 2.3.2 and incubated with appropriate primary and secondary antibodies in tubes on a rotating wheel for 2 hours at 4°C. The immune complexes were collected by the addition of either Protein A Sepharose CL4B or Protein G Sepharose Fast flow (Pharmacia). The immunoprecipitates were collected by low speed centrifugation (2000g) and washed twice with 1ml ice-cold lysis buffer.
The samples were washed as required for further analyses or SDS-PAGE sample buffer was added to immobilised immunoprecipitates, followed by heating at 95°C for 5min and electrophoresis on an SDS-PAGE gel.

2.3.4 SDS polyacrylamide gel electrophoresis (PAGE)

SDS PAGE was performed by a procedure modified from Laemmli (1970). Gels were 0.75mm thick and comprised a 375mM Tris HCl pH8.8 resolving gel with an acrylamide:bisacrylamide ratio of 30:0.5 and a 125mM Tris HCl pH 6.8 stacking gel with an acrylamide:bisacrylamide ratio of 30:0.75. Electrophoresis was carried out in electrophoresis buffer (200mM glycine, 25mM Tris-HCl pH 8.3, 0.1 % (w/v) SDS), typically at 40-100V.

2.3.4.1 Coomassie Blue Staining of SDS-PAGE gels

The presence of proteins in SDS-PAGE gels was visualised by soaking the electrophoresed gel in Coomassie blue stain (45% (v/v) methanol, 10% (v/v) acetic acid, 0.25% (w/v) Coomassie blue) for 20min, followed by destaining in 20% (v/v) methanol, 14% (v/v) acetic acid. The gel was then dried under vacuum for 40min at 80°C.

2.3.4.2 Silver Staining of SDS-PAGE gels

Silver staining of SDS-PAGE gels was used to visualise amounts of protein in the nanogram range. Following electrophoresis, the proteins were fixed in the gel for 1hour using 25% (v/v) propan-2-ol, 10% (v/v) acetic acid, followed by incubation in 10% (v/v) glutaraldehyde for 30min. The gel was then repeatedly washed in water over a period of 2 hours before being sensitised in 5μg/ml DTT. This was followed staining in 0.1%(w/v) AgNO₃ for 30min. The gel was then washed briefly in water then 3% sodium carbonate/0.05% (v/v) formaldehyde was added to develop the bands. Once protein bands were visible, the reaction was terminated by the addition of 25ml of 2.3M sodium citrate.

2.3.5 Estimation of protein concentration

Protein concentrations were estimated in solution using a Coommasie Blue based assay (Pierce) according to the manufacturer's instructions. Briefly, 0.75ml of reagent was mixed with 0.75ml protein solution and the absorbance measured at 595nm. The protein concentration was then determined by comparison with a bovine serum albumin standard curve.
2.3.6 Electroblotting SDS-PAGE-resolved proteins

Following electrophoresis, separated proteins were electrophoretically transferred onto an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore Corporation) in 50mM Tris, 380mM glycine, 0.1% SDS and 20% methanol at 0.95 A for 1 hour or at 25V for 16 hours. The polyvinylidene difluoride (PVDF) membrane was pre-soaked in methanol, then washed with water and transfer buffer prior to use.

2.3.7 Western blotting

All western blot analysis was performed using enhanced chemiluminescence (ECL) reagents (Amersham Life Sciences). All incubations were performed on an orbital shaker and at room temperature unless otherwise specified. PDVF membranes were blocked for 1 hour in 5% (w/v) low fat milk powder (Marvel, Premier Brands UK Ltd.) in PBST (20mM phosphate pH7.4, 150mM NaCl, 0.05% Tween 20), then incubated with the primary antibody diluted in PBST containing 3% (w/v) low fat milk powder for 2 hours. Unbound primary antibody was removed by rinsing twice and washing (3x5min) in the same buffer, then the blots were incubated for 1 hour in the appropriate horseradish peroxidase conjugated secondary antibody (1/1000 dilution) (Pierce and Warriner (UK) Ltd) in PBST. The blots were rinsed in PBST buffer, developed with ECL reagents (Amersham Life Sciences) and autoradiographed upon x-omat XAR film, as directed in the manufacturer's instructions.

2.3.7.1 Stripping and Reprobing PVDF membranes

Membranes were stripped of bound antibodies by immersing the membrane in stripping buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH6.7). Following incubation at 65°C for 30min with occasional agitation, the membranes were washed in large volumes of PBST to remove the mercaptoethanol and blocked Membranes were then blocked and reprobed (as described in section 2.3.7).

2.4.1 Purification of native p85α

2.4.1.1 Preparation of phosphotyrosine affinity matrix

10mls of Actigel (Sterogene) was equilibrated in 100mM phosphate buffer pH 7.8. O-phospho-L-tyrosine (Sigma) (50mgs) was dissolved in 50mls of the same buffer and coupled to Actigel at 4°C for 4-6hrs. The resin was washed, then unreacted sites were blocked using 100mM Tris pH 8.0 for a further 2-4hrs. The affinity matrix was
washed several times in PBS (20mM phosphate pH 7.4, 150mM NaCl) and stored at 4°C with 0.02% sodium azide.

2.4.1.2 Phosphotyrosine Affinity Purification
The method described here utilises the inherent affinity of the two SH2 domains of p85α for phosphotyrosine (Koegl et al., 1994), and was therefore applicable to purifying p85α or any SH2-domain containing protein, provided that the SH2 domain was not blocked due to being involved in another inter- or intra-molecular interaction.

Between 5x10⁸ and 2x10⁹ Sf9 cells were infected with a predetermined volume of baculovirus encoding p85α so as to give a multiplicity of infection (moi) of between 5 and 10. Cells were harvested 2.5 days post infection, by centrifugation at 800 g for 20min, washed in ice-cold PBS and then re-pelleted by centrifugation. The Sf9 cell pellet was resuspended in FPLC buffer A (10mM HEPES pH7.5, 5mM NaF, 0.2mM sodium orthovanadate, 2mM EDTA, 10mM Benzamidine, 50mg/ml phenylmethylsulfonyl-fluoride [PMSF] and 5mM DTT), then the cell membranes disrupted by dounce homogenisation. After 30min incubation on ice, the lysate was cleared of particulate material by centrifugation at 35,000rpm for 45min at 4°C. All subsequent procedures were carried out at 4°C. The supernatant was loaded onto a 10ml phosphotyrosine affinity column containing 5mg phosphotyrosine per ml of matrix that had been pre-equilibrated in FPLC buffer A. The column was washed with FPLC buffer A until elution of unbound material was no longer detected by absorbance at 280nm. Bound protein was eluted at a flow rate of 2ml/min, and 2.5ml fractions were collected. Using SDS-PAGE, all fractions were assayed according to procedures described in section 2.3.4. The fractions containing p85α were pooled and buffer exchanged into 20mM Tris pH8, 5mM DTT and immediately applied on to a 4.6x100mm Poros 20 HQ column (PerSeptives Biosystems). After washing in the same buffer, bound protein was eluted with 30ml 20mM Tris pH8, 5mM DTT followed by a 30ml linear gradient from 0 to 0.5M NaCl at a flow rate of 2ml/min. Fractions (2.5ml) containing p85α were exchanged into storage buffer (50% ethylene glycol, 40mM Tris, 10mM EDTA, 10mM benzamidine and 5mM DTT) by using prepacked Sephadex G25 columns (PD10, Pharmacia) then concentrated using Centriprep microconcentrators (Amicon) with a membrane that had a molecular weight cut off of 10kDa. Purified and concentrated protein was stored at -20°C.

2.4.2 Purification of p85 mutants
Mutants of p85α with two SH2 domains were purified using the method just described (section 2.4.1.2) however, in some cases anion exchange chromatography was not used, as affinity purification gave adequate levels of purity for biochemical analyses.
2.4.5 Preparation of glutathione S-transferase-fusion proteins

Glutathione S-transferase (GST) fusion proteins were expressed using the pGEX expression vectors (Pharmacia) in *E.coli*. Subcloning of the desired p85α mutant in frame with the ATG of GST allowed expression of a fusion protein with a thrombin cleavage site just carboxy terminal to the GST. GST fusion proteins were purified by a modification to the procedure previously described (Smith and Johnson, 1988). Bacteria were lysed in lysis buffer (50mM Tris, 1% TritonX-100, 150mM NaCl, 1mM EDTA, and 1mM NaF) containing 10μg/ml leupeptin and 10μg/ml pepstatin. The GST fusion proteins were purified using glutathione-Sepharose-4B (Pharmacia), and eluted using 10mM glutathione according to the manufacturer’s instructions. Where required, proteins were cleaved with thrombin (Sigma chemicals) to remove the GST portion. Both the GST fusion proteins and the cleaved proteins were dialysed into 50% glycerol, 20mM Tris pH 8, 150mM NaCl and 1mM DTT and stored at -20°C.

2.5 Size Determination Methods

2.5.1 Size-Exclusion Liquid Chromatography

The apparent molecular weight, or more precisely, the Stoke’s radius of a protein, can be measured as a function of its elution time from a calibrated analytical size-exclusion column.

Size-exclusion chromatography was performed on a 10x300mm Superose 12 column (Pharmacia) connected to an "Integral" workstation (PerSeptive BioSystems). The column was equilibrated in 20mM Tris pH 8, 150mM NaCl that had been filtered through a 0.2μm filter (Millipore) The column was calibrated using a mixture of protein standards of known molecular weight (BioRad). The sample volume injected was between 10μl- 200μl and 1min fractions were collected where necessary at a flow rate of 300μl per minute. Absorbance was monitored at 220nm and the elution time of the maximum absorbance used to determine the apparent molecular weight of purified recombinant proteins.

2.5.1.1 Sample preparation for size exclusion chromatography

Recombinant proteins were prepared as described in section 2.4. COS7 cells were harvested by trypsinisation then dounce homogenised in hypotonic lysis buffer (5mM Tris HCl pH 7.5, 2.5mM KCl, 1mM DTT, 1mM EDTA, 1mM PMSF, 10μM leupeptin, 10μM pepstatin, 1mM 1,10 phenanthroline, 1mM Na₃VO₄). The cytoplasmic fraction was clarified by centrifugation at 100,000g at 4°C for 45min. The
supernatant was then made isotonic by dilution with an equal volume of lysis buffer containing 20% sucrose. Following centrifugation at 100,000g at 4°C for 45min, the supernatant was applied to the size-exclusion column described in section 2.5.1. Total protein in each fraction was precipitated using 10% trichloroacetic acid and analysed by SDS-PAGE and western blotting.

2.6 Analysis Of Protein Phosphorylation And Kinase Activity

2.6.1 In vitro protein kinase assay

Affinity-purified proteins were immobilised on an appropriate affinity matrix and equilibrated in kinase buffer (50mM HEPES pH 7.4, 50mM NaCl, 2% (v/v) glycerol, 12mM MnCl₂, 2mM MgCl₂, 0.1% (w/v) Triton X-100) then resuspended in a final volume of 30μl of kinase buffer containing 0.3μCi of [γ³²P]ATP. The kinase reaction was allowed to proceed for 15min at room temperature, unless otherwise stated, after which time it was terminated by the addition of SDS-PAGE sample buffer. Phosphorylated proteins were then separated by SDS-PAGE and detected using autoradiography or exposure to a phosphorscreen (Molecular Dynamics).

2.6.2 Immunodetection of phosphotyrosine

Tyrosine phosphorylated proteins were identified by immunoblotting with antiphosphotyrosine antibodies according to a previously described method (Kamps and Sefton, 1988). Proteins were separated by SDS PAGE and transferred to a PVDF membrane by Western blotting as described in section 2.3.7 but with the addition of 7.5% (w/v) sodium orthovanadate to the transfer buffer. The membrane was incubated for 2hrs in blocking buffer at room temperature (section 2.3.7) then with a 1:1000 dilution of PY20 anti-phosphotyrosine antibody (Transduction Laboratory) for 2hours at room temperature in blocking buffer. The blot was washed 3 times for 5min in PBST and then incubated for one hour with secondary antibody before being processed using the ECL kit (Amersham) as previously described.

2.6.3 PI3K assay

PI3K assays were carried out essentially as described previously (Whitman et al., 1987) in a total volume of 50μl containing 50mM HEPES pH7.4, 100mM NaCl, 1mM DTT, 0.5mM EDTA, 5mM MgCl₂, 100μm ATP (plus 0.5μCi of [γ-³²P]ATP/assay), and 200 μg/ml phosphatidylinositol. Recombinant PI3K was immobilised using an
appropriate affinity matrix and added to the kinase reaction mixture and incubated for 15 min at room temperature. The reaction was terminated by the addition of 100μl 1M HCl and 200μl of chloroform/methanol (1:1). The mixture was vortexed, the upper phase discarded and the lower organic phase washed with 80μl of methanol/1M HCl (1:1). After centrifugation, the upper phase was again discarded and the lower phase containing radiolabelled lipids was evaporated to dryness. Reaction products were resuspended chloroform/methanol (4:1) and loaded onto thin layer Silica Gel-60 plates (Whatman) [pre-treated with 1% (w/v) oxalic acid, 1mM EDTA in water/methanol[6:4]] and developed in a mixture of chloroform, methanol, and 4M ammonia (9:7:4).

2.7 Analysis Of Protein-Protein Interactions

2.7.1 Affinity Purification of p85 domain-binding proteins

Affinity resins for the isolation of p85 domain binding proteins were prepared by immobilising GST fusion proteins using glutathione Sepharose CL6B (Pharmacia) as described (section 2.4.3). Affinity matrices were incubated with 1% Triton X-100 extracts of rat brain, rat liver, Jurkat or HL60 cells for 2h at 4°C. After extensive washing, proteins bound to the resins were released by boiling in SDS PAGE sample buffer, proteins were separated by SDS-PAGE, and visualised by silver staining.

2.7.1.1 Biotinylation of Proteins and peptides

Proteins were biotinylated in 100mM sodium phosphate pH 6.5-8.0. Approximately 20μg of protein was incubated with a 10-fold molar excess of N-hydroxysuccinimidebiotin (Pierce) for 1.5h at room temperature. To block unreacted sites, 0.2M Tris pH 8 was added as a source of amino groups, and incubation was continued for 1hour at 4°C. Unincorporated biotin was removed using a PD10 column (Pharmacia) and the biotinylated protein was exchanged into a storage buffer containing 20mM Tris pH7.5, 150mM NaCl and 50% glycerol, and stored at -20°C.

2.7.2 GTPase activity assays

The Rho family members, Rac1, Cdc42 and RhoA (gifts from Dr Alan Hall) were expressed as GST fusion proteins as described in section 2.4.3.

The apparent loss of γ-32P as an outcome of the hydrolysis of the radiolabelled [γ-32P]GTP bound to the recombinant Rho protein was determined by measuring the [γ-32P]GTP remaining on nitrocellulose filters at 22°C in the presence of a GAP. For GTPase assays under subsaturating conditions, the Rho proteins (30ng) were pre-
loaded with [\(\gamma^{32}\)P]GTP (10\(\mu\)Ci, 6,000 Ci/mmol) in 20\(\mu\)l of 20mM Tris-HCl pH 7.6, 0.1mM DTT, 25mM NaCl and 4mM EDTA for 10min at 30°C. Exchange was stopped by the addition of MgCl\(_2\) (to a final concentration of 17mM) and the protein was kept on ice. Preloaded protein (3\(\mu\)l (3.5ng)) (to give a final concentration of 6nM) was diluted with 20mM Tris-HCl pH 7.6, 0.1mM DTT, and an aliquot of the GAP protein to a final volume of 30\(\mu\)l. NaCl was added to a final concentration of 10mM (low ionic strength) or 150mM (high ionic strength) and the mixture incubated at 20°C. 5\(\mu\)l samples were removed at 0, 5, 10, and 15min, diluted into 1ml of ice-cold buffer A (50mM Tris-HCl pH 7.6, 5mM MgCl\(_2\), and 50mM NaCl) and filtered through nitrocellulose filters (prewetted with buffer A). The filters were washed three times with ice-cold buffer A, and the amount of [\(\gamma^{32}\)P]GTP remaining bound to the filters was measured by scintillation counting.

2.7.3 Protein interaction analysis in real-time using the BIAcore biosensor

An optical biosensor (BIAcore, Pharmacia) was used in binding studies to measure relative levels of protein-protein interactions. This method uses the phenomenon of surface plasmon resonance (SPR) to detect interactions between macromolecules. In order to measure these interactions, one of the interacting molecules was immobilised on a dextran layer, which is in turn mounted on a gold surface. A monochromatic wedge of light is shone on this surface at a particular angle, resulting in deflection at a particular angle. SPR causes one component of the light, termed the evanescent wave, to interact with the electrons on the gold surface, resulting in a dip in the intensity of the reflected light. The angle at which this dip is observed (resonance angle) changes with the refractive index of the medium. The binding partner is injected over this surface and as binding occurs, the refractive index of the medium is increased which in turn is proportional to the amount and molecular weight of the macromolecules bound at any time. The resonance angle is measured by the instrument and converted to a plot of resonance units (RU) versus time. The RU value is proportional to protein at the dextran matrix.

The basic operating procedures of the Biosensor were followed as previously described (Jonsson et al., 1991). Binding was measured in real time under accurately controlled conditions, including temperature and flow rate. The BIAcore buffer used in all experiments consisted of 20mM HEPES pH 7, 150mM NaCl, 3.4mM EDTA, 0.005% Tween 20 and 4mM DTT. All proteins were exchanged into this buffer using PD10 columns (Pharmacia). Peptides (Alta Bioscience) were purified by reversed-phase
high-performance liquid chromatography (RP-HPLC). Purified peptides were dried to remove organic solvent then resuspended in 100μl of 0.1 M phosphate buffer, pH7.8, and biotinylated as described in section 2.7.1.1. The biotinylated peptides were separated from non-biotinylated peptides and unreacted biotin by RP-HPLC and their molecular weight determined by time-of-flight laser desorption mass spectrometry in order to confirm that biotinylation had occurred.

The dextran layer on the biosensor was activated with a mixture of N-hydroxysuccinimide and N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Pharmacia) to enable it to bind free amino groups. Avidin (Boehringer Mannheim) at 50μg/ml in 29mM sodium acetate pH 4.0, was immobilised on the sensor chip surface and unreacted groups were blocked using 1M ethanolamine pH 8. The biotinylated peptides were bound to avidin at a flow rate of 5μl/min. GST fusion proteins were then injected over the surface at 5μl/min at a constant temperature of 25°C, and the amount of binding, as measured by the change in RU was determined. Bound protein was removed using BIAcore buffer or by competition using an excess of non-biotinylated peptide. Any material remaining bound to the surface after analysis was removed with 4μl 0.05% SDS.

2.7.4 In vitro activation of protein-tyrosine kinases and substrate association

This assay was performed essentially as previously described (Kazlauskas and Cooper, 1990). Sf9 cells were infected with a baculovirus encoding the desired kinase as described and harvested 48 h after infection as described in section 2.2.1. The relevant tyrosine kinase was immunoprecipitated from the lysate using an appropriate antibody and protein collected on Protein A-Sepharose CL4B. The immunocomplex was then subjected to extensive washing with lysis buffer, followed by two washes with kinase buffer (section 2.6.1).

In vitro phosphorylation was carried out in the presence of 20μM ATP at room temperature for 20 min. The immunoprecipitates were washed with lysis buffer again to remove free ATP and incubated for 2hrs at 4°C together with the substrate. The immune complexes were washed again with lysis buffer to remove free unlabelled ATP and subjected to an in vitro kinase assay (section 2.6.1) or assayed for associated PI3K activity (section 2.6.3).
Chapter 3: CONSTRUCTION AND EXPRESSION OF THE p85α MUTANTS

3.1 Aims

The p85α adaptor subunit is a large multidomain protein. In order to investigate the structural and functional roles of individual domains and interdomain interactions, it was necessary to introduce a range of point mutations and deletions in the p85α wild type cDNA. This chapter describes first, the cDNA manipulation strategies involved in constructing these mutants, and secondly, the expression and purification of the encoded proteins from bacteria (E.coli), insect (Sf9) and mammalian (COS7) cells. A summary of all these constructs is shown in Figure 3.1. Each expressed recombinant protein will be referred to by the nomenclature assigned in Figure 3.1. The amino acid boundaries of each mutant are described in Table 3.1.

3.2 Results

3.2.1 Construction and Expression of bacterial p85α GST fusion proteins

cDNAs encoding various domains of bovine p85α were amplified by PCR using wildtype p85α cDNA as a template and subcloned into the pGEX2T expression vector (Pharmacia). To facilitate sub-cloning, BamH1 and EcoR1 sites were introduced 5’ and 3’ ends of the cDNA respectively using oligonucleotides with appropriate sequences. Termination codons were also inserted at the 3’ end of each coding sequence internal to the EcoRI site. DNA sequence was determined for resultant clones (ABI) and appropriate constructs were selected for subsequent protein expression. Figure 3.2 outlines the strategy employed to subclone these mutants of p85α into the pGEX-2T expression vector.

The resultant GST fusion proteins were purified as described in section 2.4.3, with yields of approximately 5-10 mgs of recombinant protein per litre culture. Recombinant proteins were utilised either as GST fusion proteins or as thrombin-cleaved preparations (denoted in text). Proteins purified in this manner were greater than 90% pure as assessed by SDS-PAGE and Coomassie blue staining, (Figures 3.4, 3.5 and 3.7), and no further purification steps were deemed necessary unless the proteins were to be used for structural studies. Figure 3.3 depicts the amino acid boundaries and molecular sizes.
of the encoded proteins. Figures 3.4 and 3.5 show Coomassie stained SDS-PAGE analysis of the purified proteins.

### 3.2.2 Site-directed mutagenesis of selected proline residues

Selected proline residues within the proline-rich motifs of p85α were mutated to alanines using a PCR based approach. Where possible, sequence alterations were introduced in conjunction with suitable restriction sites to permit sub-cloning at the ends of the targeted sequence. Site-directed mutagenesis by overlap extension using PCR was employed to generate point mutations (Ho et al., 1989). This method allows the introduction of specific mutations into the nucleotide sequence directly from a cDNA in its original vector, and without a requirement for digestion by restriction endonucleases or ligation with DNA ligase. The method is illustrated schematically in Figure 3.6.

Briefly, two PCR reactions are performed to amplify the two fragments of the target gene sequence. Each reaction requires one flanking oligonucleotide that hybridises at one end of the target sequence (primer ‘x’ or ‘y’ in Figure 3.6.), and with one internal oligonucleotide that hybridises at the site of the mutation and contains the mismatched bases (primer ‘y’ or ‘z’ in Figure 3.6). The product generated by PCR incorporates the primers, and therefore the wild type sequence will not be amplified. By using two internal primers that overlap, the two fragments CB and EF, generated in the first PCR, can be fused by denaturing and annealing them in a subsequent primer extension reaction. The overlap generated allows one strand from each fragment to act as a primer on the other, and extension of this overlap results in the mutant cDNA product. Even though the annealing of the short overlap between the two fragments may occur at low frequency, the inclusion of additional flanking primers (‘x’ and ‘y’ in Figure 3.6.) allows the ‘fusion’ product that is formed to be amplified by PCR. p85αΔP1a, p85αΔP2, and p85αΔP1aP2 were generated by this method (Figure 3.7).

### 3.2.3 Deletion of domains in the p85α protein

#### 3.2.3.1 p85αΔSH3

The SH3 domain is located at the amino-terminus of the p85α protein and is encoded by DNA containing a unique KpnI site 18bp after the ATG. In order to introduce convenient restriction sites to aid cloning, the p85α cDNA was sub cloned into the mammalian expression vector PMT2SM, which was derived from pMT2 by insertion of an oligonucleotide containing an engineered multiple cloning site (Kaufman et al., 1989). A 767bp BglII-EcoRV fragment of p85α was amplified by PCR. The 5' oligonucleotide was used to incorporate an additional KpnI site. This allowed the
fragment to be digested with KpnI and EcoRV and ligated into the p85αpMT2SM cDNA. A schematic flow diagram of the cloning strategy is shown in Figure 3.8(i).

3.2.3.2 p85αΔBH
Amino acids 134-302 of p85α, corresponding to the BH domain were deleted from the p85α cDNA using a PCR based approach (Imai et al., 1991). A fragment of p85α cDNA, corresponding to bp 241-1020, was cloned into pGEM-1 (Promega). Oligonucleotide primers were designed in inverted tail to tail directions to amplify the cloning vector together with the target sequence. The deletion was generated by amplification with primers that have a corresponding gap between their 5’ ends. The amplified linear DNA generated was self-ligated and used to transform appropriate DNA. The new insert was digested from the self-ligated pGEM-1 with BgIII and EcoRV and subcloned into the same sites in the pGEX2Tp85α construct. The strategy used is outlined in Figure 3.8 (ii). Figure 3.7 shows a Coomassie Blue stained SDS-PAGE of purified GST p85αΔSH3 and p85αΔBH.

3.2.3.3 p49α and p55γ
p49α is an amino-terminal truncation of p85α and contains both the SH2 domains and the inter-SH2 region (a gift from Mr Jeff Linacre). The cDNA encoding this truncated p85α was cloned into the PET bacterial expression vector. A carboxy-terminal hexahistidine tag (histag) was incorporated using PCR with appropriate oligonucleotides, to aid purification. The same construct was also subcloned into a baculovirus transfer vector (pBlueBac4) such that p49α could be co-expressed with p110α the catalytic subunit of PI3K, in insect cells. p55γ is a naturally occurring isoform of the bovine p85α which lacks the amino-terminal SH3 and BH domains (a gift from Dr Françoise Pages). It is homologous to p49α and differs only by an amino-terminal extension of 34 amino acids, which contains a proline-rich motif with a consensus sequence similar to that of the second proline-rich motif of p85α (Figure 3.11). p55γ was subcloned into a baculovirus transfer vector (PVL1393) and expressed in Sf9 cells. An amino acid sequence alignment between p85α, p85β and p55γ is shown in Figure 3.12. p49αΔ110BS is essentially p49α with a deletion within the inter-SH2 domain of 45 amino acids (residues 478-513) corresponding to the p110 binding site (a gift from Dr Ritu Dhand).
3.2.4 Expression of p85 mutants in insect (Sf9) cells

Expression of mammalian proteins in insect cells provides a cellular environment with similar protein translation machinery to a mammalian cell and therefore over-expressed recombinant proteins are correctly folded, have correct disulphide bonds and oligomerisation properties and, additionally, are post translationally modified. Compared to other higher eukaryotic expression systems, the unique feature of the baculovirus expression system is its potential to achieve high levels of expression of a cloned gene. For this reason, expression of large, multisubunit and post translationally modified proteins is often more successful than in bacterial cells. The baculovirus that naturally infects insect cells has been modified in order to facilitate the incorporation of the gene of interest into the genome of the insect cell.

The baculovirus expression system generally expresses recombinant proteins at a high level compared to mammalian expression systems. High level expression was achieved by placing the coding region of a cDNA under the control of the strong promoter, known as the polyhedron promoter. Several longer p85α mutants were sub-cloned downstream of the polyhedron promoter using the BamHI and EcoRI sites of the pVLI393 baculovirus transfer vector (Invitrogen). Several clones containing inserts were identified by subsequent digestion of miniprep cDNA. Two clones for each mutant were transfected into Sf9 cells where they underwent cDNA recombination with co-transfected baculoviral DNA (section 2.2.1.2) and passaged until a high titre viral stock was obtained (on average at passage four). The viruses were then further characterised by determining the optimal harvest time and the optimal titre of virus required to maximise protein expression levels. Figure 3.9 outlines the cloning strategy, and Figure 3.10 shows a Coomassie-Blue stained SDS-PAGE analysis of the expression levels of the p85 baculovirus proteins at various times post-infection. The proteins were immunoprecipitated from a TritonX-100 lysate of baculovirus infected Sf9 cells using phosphotyrosine covalently coupled to Actigel (Sterogene) and analysed by SDS-PAGE. Maximal expression was observed between day 2 and day 3 post infection. Around day 4, the virus enters its lytic phase and the protein begins to degrade.

3.2.4.1 Purification of recombinant p85 mutant proteins from insect cells

Recombinant p85α, p85αΔP1, p85αΔP2, p85αΔP1P2, p85αΔSH3 and p85αΔBH were produced in Sf9 cells as described in section 2.2.1.5 and purified as described in section 2.4.1.2. The relevant protein-containing fractions were detected by a protein
assay (section 2.3.5), were pooled, buffer-exchanged into storage buffer and stored at -20°C as described in section 2.4.1.2. Figure 3.11 shows a Coomassie Blue stained SDS PAGE analysis of the p85α mutants expressed in Sf9 cells, and a typical chromatographic profile of absorbance at 280nm versus time obtained during purification of p85α.

3.2.4.2 Additional constructs

Other constructs described in this thesis include the amino terminal SH2 domain of p85α, (p85αN-SH2) and the carboxy terminal SH2 domain of p85α, (p85αC-SH2), both were a gift from Mr Jeff Linacre. p85β was a gift from Dr Ivan Gout and p110α glutag was a gift from Dr Meredith Layton.

Although the p85α mutants varied in their level of expression, they were all soluble and the concentrations required for the experiments presented in this thesis were achieved. The bacterial fusion proteins could be produced in milligram quantities and maintained their structural integrity during storage for several months. However, proteins expressed in Sf9 cells were more susceptible to degradation, thus preparations were stored for a maximum of eight weeks.
Figure 3.1 Schematic summary of p85α and the expression systems used to produce them.
Table 3.1 Amino acid sequence specifications for p85α and p85 mutants. Amino acid residue numbering is derived from that of bovine p85α. Amino acid sequences are designated according to the following examples: 86-742 contains residues 86-742 from the p85α sequence; P96A indicates that the proline residue at position 96 has been mutated to an alanine residue.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Amino Acid Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>p85α</td>
<td>1-742</td>
</tr>
<tr>
<td>p85αΔSH3</td>
<td>86-742</td>
</tr>
<tr>
<td>p85α ΔP1a</td>
<td>1-742,P96A,P99A</td>
</tr>
<tr>
<td>p85α ΔP2</td>
<td>1-742,P308A,P311A</td>
</tr>
<tr>
<td>p85α ΔP1aP2</td>
<td>1-742,P96A,P99A,P308A,P311A</td>
</tr>
<tr>
<td>p85αΔBH</td>
<td>1-133,303-742</td>
</tr>
<tr>
<td>p49α</td>
<td>333-742</td>
</tr>
<tr>
<td>p85αSH3-BH-SH2</td>
<td>1-446</td>
</tr>
<tr>
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<td>1-339</td>
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<td>1-106</td>
</tr>
<tr>
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<td>1-112, P96A,P99A</td>
</tr>
<tr>
<td>p85αSH3ΔP1b</td>
<td>1-112, P91A,P92A,R90Q</td>
</tr>
<tr>
<td>p85αSH3</td>
<td>1-86</td>
</tr>
</tbody>
</table>
Digest p85 inserts and pGEX2T with Bam and EcoRI
Ligate
Transform
Select positives
Sequence clones
Replicate in E.coli
Produce recombinant protein

Figure 3.2 Cloning and expression of p85 mutants in the pGEX-2T expression system
* represents proline to alanine point mutations. ■■ represents a deleted domain
Figure 3.3 p85α GST fusion proteins

The numbers beneath p85α full length (I) indicate the residue number at which each domain or motif begins and ends. The sizes are the predicted molecular weights as determined from the amino acid sequence.
Figure 3.4 Purified amino-terminal p85α mutants expressed in E.coli

Proteins were resolved on a 15% SDS-PAGE gel and stained with Coomassie Blue
Lane 1 GSTp85αSH3-P1, Lane 2 p85αSH3-P1, Lane 3 GSTp85αSH3-BH,
Lane 4 p85αSH3BH, Lane 5 GSTp85αSH3-BH-SH2, Lane 6 p85αSH3-BH-SH2
Fig 3.5 Coomassie stained SDS-PAGE gels of recombinant p85α proteins

(A) GSTp85α fusion proteins, (B) Thrombin-cleaved fusion proteins
Figure 3.6 Schematic diagram of site-directed mutagenesis by overlap extension

The synthetic oligonucleotides are represented by arrows indicating the 5′-to-3′ orientation. The point mutations are indicated by *. Oligonucleotide primers are denoted by lower-case letters and PCR products are denoted by pairs of uppercase letters corresponding to the oligonucleotide primers used to generate that product. The boxed portion of the figure represents the proposed intermediate steps taking place during the course of reaction (3), where the denatured fragments anneal at the overlap and are extended 3′ by DNA polymerase (dotted line) to form the mutant fusion product. By adding additional primers 'c' and 'b' the mutant fusion product is further amplified by PCR.
Figure 3.7 Coomassie Blue stained SDS-PAGE of purified GST fusion proteins of p85α mutants
Panel (I) Starting from the end of the coding region of the SH3 domain, a 767 bp fragment was amplified by PCR and digested and ligated back into p85αpMT2SM. Panel (II) solid lines, open boxes and small arrows indicate plasmid DNAs, target sequences and PCR primers, respectively. PCR in inverted directions is done with primers that have a gap between their 5' ends, and the resulting deleted DNA is self-ligated to transform *E. coli.*
Figure 3.9 Cloning and Expression of the p85 mutants in the baculovirus expression vector system.
Figure 3.10 Expression of p85α mutants in Sf9 cells

Sf9 cells were infected with recombinant baculovirus encoding each of the p85α mutants. The infected cells were harvested at the times indicated and precipitated with phosphotyrosine-agar. The bound proteins were resolved by SDS-PAGE (10% acrylamide), and detected by staining with Coomassie Blue.
Figure 3.11 (A) Representative chromatographic profile of a baculovirus expressed p85α affinity purified using phosphotyrosine-actigel. (B) Coomassie stained SDS-PAGE gels of purified proteins.
Figure 3.12 Comparison of sequence homologies between bovine p85α, p85β and p85γ. Pink (SH3 domain), Red (proline-rich regions), Blue (BH domain), Green (SH2 domains) and Cyan (inter-SH2 domain).
Chapter 4: STRUCTURAL AND FUNCTIONAL STUDIES OF THE BH DOMAIN IN THE p85 SUBUNIT OF PI3K.

4.1 Introduction

The BCR homology (BH) domain in the p85α and p85β regulatory subunits of PI3K was first identified from the deduced amino acid sequence of bovine p85 cDNA clones (Otsu et al., 1991). The region is flanked by two proline-rich sequence motifs and is located between the SH3 domain and the amino-terminal SH2 domain (section 1.2 Figure 1.23). The amino acid sequence alignment of the α and β isoforms of p85 shows that the least sequence identity occurs within this domain (42%). This observation may suggest that the BH domain is involved in a regulatory function specific for each isoform. There is one report that p85α and p85β are involved in separate signal transduction pathways emanating from the T cell receptor (Reif et al., 1993). Alternatively, the BH domains of p85α and p85β may confer localisation within different compartments of the cell. Interestingly, several other adaptor subunits for the mammalian class Iα PI3Ks have now been identified (Antonetti et al., 1996; Fruman et al., 1996; Inukai et al., 1996; Pons et al., 1995). In each of these isoforms, the SH3 domain, the first proline-rich region and the BH domain present in p85α and p85β are replaced by short amino-terminal extensions (section 1.4.7.3).

BH domains in several other proteins have been demonstrated to have GAP activity towards the Rho-subfamily of GTPases (section 1.3.4.2), but it has not been possible to demonstrate that the p85 BH domain has GAP activity for this subfamily of GTPases (Ahmed et al., 1994). However, PI3K has been implicated in the pathways leading to cytoskeletal reorganisation which are known to involve the Rho-family GTPases in a number of different cell types. In human platelets, it has been reported that levels of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ were elevated in response to thrombin or GTPγS (Zhang et al., 1992). Exposure of platelets to thrombin led to the activation of PI3K, and concomitant translocation of this enzyme to the platelet membrane and rapid cytoskeletal re-organisation. Activation of PI3K by GTPγS was blocked by C3 transferase, a specific inhibitor of Rho. In addition, Rho was translocated to the cytoskeleton in platelets exposed to thrombin (Zhang et al., 1993). The mechanism by which Rho-GTPγS stimulates PI3K has yet to be elucidated, but the presence of GAP activity in BH domains of other proteins may implicate the BH domain of p85 as a potential effector of this biological response.
More recently it has been reported that GTP-bound Cdc42 or Rac, but not GTP-bound Rho, has the ability to bind recombinant p85 through its BH domain (Bokoch et al., 1996; Zheng et al., 1994). There is also evidence that both Rac and Cdc42 co-precipitate with PI3K lipid kinase activity in fibroblasts (Tolias et al., 1995). This association of Rac with PI3K in vivo was shown to be regulated by PDGF, implicating it in the PDGF or insulin induced Rac signalling pathways which lead to membrane ruffling (Hawkins et al., 1995; Kotani et al., 1994; Nobes and Hall, 1995; Wennstrom et al., 1994).

The three dimensional structure of the p85α BH domain has recently been determined by x-ray crystallography (Musacchio et al., 1996) (Figure 4.1). The domain is made up of seven α helices and represents a previously undescribed protein fold (Orengo et al., 1994). A four helix bundle contains most residues that are conserved in the BH family, and there is a projection composed of three more α helices. Interestingly, the crystals of the BH domain contain two monomers in the asymmetric unit that interact with each other, thereby creating a dimeric interface.

At present it is unclear as to whether it is the BH domain in p85 that is involved in the association of PI3K activity with Rho-family GTPase mediated responses. As yet, a biological function for this domain has yet to be defined.

4.1.1 Aims

At the time of this investigation, a three-dimensional structure had not been determined for any BH domain. The aim of this study was to purify the BH domains of both p85α and p85β from E.coli, and to produce sufficient pure protein for structural studies. As discussed in section 1.3.4, the BH domain of p85α has been reported to have no GAP activity for the known Rho-subfamily GTPases in vitro, however the BH domain of p85β had not yet been examined. The lack of reported GAP activity of the p85α BH domain may be because it is a GAP for a GTPase subfamily other than that of the Rho-related GTPases, or it may participate in a different signalling pathway. Mammalian cells and rat tissue lysates were examined for BH domain-associated proteins that may give clues as to a role for this domain. Three domains were examined in this way, p85α BH domain, p85β BH domain and a larger construct comprising both the BH and the SH3 domains and the two proline rich sequences. Use of the p85α SH3-BH construct allowed a comparison between proteins that may additionally interact with or cease to interact with the BH domain in the presence of the neighbouring SH3 domain.
4.2 Results

4.2.1 Expression and Purification of the p85α BH domain

During the course of this study, three constructs of different lengths were made in order to assess the effect of extending the amino- or carboxy-termini on the expression level and solubility of the recombinant protein. Figure 4.2 shows the amino acid sequence of the BH domain of p85α and p85β. The arrows indicate at which residue each construct starts and ends. The domains were expressed as GST fusion proteins to facilitate affinity purification. Table 4.1 summarises the expected molecular weight of the recombinant proteins before and after separation from the GST moiety by cleavage with thrombin.

4.2.1.1 Expression of BHα1

Amino acid sequence alignment of known BH domains suggested that the greatest homology lay between residues 130 and 270, (amino acid numbering from Figure 3.12), suggesting that these residues may be the boundaries of the BH domain. BHα1 was constructed so as to begin and end at these proposed boundaries. BHα1 was expressed as a GST fusion protein in E.coli (Figure 4.3) and expression levels were approximately 8 mg BHα1 per litre of culture. However, the majority of the protein was insoluble in a lysis buffer containing 1% Triton X-100 and formed inclusion bodies. Consequently, the yield of soluble protein was low and insufficient for structural studies. Lysis buffers containing various detergents were used in an attempt to try and solubilize BHα1 from inclusion bodies. Three different lysis buffers were compared: lysis buffer containing 0.5% NP40, lysis buffer containing 0.5% deoxycholate and RIPA buffer (50mM Tris pH8, 150mM NaCl containing 1% NP40, 0.5% deoxycholate and 0.1% SDS). Each of these buffers failed to solubilize BHα1 (data not shown) suggesting that the protein was not correctly folded and was forming insoluble aggregates.

4.2.1.2 Expression of BHα2

As more BH domain sequences became available, amino acid sequence alignments suggested that the domain was larger than first assumed. Sequence alignment of BH domains (section 1.3.4.4.2) suggested that leucine 118 and proline 129 were also conserved amongst the known family members. Based on this assumption, a second construct was made and designated BHα2. BHα2 had eighteen amino acids corresponding to residues 113-130 of the p85α sequence added to the amino-terminus and four amino acids corresponding to residues 276-279 added to the carboxy-terminus compared to BHα1. Recombinant BHα2 was found to be more soluble in 1% Triton
X-100 buffer and therefore amenable to large scale purification for structural studies using glutathione affinity chromatography (Figure 4.4).

However, after thrombin cleavage in order to release BHα2 from the GST-BHα2 fusion protein, a 70 kDa band was observed by SDS-PAGE, as well as the expected 19 kDa band (Figure 4.5). In addition, several protein samples were contaminated with GST and several contained low molecular weight bands on SDS-PAGE, which were presumed to be degradation products (data not shown). BHα2 cDNA was transformed into protease deficient strains of *E. coli* (Lon' and BL21) in order to reduce the levels of apparent protein degradation, however little difference was observed in the purity of BHα2 expressed in various strains of *E. coli* (data not shown).

The 70 kDa band observed by SDS-PAGE was presumed to be Hsp70, a bacterial heat shock protein involved in protein folding, and commonly associated with recombinant proteins over-expressed in *E. coli* (Craig *et al.*., 1993). Two strategies were devised to separate BHα2 from bound Hsp70. Ion exchange chromatography was used to try and separate these two proteins on the basis of charge, however they co-migrated on a range of ion-exchange matrices including Mono Q (Pharmacia), Mono S (Pharmacia), DE52 (Whatman) and Heparin Sepharose CL6B (Pharmacia) (data not shown). Ion exchange chromatography was carried out under native conditions, thus BHα2 and Hsp70 probably remained associated with each other while bound to the ion-exchange matrix, thus leading to co-elution of these two proteins.

In contrast, chromatography under dissociating conditions was able to successfully separate BHα2 and Hsp70. Under reversed phase conditions, BHα2 was eluted from a C4 matrix in 60% acetonitrile 0.1% TFA, and was separated from the 70kDa band (data not shown). However, as there was no biological assay available to test the integrity of the structure of BHα2 that had been exposed to organic solvent and low pH, this method of separation was not pursued.

A third strategy was therefore used to try to separate BHα2 and Hsp70. Size exclusion chromatography of this mixture under native conditions using a calibrated Superose-12 column (Pharmacia) resulted in the elution of two peaks (Figure 4.5). The first, which eluted with an apparent molecular weight of 90 kDa, probably comprised the BHα2/Hsp70 complex. The second, which eluted with an apparent molecular weight of 20 kDa comprised BHα2 of approximately 95% purity. Fractions containing BHα2 were dialysed into 10 mM Tris pH7.0 and analysed by circular dichroism (CD) (Figure 4.6). The characteristics of these spectra suggested that BHα2 was predominantly α-helical and therefore correctly folded.
4.2.1.3 Expression of BHα3 and BHβ

In order to improve protein stability, the boundaries of the domains were further extended up to, but not including the proline-rich motifs that lie either side of the BH domain. BHα3 had an extra eight amino acids, corresponding to residues 105-112 of p85α, added to the amino-terminus, and sixteen amino acids, corresponding to residues 275-312 of p85α, added to the carboxy-terminus relative to BHα2. The homologous region of p85β was used to construct the BHβ (Figure 4.2). Both proteins were expressed at levels of approximately 15mg/l, were soluble and showed few signs of degradation. These proteins were purified firstly by affinity chromatography using glutathione Sepharose CL4B, followed by size-exclusion chromatography of the products of thrombin cleavage of the GST-fusion proteins (data not shown). Fractions containing BHα3 or BHβ were pooled and dialysed into an appropriate buffer and preliminary structural analysis was performed.

4.2.2 Preliminary 1-d and 2-d proton NMR spectra of BHα3 and BHβ

In order to assess suitability of BHα3 and BHβ for structural analysis by NMR, 1-d NMR experiments were performed in order to determine whether these proteins were correctly folded. The 1-d NMR spectrum of BHα3 (Figure 4.7) suggested that the domain was folded. There is a good dispersion of signals in both the aliphatic region (0-4 ppm) and the aromatic region (6-7.5 ppm). The signal at 14 ppm is likely to be that of a histidine side chain proton and implies that there is strong hydrogen bonding around the histidine ring. Resonance shifts up field of 0.7 ppm (at chemical shifts below 0.7 ppm) suggest the presence of aliphatic protons in close contact with an aromatic ring. Inspection of 1-d spectra can also give some indication of secondary structure. The absence of α-proton resonances shifted above the water line at 4.7 ppm implies that BHα3 contains little or no β-sheet structure. The large number of resonances between 3.5 and 4.2 ppm is consistent with a primarily α-helical and random loop structure.

The 1-d NMR spectrum of BHβ (Figure 4.8) also suggested that the domain was folded. The dispersion of signals was similar to that of BHα3. The presence of one or two peaks above the water signal possibly implies some β-sheet content in addition to the α-helices. A different pattern of peaks is observed under the methyl envelope (between -1 and 1.5 ppm), which is indicative of a different hydrophobic core structure between the two domains. The signal tentatively assigned to a histidine side chain proton at 14ppm in BHα3, is absent in the spectrum of BHβ. Comparison of the
aligned amino acid sequences of BHα3 and BHβ showed that three out of four histidines in BHα3 were substituted for different residues in BHβ.

A heteronuclear single quantum coherence (HSQC) spectrum correlates nitrogen nuclei with bound protons in an uniformly 15N labelled sample. Most backbone NH or sidechain NH2 groups give a signal in this spectrum. F1 is the 15N axis and F2 is the 1H axis. An HSQC spectrum of BHα3 (Figure 4.9) showed that the central region of the spectrum was well dispersed, indicating the protein was folded. Peaks in the central region were broad and overlapped, consistent with the relatively high molecular mass of this protein with respect to the size limits imposed by NMR.

4.2.3 The search for GAP activity of BHα3 and BHβ towards the Rho family GTPases

4.2.3.1 Scrape Loading of the p85α BH domain into Swiss 3T3 cells

In order to determine a putative biological role for the BH domain of p85α, the protein was introduced into cells by scrapeloading. Scrape loading of Rho family GTPases had previously been used to demonstrate stimulation of lamellipodia formation by Rho and Rac (Flinn and Ridley, 1996), and was found to be a viable alternative to over-expression by transfection of cDNAs encoding a particular protein. The short term effects of scrape loaded BHα3 on cell morphology and actin cytoskeletal organisation were determined immunocytochemically.

Quiescent, serum-starved Swiss 3T3 cells were scrape loaded with scrapeloading buffer (SL) buffer alone, the recombinant, constitutively activated form of Rho, V14RhoA (Paterson et al., 1990), the recombinant, activated form of Rac, V12Rac (Ridley et al., 1992) and BHα3. In these experiments, V12Rac and V14RhoA served as positive controls so that the effects of scrapeloading BHα3 could be compared and interpreted. In accordance with previous reports (Flinn and Ridley, 1996), a striking difference in the organisation of the actin cytoskeleton was observed between control and V14RhoA and V12Rac scrape loaded cells seeded on PLL. (Flinn and Ridley, 1996) (Figure 4.10). Within 10 minutes, greater than 80% of cells had become adherent to the PLL and were therefore viable. Cells scrape loaded with buffer alone immediately started to spread out, and by 30 minutes had a large, round, flattened appearance, with an increased concentration of actin filaments in lamellipodia at the leading edge of the cell membrane (Figure 4.10A). In contrast, cells scrape loaded with V14RhoA had a more contracted morphology with long cables or spikes of polymerised actin extending radially from the periphery of the nucleus (Figure 4.10B). Cells scrape loaded with
V12Rac were more rounded and had an accumulation of polymerised actin in ruffles at the plasma membrane. (Figure 4.10C). The morphology of cells scrape loaded with BHα3 was more similar to those scrape loaded with V14RhoA, in that the cells were contracted, but there were no obvious extended cables of actin filaments (Figure 4.10D). This change in morphology of cells scrape loaded with BHα3 compared to control cells suggested that the BH domain of p85α may have a role in alteration of cell morphology via the Rho subfamily signal transduction pathway.

In order to determine whether the predicted role of BHα3 in actin rearrangement involved GAP activity, BHα3 and p85α were tested for GAP activity by determining the level of Rac1 or Rho bound \( [\gamma^{32}\text{P}]GTP \) converted to GDP over a 20 minute time course in the presence of either 20\( \mu \)g/ml or 200\( \mu \)g/ml of wildtype p85α or BH domain protein. Unlike RhoGAP, a known GAP for both Rac and Rho (Diekmann et al., 1991; Lancaster et al., 1994), neither BHα3, BHβ or p85α could stimulate the GTPase activity of RhoA or Rac1 under these conditions (Figure 4.11). It has been suggested that altering the ionic strength in a GAP assay differentially affects RhoGAP stimulation of Rho, Rac and Cdc42 (Lancaster et al., 1994), however it is unlikely that significant changes in ionic strength occur in vivo. Despite this report, the ionic strength was not varied in the above experiments and this reported aspect of Rho signalling was not tested further. However, our results are in agreement with several others who have been able to show no GAP activity of the BH domains of p85α or p85β towards the three best characterised GTPases in the Rho subfamily: RhoA, Rac1 and Cdc42 (Bengt Halstrom and Sohail Ahmed, personal communications).

### 4.2.4 Identification of BH domain-binding proteins

#### 4.2.4.1 Binding of \( ^{35}\text{S} \) methionine labelled mammalian cells to p85α-GST fusion proteins

Since no GAP activity was detected for the BH domains of p85α, but binding of GAPs to GTPases has been implicated in several physiological processes, cell lysates were examined to find binding partners for BH domains of p85α. GST fusion proteins of BHα3, p85αSH3 and p85αSH3-BH domains of p85α were immobilized on glutathione Sepharose CL4B and used to probe a 1.0% Triton X-100 extract of \( ^{35}\text{S} \) methionine labelled Jurkat or HL60 cells. Bound radiolabelled proteins were separated by SDS-PAGE and visualised by autoradiography (Figure 4.12).

While several bands were associated with all three GST-fusion proteins but not GST alone, others were specific for only one. Little difference was seen between lysates from HL60 or Jurkat cells. GST-p85αSH3 bound both 100 kDa (p100) and 68 kDa
proteins, but neither of these proteins bound to GST-p85αSH3-BH. This observation and its implications are explored in more detail in chapter 5.

GST-BHα3 was found to bind a 45 kDa (p45) and a 33 kDa (p33) protein. Interestingly, p45 did not bind GST-p85αSH3-BH, but p33 was found to be more strongly associated with GST-p85αSH3-BH than the GST-BHα3 domain alone. Both GST-BHα3 and GST-p85αSH3-BH also associated with a band in the 80-90 kDa range.

It is therefore clear that a number of proteins demonstrate differential binding to the amino-terminal domains of p85α. p85αSH3-BH contains two proline rich motifs which are docking sites for SH3 domain-containing proteins, and therefore it may be expected that this protein would have more potential binding partners in the cell than either of the individual domains. However, several proteins bound the SH3 and BH domains but not p85αSH3-BH, which suggests that these binding sites become inaccessible in p85αSH3-BH due to either a conformational change imposed by the surrounding domains or intramolecular or intermolecular interactions which block the ability of exogenous proteins to bind the amino-terminal domains of p85α.

4.2.4.2 Identification of BHα3-associated proteins in rat tissues using a far-Western assay

Rat brain and liver tissues were removed and homogenised as described in section 2.3.2. The clarified homogenates were incubated with immobilized GST fusion proteins of p85αSH3, p85αSH3BHα3 and p85α. Samples were divided into two and separated by SDS-PAGE. One gel was silver stained and the other was electroblotted onto a PVDF membrane and probed with biotinylated BHα3 (described in section 2.7.1.1). In this way, bands shown to be positive in the far western blot can be matched with bands on the silver stained gel (Figure 4.13).

The overlay assay with biotinylated BHα3 showed a single band of 80 kDa that was present in GST-p85αSH3, GST-p85αSH3-BH and GST-p85α precipitates, and was most strongly associated with GST-p85αSH3-BH. This 80 kDa band therefore seems to bind the BH domain as well as the SH3 domain, although with a lower affinity.

This band was not clearly distinguishable on the silver stained gel due to degradation of the GST-fusion proteins, co-precipitation of endogenous GST and overloading of the GST-fusion proteins. A similar band in the same size range was shown to co-precipitate with GST-p85αSH3-BH in Jurkat cells. Attempts were made to purify the 88 kDa band for amino acid sequence analysis. 1μg GST-p85αSH3-BH immobilized
on glutathione sepharose CL6B was used to co-precipitate this protein from the lysate of approximately $10^9$ Jurkat cells and was clearly visible on a silver stained SDS-PAGE gel (Figure 4.14). However, precipitation from $5\times10^9$ Jurkat cells did not produce enough protein for amino acid sequence analysis.
4.3 Discussion

The aim of this study was to express and purify proteins corresponding to the BH domains of p85α and p85β for structural and biochemical analyses. The folding and stability of the recombinant domain was governed not only by the conserved regions of the domain but also by flanking sequences. By extending the amino-terminal and carboxy-terminal ends of the domain, the expressed protein was stable and could be purified in the amounts required for three-dimensional structure determination by x-ray crystallography or NMR.

The main difficulty in the purification of the BH domain of p85α was that there was no biological assay with which to ascertain the correct folding of the domain. BHα1 was insoluble, but the addition of 18 amino acids to the amino-terminus made the protein soluble and improved expression levels (BHα2). Amino acid sequence alignments of BH domains from a range of proteins suggested that the conserved residues absent in this construct were leucine at position 118 and proline at position 129. The main difficulty in the purification of BHα2 was the co-purification of p70, whose identity may be Hsp70, again suggesting incorrect folding of BHα2. Further extending the domain solved this problem, implicating residues outside the consensus sequence of the BH domain in domain stability and solubility (Musacchio et al., 1996). Interestingly, the form of RhoGAP which crystallised and allowed determination of its three-dimensional structure was a protein containing 204 amino acids, much larger than the boundaries of the domain required for GAP activity (Ahmed et al., 1994). The form of the p85αBH domain that was crystallised was a construct encoding residues 105-319 of p85α, which is larger than BHα3 (Musacchio et al., 1996). Three blocks of sequence conservation have been identified in the BH domain family (section 1.3.4.4.2). These regions roughly coincide with the four α-helical bundle core of the domain. However, the three-dimensional structure of p85αBH showed the boundaries of the domain extend beyond these conserved regions. At the amino-terminus, the side chains of Leu 118, Gln 121, and Phe 122 are buried within the hydrophobic core of the molecule and appear to be important for protein stability. The carboxy-terminus, also corresponding to a region of poor sequence conservation, plays an essential role in the packing of the domain. Thus, poorly conserved regions at both termini are part of the integral structural components of the BH domain, which explains why BHα3 expressed at the highest level and was the most stable of the BHα domain proteins. The three-dimensional structure of the BH domain of p85α was determined by x-ray crystallography during the course of this study, thus it was decided not to pursue structural studies of this domain beyond a preliminary characterisation of folding and
secondary structural content by CD and 1-d NMR, which confirmed that BHα3 represented a stable, folded domain.

As previously described (section 1.3.4.4.2), a number of BH domain containing proteins possess GAP activity for specific members of the Rho subfamily of GTPases. As a consequence of this activity, BH domain containing proteins have been shown to inhibit cytoskeletal rearrangements induced by activated Rho proteins. Numerous studies have implicated PI3K and its phosphorylated lipid products in regulation of cytoskeletal rearrangements. The presence of a BH domain in the p85 regulatory subunit supports this association. To address the possible biological role of this domain, the BHα3 protein was introduced into quiescent Swiss 3T3 cells by scrape loading. The preliminary results shows the domain may cause a change in cell morphology, and the cells looked similar to those scrape loaded with V14RhoA. Further work is needed to characterise the change in morphology and establish a pathway in which the p85αBH domain may function. Many BH domain-containing proteins are involved in signalling pathways that ultimately act on the cytoskeleton, for example the RhoGAP, p190 and BCR BH domains inhibit stress fibre formation or membrane ruffling when microinjected into Swiss 3T3 cells (Ridley et al., 1993). The chimaerin BH domain also inhibits stress fibre formation, but microinjection of full length chimaerin induces the formation of filopodia and lamelipodia (Kozma et al., 1996).

It had been proposed that GAPs stimulate the intrinsic GTPase activity of the Rho and Ras family proteins, either by contributing residues that participate directly in catalysis or by performing an allosteric function (Ahmed et al., 1994). This issue has been resolved through a combination of mutagenesis studies (Ahmed et al., 1994) and the elucidation of the three dimensional structure of RhoA in complex with RhoGAP. (Rittinger et al., 1997; Rittinger et al., 1997), which suggest that the GAP contributes residues that actively participate in the GTPase reaction to make it more efficient. An analysis of the n-chimaerin GAP domain by mutagenesis suggest that binding to a RhoGAP family member and stimulating its GTPase activity were two separate events (Ahmed et al., 1994). This study mapped residues that were essential for GAP binding, and separate residues essential for GAP activity. Furthermore, there was evidence that a mutant of chimaerin (section 1.3.4.4.2) that lacked GAP activity but was still able to bind the GTPase, induced the formation of lamelipodia (Kozma et al., 1996). This may indicate a general mechanism in which the binding of a GTPase to its GAP is an important step in cytoskeletal organisation, which is separate from conversion of the GTPase to its GDP bound form by the GAP. The BH domains of
p85α and p85β do not possess GAP activity for known Rho family GTPases. PI3K has been shown to bind the GTP-bound form of Rac and Cdc42, and it is possible that binding to these GTPases without concomitant GAP activity is sufficient to affect cytoskeletal morphology. Deletion analysis of particular motifs along the length of the chimaerin GAP domain was used to obtain an overview of the importance of certain regions for GAP activity (Ahmed et al., 1994). Deletion of the motifs EIE in block 1 and YRV and LKLY in block 2 (section 1.3.4.4.2) caused complete loss of GAP activity. Neither p85 isoform contains these EIE or YRV motifs, and this may explain the absence of GAP activity of the BH domains of these proteins (Barrett et al., 1997). Furthermore, the three-dimensional structure of the RhoGAP alludes to five residues conserved in most GAP domains that promote GTP hydrolysis, but which are not conserved in either p85α or p85β (Barrett et al., 1997).

The three-dimensional structure of the Cdc42-RhoGAP complex in the ground state with the non-hydrolysable GTP analogue GMMNP has been compared to the same complex with the transition state analogue GDP.AIF^-4, which is thought to mimic the transition state of a phosphoryl-transfer reaction (Rittinger et al., 1997; Rittinger et al., 1997). There is a rotation of 20 degrees between the Rho and RhoGAP proteins in this complex when compared with the ground state complex. Consequently, in the transition state complex but not in the ground state, the RhoGAP domain contributes one residue, Arg 85, directly into the active site of the G protein. This residue acts to stabilise the transition state of the GTPase reaction. Indeed, the movement of Arg 85 during the catalytic cycle appears to be the key to the function of RhoGAP. Interestingly, this arginine residue is conserved in both the p85α and p85β BH domains, suggesting that they may have the potential to act as GAPs. With the growing number of Ras and Rho family GTPases, many of which have not been characterised, it is also possible that the BH domains of p85α and p85β have GAP activity for a GTPase not yet known or investigated.

It has been reported that PI3K activity associates with GTP-bound Rac and Cdc42 in vitro, and that this interaction occurs through the BH domain of p85 (Tolias et al., 1995; Zheng et al., 1994), thus it is also possible that p85 may be a target protein of GTPases rather than a GAP that down-regulates GTPases. The cycling of Rho GTPases requires tight regulation to prevent permanent deactivation, but the nature of this regulation is unclear at present. The BH domain may bind GTP-bound Rac or Cdc42 and undergo a conformational change so that it can interact with another protein, perhaps its target.
It has been shown recently that Ras transformation and membrane ruffling requires PI3K (Rodriguez Viciana et al., 1997). A dominant-negative mutant of Rac has been shown to block the induction of ruffling by Ras and by growth factors. Therefore, it is likely that Rac functions downstream of Ras and growth factors in the pathway leading to actin rearrangement (Ridley and Hall, 1992). To determine the effect of inhibiting PI3K on cell transformation induced by a constitutively active form of Ras(V12Ras), the dominant-negative mutant of p85α which lacks the putative p110 binding site (Δp85) and mutants of p85α described in this thesis, were expressed in V12Ras transformed cells in collaboration with Dr. Pablo Rodriguez-Viciana. Full-length p85α, Δp85 and full length p85α with the BH domain deleted (p85αΔBH) were able to inhibit V12Ras induced transformation, suggesting that the direct interaction of the BH domain of p85α with Rac or Cdc42 was not likely to be a critical part of the mechanism of cell transformation by Ras. Similarly, V12 Ras-induced membrane ruffling was inhibited by p85αΔBH to the same extent as p85, but had no effect on ruffling induced by constitutively active V12 Rac. Thus it is unlikely that direct interaction of p85 with GTP-bound Rac or Cdc42 is responsible for this inhibition. The exact mechanism by which overexpression of p85 mutants interferes with Ras-induced transformation has not been defined and awaits further study. Another study examined the overexpression of selected domains of p85 in NIH 3T3 fibroblasts transformed by the v-Ha-Ras oncogene. The inter-SH2 domain and the SH3 domain were sufficient to reverse the transforming effects of v-Ha-Ras, but over expression of the BH domain had no effect on v-Ha-Ras transformation (Zhang et al., 1996). Together, these data suggest the Ras pathway is not likely to directly involve the BH domain, and distinguishes between PI3K-dependent Ras and Rac induced cytoskeletal rearrangements, one of which may involve p110 and one of which may involve binding the BH domain of p85.

Two aspects of function of p85BH domains require further investigation. Firstly, it is important to discover whether they have GAP activity for other GTPases. Secondly, naturally occurring homologues of p85α, p55γ, and p55α and p50α, have no BH or SH3 domains. The differences in the amino-terminal region observed among the regulatory subunit isoforms may contribute to differences in subcellular distributions or to varying degrees of PI3K activation in response to various growth factors and oncogenic products (Inukai et al., 1996; Pons et al., 1995). In contrast, the absence of the BH domain in this form of PI3K, would result in the inability of Cdc42 or Rac to regulate PI3K. p55α, also known as p55PIK, has been shown to be efficient for the regulation of PI3K by IRS-1, therefore it can be presumed that this pathway does not require the SH3 or BH domains (Inukai et al., 1997). Although p85β is 62% identical to p85α at the amino acid level, it is only 62% identical within the BH domain.
this domain may confer a different function on p85α versus p85β, such as responsiveness to different agonists.

In order to search for putative binding partners for the BH domain that may give clues to its role in a signalling pathway, Jurkat and HL60 cells were examined for associated proteins. Using affinity purification, the domain bound a number of proteins including a protein in the 80-90 kDa range. A protein of the same size was also identified in rat tissues using a far-Western assay, strengthening the hypothesis that it may be a binding partner for the BHα domain. As yet, the identity of this protein is unknown due to difficulty in purifying enough material for amino acid sequence analysis.

The BH domain of p85α, like other BH domains may therefore be involved in regulating the actin cytoskeleton. Unlike other BH domains however, the p85α BH domain may not have GAP activity but, as discussed, the definition of different residues involved in GAP activity and GTPase binding in chimaerin provide a precedent for this observation. As yet, biological function is hinted at, but not defined, by the lack of homology of the BH domain between p85α and p85β. Furthermore, the absence of this domain in p55α, p50α and p55γ suggest the possibility of differential regulation of PI3K by different isoforms of p85.
Figure 4.1 The three dimensional structure of the BH domain from p85α.

(A) Ribbon representation of the BH domain from p85α (Mussachio et al., 1996), Proc. Natl. Acad. Sci., 93, 14373-14378). Crystals of the BH domain contain two monomers (I and II) in the asymmetric unit. Positions of the amino-(N) and carboxy-(C) termini are indicated, and the seven α-helices are labelled αA to αG. (B) Amino acid sequence alignment of the BH domains from p85α (red) and RhoGAP (blue). (C) Superimposed Cα representation of the three dimensional structures of the BH domain from p85α (red) and RhoGAP (blue).
Table 4.1 Predicted molecular weights of the recombinant p85 BH domain proteins

<table>
<thead>
<tr>
<th>CONSTRUCT</th>
<th>Molecular weight (kDa) GST-FUSION PROTEIN</th>
<th>Molecular weight (kDa) CLEAVED PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHα1</td>
<td>42</td>
<td>16</td>
</tr>
<tr>
<td>BHα2</td>
<td>45</td>
<td>19</td>
</tr>
<tr>
<td>BHα3</td>
<td>48</td>
<td>22</td>
</tr>
<tr>
<td>BHβ</td>
<td>45</td>
<td>19</td>
</tr>
</tbody>
</table>
Figure 4.2 Amino acid sequence of the BH domain from (A) p85α and (B) p85β. Highlighted residues indicate the beginning and end of each construct.

- **A**
  - PSKTEADSEQ  QASTLPDLAE  QFAPPDVAPP
  - LLIKLVEAIE  KKGLECSTLY  RTQSSSNPAE
  - LRQLLDCDTA  SLDEMFVH  VLADAFKRYL
  - LDLPNPVIPV  AVSSESIISLA  PEVQSSEEEYI
  - QLLKKLIRSP  SIPHQYWLTL  QYLLKHFKKL
  - SQTSSKNLLN  ARVLSELFSP  LLFRFPAAASS
  - ENTEHLIKII  EILISTEWNE  RQPAPALPPK
  - PPKP

- **B**
  - RDGPPPEPGLT  LPDLPEQFSP  PDVAPPILVK
  - LVEAIERTGL  DSYRPEPPAV  RTDWSLSDVE
  - QWDAAALSDG  VKGFLLLALPA  PLVTPEAAAAE
  - AHRALREAAG  PVGPALEPPT  LPLHHALTLR
  - FLQHLLGRVA  GRAPAPGPAV  RALGATFGPL
  - LLRAPPTEPS  PPGAPDGTE  PTPDFPALLV
  - EKLLQEHLEE  QEVE

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**Figure 4.2** Amino acid sequence of the BH domain from (A) p85α and (B) p85β. Highlighted residues indicate the beginning and end of each construct.

- **BHα1.**
- **BHα2.**
- **BHα3.**
- **BHβ**
Figure 4.3 Affinity purification of GST BHα1 from *E.coli*

The cells were lysed and sonicated, and the supernatant and pellet fractions were separated by centrifugation at 14000rpm. The supernatant was applied to a glutathione Sepharose CL6B column, unbound proteins removed by washing with 150mM NaCl in 50mM Tris pH 8 and the fusion protein was eluted from the column using 20mg/ml glutathione in Tris pH 8. Fractions (0.5mls) were collected and 10μl of each of fractions 1-9 were analysed by Coomassie Blue stained SDS-PAGE.
Figure 4.4 Affinity purification of GST BHα2 from *E.coli*

Each step of the purification was analysed using Coomassie Blue stained SDS-PAGE.

Lane 1, Uninduced cells, Lane 2, Induced cells, Lane 3, Whole cell lysate,
Lane 4, 1% Triton X-100 soluble supernatant, Lane 5, 1% Triton X-100 insoluble pellet,
Lane 6, Protein not bound to glutathione Sepharose CL6B, Lanes 7-13, Fractions containing GST BHα2 eluted from glutathione Sepharose CL6B using 20mg/ml glutathione in 50mM Tris pH 8.
Figure 4.5 Purification of BHα2 by size exclusion chromatography using a Superose 12 column. (A) Coomassie Blue stained SDS-PAGE analysis of 10µl from each 2ml fraction. (B) Representative chromatographic profile of the purification of BHα2 using a Superose12 column. The column was run in 20mM Tris pH 8, at a flow rate of 0.2ml/min.
Figure 4.6 Analysis of the secondary structure of BHα2 by circular dichroism (CD)

(A) CD spectra of purified BHα2. The $\Delta\varepsilon$ of 0.2 mM BHα2 in 10 mM Tris pH 7.0 was measured at wavelengths between 190 and 260nm.

Figure 4.7 1-d proton NMR spectrum of the BHo3
Figure 4.8 1-d NMR spectrum of BHβ
Figure 4.9 2-d Heteronuclear Single quantum Coherence (HSQC) spectrum of BHκ3
Fig 4.10 Actin re-organization in Swiss 3T3 cells

Quiescent serum-starved Swiss 3T3 cells were scrape loaded with (A) SL buffer alone, (B) 20µg/ml V14RhoA, (C) 20µg/ml V12Rac and (D) 20µg/ml BHz3. Scraped loaded cells were washed with serum-free media and added to coverslips coated with poly L-lysine (PLL). After incubation for 30 minutes at 37°C and 10% CO2, the cells were fixed, permeabilized and stained with TRITC-labelled phalloidin to visualise actin filaments.
Figure 4.11 In vitro GAP activity of BHα3, BHβ and p85α WT

Purified recombinant Rac1 (A and B) or Rho (C and D) (6nm) was bound to [γ-32P]GTP (Amersham) and mixed with GST, RhoGAP, BHα3, BHβ or p85α. The loss of the bound radioactivity (32P) over the indicated time at 25°C was measured by a filter binding method. The % of [γ-32P]GTP remaining is plotted relative to the radioactivity bound Rac1 and Rho at time zero.

- □ GST
- ○ RhoGAP
- ◦ BHα3
- ▲ p85α
- ♦ BHβ
Figure 4.12 SDS-PAGE analysis of proteins from $^{35}$S-methionine labelled Jurkat (J) and HL-60 (H) cell lysates that bound to p85α mutants.
Figure 4.13 Identification of BH\(\alpha\)3 binding proteins from rat brain and liver by far-Western Assay.

GST fusion proteins were immobilised and incubated with lysates of rat brain or liver. (A) Silver-stained SDS-PAGE analysis of the differential binding of GST-p85\(\alpha\) mutants to rat brain and liver proteins. Lanes 2, 6 and 10, GST-p85\(\alpha\)SH3, Lanes 3, 7 and 11, GST-p85\(\alpha\)SH3-BH, Lanes 4, 8 and 12, GST-p85\(\alpha\) and Lanes 1, 5 and 9, GST. (B) Detection of BH\(\alpha\)3 binding proteins by far-Western assay. The Western blot was probed with biotinylated BH\(\alpha\)3 and horseradish peroxidase-linked avidin.
Figure 4.14 An 88kDa protein from Jurkat cell lysates specifically bound
GST-p85αSH3BH. Silver-stained SDS PAGE analysis of GST-p85αSH3BH (Lane 1)
and proteins from Jurkat cell lysates bound to 1μg of GST-p85αSH3BH (Lane 2).
Chapter 5: THE SH3 DOMAIN AND PROLINE RICH MOTIFS OF p85

5.1 Introduction

The SH3 domain of the p85 regulatory subunit of PI3K is at the amino-terminus and is directly followed by the first proline-rich motif (P1). As discussed in Chapter 4, several isoforms of p85α, for example p55πIK, p55γ and p50α, do not contain an SH3 domain, suggesting a role for the domain in the differential signalling pathways of various p85 isoforms. The best characterised binding interaction of the p85α SH3 domain to date is its in vitro interaction with dynamin, a GTPase implicated in membrane invagination during the initial stages of endocytosis (reviewed in Urrutia et al., 1997). The p85α SH3 domain has been shown to activate the GTPase activity of dynamin by binding to an amino-terminal proline-rich motif (Gout et al., 1993), although no association of the two proteins has yet been observed in vivo.

The SH3 domains of Lyn and Fyn have been shown to bind to the first proline rich motif (P1) in the p85α subunit, leading to a five to seven-fold increase in the specific activity of PI3K immunoprecipitated from a B cell lymphoma cell line (Pleiman et al., 1994). Stimulation of the B cell receptor in this cell line leads to the activation of PI3K, and this activation can be blocked by a peptide corresponding to the P1 amino acid sequence. The SH3 domains of the Src family tyrosine kinases have the ability to bind directly to the P1 motif of p85α, as does the p85α SH3 domain (Kapeller et al., 1994), and it has been postulated that the interaction of the SH3 domain of p85α with the p85α P1 motif may constitute a regulatory mechanism. The ability of the SH3 domain to bind to and activate a range of substrates implies that it has a role in the myriad of signal transduction pathways emanating from PI3K.

5.1.1 The three-dimensional structure of the p85α SH3 domain

The three dimensional structure of the p85α SH3 has been determined both with (Yu et al., 1994) and without a bound proline-rich peptide ligand (Booker et al., 1993), (Koyama et al., 1993; Liang et al., 1996), (Figure 1.6). Structures determined using x-ray crystallography or NMR are essentially identical. The p85α SH3 domain is structurally homologous to other known SH3 domains, comprising 5 β strands arranged as two orthogonal β-sheets, a long hairpin loop and a carboxy-terminal 310
helix. These arrange to form the characteristic β barrel tertiary fold. Furthermore, the amino acid residues that are conserved in all known SH3 domains (L11, Y12, Y14, D21, W55, P70, Y73 and V74; using the p85α numbering system), are involved in forming the solvent-exposed, hydrophobic peptide binding surface and are present in the p85α SH3 domain. Unlike most SH3 domains, the p85α SH3 domain also contains a 15-residue insertion in the n-Src loop that connects the second and third β strands. The corresponding loop of the Src SH3 domain is much shorter, but is nevertheless important for binding. It is named the n-Src loop because the neuronal isoform of Src also has an insertion in this position, which alters the binding specificity of the n-Src SH3 domain (Feller et al., 1994).

In p85α, the n-Src loop is involved in the formation of two hydrophobic pockets on the SH3 domain surface. Both pockets are relatively deep and can accommodate leucine residues. This feature distinguishes the p85α SH3 domain from other SH3 domains and may play a role in its binding specificity. Screening of phage display libraries that contain peptides with the sequence XXXXXRPLPPLPPP (X stands for any amino acid), demonstrated that the peptides with the consensus sequence CLXCXRPLPPLPPP bound the p85α SH3 domain with the highest affinity (Rickles et al., 1995), supporting the hypothesis that p85α SH3 domain binding pockets can accommodate bulky residues such as leucine. The solution structure of the p85α SH3 domain in complex with one of these ligands, a peptide with the sequence RKLPPRSK (Yu et al., 1994), (Figure 1.6 B) showed that the complex was stabilised predominantly by hydrophobic contacts and by electrostatic interactions between the arginine residues of the peptide and residues D21 and E51 of the SH3 domain.

5.1.2 Aims

This chapter attempts to identify the role of the p85α SH3 domain in signalling pathways that involve PI3K and to examine the nature of the interactions of this domain with other cellular proteins, as well as with p85α itself. Using site-directed mutagenesis, the role of the proline-rich sequences of p85α is also investigated. Furthermore, the ability of the p85α SH3 domain to bind to both intramolecular and intermolecular substrates is investigated, as well as the possible significance of these interactions with respect to regulation of the enzymatic activity of PI3K.

5.2 Results
5.2.1 Affinity purification of p85α SH3 domain-binding proteins

An immobilised GST fusion protein of the p85α SH3 domain (GST-p85αSH3) was used to identify potential SH3 domain-binding proteins in detergent extracts of bovine brain. They were compared to proteins co-precipitated with GST fusions of a number of other SH3 domains, including the amino-terminal SH3 domain of p67 (GST-p67-NSH3), the carboxy-terminal SH3 domain of p67 (GST-p67-CSH3), the carboxy-terminal SH3 domain of p47 (GST-p47-CSH3), the SH3 domain from the neuronal isoform of Src (GST-n-Src-SH3), the SH3 domain from c-Src (GST-c-Src SH3), the SH3 domain from PLCγ (GST-PLCγ-SH3) and the SH3 domain from RasGAP (GST-RasGAP-SH3). The associated proteins were eluted from glutathione sepharose using SDS-PAGE sample buffer, resolved by SDS-PAGE and visualised by silver staining (Figure 5.1). GST-SH3 domains, but not GST alone, specifically associated with a number of proteins. p85αSH3 (Lane 8, Figure 5.1) bound several proteins including a 100 kDa protein, p100, a 70kDa protein, p70, a 55 kDa protein, p55, and a 150 kDa protein, p150. These proteins were also bound to the GST-SH3 domains of PLCγ (Lane 6), c-Src (lane 5) and, to a lesser extent, the carboxy-terminal SH3 domain of p67 (Lane 1). There were no detectable binding proteins for the GST-SH3 domains of RasGap (lane 7), n-Src (lane 4), p67 (amino-terminal) (lane 2) and p47 (carboxy-terminal) (lane 3). There are, therefore, different binding patterns for a subset of recombinant SH3 domains in a brain lysate, suggesting that SH3 domains display specificity in their ligand binding.

Some of these associated proteins (p55, p70, p100 and p150) were further purified in quantities sufficient for protein sequencing. Due to the relative abundance of p100 and p70 as observed by silver stained SDS-PAGE, it was decided to characterise these proteins further.

Amino-acid sequencing of p100 gave sequences for 7 tryptic peptides (GISPVPINLR, VPVGDQPP, DIEFQIR, GYIGVVN, FFLSHPSYR, LQSQDLLSIEK, YMLSVDNLK and HIFALFNTEQ). These peptides were used to search the protein sequence data bases. All seven peptides were identical to the sequence of rat dynamin (Obar et al., 1990), thus identifying the p100 GSTp85αSH3 domain-binding protein as bovine dynamin. 5 tryptic peptide sequences were generated for p70 (NVGSLLLTPQ, ENESLFTFLGK, LLXEQELYNNFVYNSP, KADIGTPSNFQHIGH AND KVIYDFIEK) which were 50% homologous with the human Wiskott Aldrich Syndrome protein (WASP), a protein that is expressed exclusively in cells of the
immune system and is mutated in patients with Wiskott Aldrich Syndrome ((Derry et al., 1994)). Subsequently, cloning of a homologue of WASP known as N-WASP has been reported ((Miki et al., 1996)). This protein has 90% identity to the peptide sequences generated, therefore bovine N-WASP is the best candidate for p70.

### 5.2.2 Analysis of proteins bound to deletion mutants of p85α

As described in section 4.2.4.2, the binding characteristics of the p85α SH3 domain, the combined SH3-BH domains and p85α suggested that a region of p85α itself may bind to the SH3 domain, preventing binding of exogenous proteins. For example, p100 (dynamin) bound to the GST-p85αSH3 but not to GST-p85αSH3-BH, GST or GST-p85α (Figure 5.2), suggesting that the SH3 domain is not accessible in GST-p85α-SH3-BH or GSTp85α as it is in GST-p85αSH3. Similarly, GST-p85αSH3 domain bound proteins from 35S-methionine labelled Jurkat and HL60 cells that GST-p85αSH3-BH and GST-p85α did not (Figure 4.12).

### 5.2.3 Site-directed mutagenesis of internal proline residues in full length p85

It was hypothesised that, in the context of the whole protein, the SH3 domain of p85α may be bound to one of its endogenous proline rich motifs which lie between the SH3 and BH domains (P1), and the BH and amino-terminal SH2 domain (P2).

In order to test this hypothesis, mutations were made in p85α with the aim of “unlocking” the putative intramolecular SH3-P1 interactions. The three-dimensional structure of the p85αSH3 domain bound to a proline-rich peptide (Yu et al., 1994), as well as several mutagenesis studies, have shown that a large number of residues in p85αSH3 are involved in binding p85αP1. Mutation of all these residues in order to destroy the p85αP1 binding site is likely to disrupt the overall structure of the p85αSH3 domain, therefore point mutations were introduced into the proline rich motifs of p85α, P1 and P2. In addition, a deletion mutant, p85αΔSH3, in which the entire SH3 domain is deleted, was also constructed.

Two proline residues, which have been shown to be critical for binding p85αSH3 (Feng et al., 1994), were mutated to alanine residues in both the SH3-binding motifs in full length p85α using a PCR -based approach (described in detail in section 3.2.2). The resulting proteins were expressed as recombinant GST fusion proteins in E.coli and as non-fusion proteins in insect cells using the baculovirus expression system (section 2.2.1.5, Figure 3.7).
In order to analyse the interaction of p85αSH3 and p85αP1, the binding of a range of mutants of p85α to proline rich peptides was studied using a biosensor. Three proline-rich peptides, p85αP1 (TPKPRPLPVAPGS), p85αP2 (WNERQQPAPALPPKPT) and a proline-rich motif from dynamin (SPTQRRAPAVPPARGS) were biotinylated at their amino-terminus and immobilised onto the dextran coated surface of the gold chip of a BIAcore 2000 (described in section 2.7.2). Several SH3 domains were passed over the surface of the chip and the degree of association was measured in arbitrary resonance units (RU) versus time. The SH3 domains of Src, p85α, as well as wild type Grb2, which contains two SH3 domains, bound to immobilised p85α P1 (Figure 5.4(A)). In contrast, neither p85αSH3 or Src SH3 bound to p85αP2 (Figure 5.4(B)) but Grb2 was able to bind, demonstrating that p85αP2 had been successfully immobilised. In addition, specificity between the interactions of various SH3 domains and proline-rich motifs was demonstrated. These data suggest that p85αSH3 is more likely to bind to its endogenous PI motif compared to its P2 motif. Interestingly, it has been demonstrated that the p85 subunit interacts directly with Grb2 both in vitro and in vivo. The association is mediated by the SH3 domains of Grb2 and the proline-rich motifs of p85α (Wang et al., 1995). A proline-rich peptide derived from the amino terminus of dynamin was also immobilised (Figure 5.5(A)). Both p85αSH3 and Grb2 were able to bind, but the Src SH3 domain did not, which is in agreement with a previous study (Gout et al., 1993).

Phosphotyrosine-containing peptides, corresponding to two autophosphorylation sites surrounding Tyr-740 and Tyr-751 on the human PDGF β-receptor, have been shown to interact with the SH2 domains of the p85 subunit of PI3K (Fantl et al., 1992; Panayotou et al., 1993). This property of p85α was used to test whether mutations in the proline-rich motifs of p85 disrupted its overall structure. The p85α mutants, p85αΔP1a, p85αΔP2 and p85αΔP1aP2, all bound to a phosphopeptide derived from the sequence surrounding Tyr 751 (DMSKDESVDPYVPLDMK) in the PDGFβR to the same extent as wild type p85α (Figure 5.5(B)) confirming that mutations in the proline-rich motifs did not have unforeseen, long-range effects on p85α structure.

In order to examine whether the SH3 domain of p85α is bound to its endogenous P1 or P2 motifs, the p85α mutants were passed over immobilised proline-rich peptides. The ability of the SH3 domain in the wild type p85α and in the mutants to bind exogenous peptides were compared with each other. Wild type p85α did not bind immobilised p85αP1 peptide but p85αΔP1a did. Similarly p85αΔP2 did not bind p85α P1 but p85αΔP1aP2 did (Figure 5.6(A)). These results suggest that the SH3 domain in p85α is bound to its own P1 motif, since mutations in this sequence allows the SH3 domain
to bind exogenous P1 peptide when it is not able to do so in the wild type p85α.
Furthermore, in agreement with the results described in Figure 5.4(B), the p85α SH3
domain is not likely to be bound to its own P2 motif since mutations in this region did
not allow the SH3 domain to bind immobilised P1. Essentially identical results were
obtained when a peptide with a sequence derived from the proline-rich motif of
dynamin was used as the immobilised peptide (Figure 5.6 (B)). Wild type p85α and
p85α ΔP2 did not bind the dynamin peptide whereas mutants in which the p85α P1
motif is disrupted, p85α ΔP1a and p85α ΔP1aP2, were able to bind the dynamin
peptide.

Thus, mutation of key residues in the proline-rich motifs of p85α has long range effects
on this protein. These mutations not only affect the p85α P1 motif itself, but also the
binding characteristics of the p85αSH3 domain. Moreover, these mutations increase
the ability of the p85α SH3 domain to bind exogenous substrates, strongly suggesting
that in wild type p85α, the SH3 domain interacts with P1, and that when this
intramolecular interaction is disrupted, it frees the SH3 domain to bind exogenous
substrates (Figure 5.7).

5.2.4 The PI3K complex can only be co-precipitated by exogenous SH3 domains when the endogenous p85α SH3 domain is deleted

Another way to disrupt the endogenous p85αSH3-P1 interaction is to delete the SH3
domain, therefore a deletion mutant of p85α lacking the SH3 domain was constructed
as described in section 3.2.3.1. In order to determine whether deleting the endogenous
SH3 domain would allow increased association with exogenous SH3 domains as
would be predicted from the model described in Figure 5.7, and whether this
phenomenon still occurred in the presence of the catalytic subunit, p85α and
p85αΔSH3 were co-expressed with p110α using the baculovirus expression system.

GST fusion proteins of the SH3 domains from c-Src and p85α were immobilised on
glutathione Sepharose CL4B and incubated with baculovirus lysates containing co-
expressed p85α/p110α or p85αΔSH3/ p110α. In addition p85α/p110α and
p85αΔSH3/p110α were immunoprecipitated alone with an antibody directed against
the SH2 domain of p85α (Lanes 1 and 4 in Figure 5.8A and B.) . In order to
determine whether the exogenous GST-SH3 domains could co-precipitate the enzyme
complex, the glutathione sepharose precipitates were resolved on SDS-PAGE (Figure
5.8(A). To examine whether the exogenous GST-SH3 domains could co-precipitate

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lipid kinase activity, the glutathione Sepharose precipitates were subjected to PI3K assays using PI as a substrate (Figure 5.8(B)). p85αΔSH3/p110α bound more efficiently than wild type p85α/p110α to the SH3 domains of Src and p85α (Figure 5.8A). Similarly, the GST-SH3 domains of Src and p85α could co-precipitate lipid kinase activity more efficiently from lysates containing p85αΔSH3/p110α compared to p85α/p110α (Figure 5.8 B). It had already been shown that GST fusion proteins of the SH3 domains of p85α and Src bind efficiently to p85α P1 (Figure 5.4(A)). These results show that deletion of the p85α SH3 domain allows exogenous SH3 domains access to the p85αP1 region. These data support the proposed model that the p85αSH3 domain is bound to its own P1 motif. Furthermore, this interaction has now been shown to occur when p85α is in complex with p110α.
5.3 Discussion

The modular structure of the non-catalytic p85 subunit of the PI3K has facilitated both structural and functional studies of this protein and has led to rapid advances in the understanding of its role in the heterodimeric PI3K complex. Despite these advances, the precise function of the SH3 domain in this adaptor molecule has remained elusive. In an attempt to address this question, GST fusion proteins of SH3 domains from p85α, and also from a subset of other signalling molecules, were used as affinity matrices to screen a bovine brain extract for proteins to which they could specifically bind (Gout et al., 1993) (Figure 5.1). Several proteins were identified, including a known 100 kDa GTP-binding protein, dynamin and a 68 kDa protein (p70). (Derry et al., 1994) Phosphorylation of membrane lipids by PI3K may play a role in some of the processes involved in endocytosis, such as membrane invagination, which is known to involve dynamin (reviewed in Urrutia et al., 1997). It is possible to speculate that PI3K provides a link between transmembrane receptors which it binds through its SH2 domain, and dynamin, which it binds through its SH3 domain, leading to receptor internalisation and down regulation.

Sequence alignment of the peptides from p70 confirmed it was 50% identical to the human Wiskott Aldrich Syndrome protein (WASP) (Derry et al., 1994). Recently, a neural form of WASP, N-WASP has been identified ((Miki et al., 1996)) which is 100% identical to the tryptic peptides of p70 that were sequenced. N-WASP is an actin-depolymerising protein that regulates the cortical cytoskeletal rearrangement in a PtdIns(3,4)P2-dependent manner downstream of tyrosine kinases. Again PI3K may provide a link between PtdIns(3,4)P2, a product of PI3K catalytic activity, and the activity of N-WASP, which it binds through its SH3 domain, on the actin cytoskeleton. The involvement of PI3K in both fluid-phase endocytosis (Li et al., 1995) and regulation of the actin cytoskeleton (Hawkins et al., 1995; Nobes and Hall, 1995) support the idea that p85α SH3 domain associates with such molecules as dynamin and N-WASP. Microinjection of the SH3 domains of Grb2 and PLCγ have shown that they localise to membrane ruffles and actin stress fibres respectively. Such a study has yet to be reported for the p85α SH3 domain, but the identification of the binding partners described above supports the hypothesis that it is involved in cytoskeletal re-arrangement and cell movement.

The data presented in this chapter strongly suggest that the SH3 domain of p85α is bound to its own proline-rich motif. The first evidence came from binding studies using a biosensor. As an isolated domain, p85αSH3 was able to bind immobilised
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p85αP1 peptide but not p85αP2 (compare Figure 5.4(A) and Figure 5.4(B)). In order to determine whether this interaction between p85αSH3 and p85αP1 occurred in full length p85α, mutants of p85α in which proline residues in the P1 and P2 motifs were mutated to alanine were constructed and the effects of these mutations on the binding of the SH3 domain of p85α to exogenous ligands was examined. Wild type p85α exhibited increased binding to exogenous P1 peptide when endogenous p85α P1 but not p85α P2 was mutated. Deletion of the SH3 domain of p85α allowed the exogenous SH3 domains of Src and p85α to bind the endogenous p85αP1 motif more efficiently (Figure 5.8). Therefore mutation of one region in p85α increased the binding affinity of another region in the same molecule.

The best explanation for this phenomenon is an interaction of p85αSH3 and p85αP1. This hypothesis also provides an explanation for the differential binding of p85αSH3, p85αSH3BH and full length p85α described in Chapter 4. For example, p85αSH3 was shown to bind dynamin (p100) from Jurkat and HL60 cell lysates, whereas p85αSH3BH and full length p85α did not (Figure 4.12). If p85α SH3 was bound to p85αP1 in p85αSH3BH and p85α, it would be less accessible to exogenous cellular binding partners. The lack of accessibility of the p85αSH3 domain also provides an explanation for the lack of reports of p85α binding partners in vivo.

It seems likely that p85αSH3 does not bind p85αP2. The naturally occurring isoform of p85α, p55γ, which lacks the SH3 domain and P1 motif but has an identical P2 sequence to p85α, could not be co-precipitated with GSTp85αSH3 (see Figure 5.11). Screening of phage display libraries also identified p85αP1 as the preferred binding partner for p85α compared to p85αP2 (Rickles et al., 1994). It has been suggested that p85αP2 is sufficiently exposed in full length p85α to bind exogenous SH3 domains (Liu et al., 1993), although it remains possible that this sequence is occluded in full-length p85α, perhaps by steric hindrance.

Since the studies reported in this thesis were carried out, there have been several reports which support the hypothesis that there is an intramolecular interaction between p85αSH3 and p85αP1. p85αP1 is reported to be the highest affinity ligand for the p85αSH3 domain in binding experiments with synthetic peptides (Y.Zvashchenko personal communication). The structural basis for the binding of proline-rich peptides to SH3 domains has been reported (Yu et al., 1994), (Feng et al., 1994). The use of biased combinatorial libraries, multidimensional NMR and structure based mutagenesis showed that only two proline residues in a proline-rich motif made direct contact with the SH3 domain, whilst the others appeared to function as a molecular scaffold,
promoting the formation of a type II polyproline helix. Three nonproline residues, often arginine or leucine, also interact with the SH3 domain and appear to confer ligand specificity. Several ligands that were selected from the peptide libraries on the basis of their ability to bind to a fluorescein-conjugated p85α SH3 domain contained the consensus sequence RXLPPRP, where X represents any amino acid except a cysteine residue. In p85α, the sequence KPRPPRPLPVAP in the P1 motif was proposed as a likely ligand for p85αSH3, as it contains two partially overlapping motifs (KPRPPRP and RPLPVAP), both of which fulfill the consensus sequence criteria for this domain (Yu et al., 1994). In addition, it has been reported that the proline-rich motifs in PI3K p85 can bind to the SH3 domains of Abl, Lck, Fyn, and p85, with the p85 SH3 domain exhibiting the strongest affinity (Kapeller et al., 1994).

Unlocking the closed conformation of p85α, in which the SH3 domain is bound to its own P1, would free the SH3 domain and allow it to bind dynamin or other exogenous proline-rich ligands. Secondly, unlocking the p85αSH3P1 interaction would allow access to the p85αP1 motif by the SH3 domains of the Src family tyrosine kinases. The locking and unlocking of this interaction may be a regulatory mechanism for PI3K, however this will require further investigation. There is, however, evidence that this may be the case, as it has been reported that the proline-rich region in p85 mediates the activation of lipid kinase activity by binding to the Lyn or Fyn SH3 domains (Pleiman et al., 1994). This type of regulatory mechanism is analogous to that reported for the Src family tyrosine kinases, in which phosphorylation of a carboxy-terminal tyrosine residue regulates kinase activity and substrate binding through co-operative intramolecular binding of the tyrosine phosphorylated tail and the SH2 domain, and a pseudo type II polyproline helix and the SH3 domain (Cooper and Kashishian, 1993; Courtneidge, 1985; Xu et al., 1997), (section 1.3.3.1.3). A similar mechanism has also been shown to be important for the regulation of the Tec family of intracellular tyrosine kinases. The SH3 domain of Itk interacts with an adjacent proline-rich region, and therefore restricts access of the SH3 domain to potential exogenous binding partners (Andreotti et al., 1997). Phosphorylation of a tyrosine residue within the SH3 domain of Btk, a member of the Tec family was reported to disrupt this intramolecular SH3-proline-rich region interaction, thereby liberating the SH3 domain to engage kinase substrates (Park et al., 1996).

As discussed in chapter 4, no GAP activity has been detected in recombinant p85α or PI3K to date. It is possible that the BH domain of p85 may be a GAP for an as yet unidentified GTPase protein, however, it is also possible that the SH3 domain interactions regulate the GAP activity of the neighbouring BH domain. A specific
p85αSH3 domain or p85αP1 binding event maybe required to unlock the intramolecular interaction before GAP activity is detectable.

The SH3 domain of p85 may therefore provide a link between receptor binding of PI3K and activation of downstream pathways involving SH3 domain and proline-rich motif containing proteins that regulate cellular functions such as cytoskeletal rearrangements and endocytosis. The interaction of p85α and one such SH3 domain containing protein, c-Src, was examined further.
5.4 SRC and PI3K

5.4.1 Introduction

PI3K was first identified in anti-v-Src immunoprecipitates from lysates of Rous sarcoma virus-infected chicken embryo fibroblasts (CEFs) (Sugimoto et al., 1984). Activation of PI3K (Fukui and Hanafusa, 1989) and physical association of this lipid kinase with the activated Src tyrosine kinase (Fukui et al., 1989) are correlated with cellular transformation by Src. PI3K activity was also found associated with RTKs in cells transformed with polyoma middle T-antigen (Bjorge et al., 1990; Fukui and Hanafusa, 1989; Kaplan et al., 1987; Whitman et al., 1985). Indeed, the transforming ability of the polyoma middle T-antigen c-Src complex was dependent on its association with PI3K (Courtneidge and Heber, 1987; Whitman et al., 1985).

In vivo, the amount of PI3K binding to v-Src was reduced by deletions in the v-Src SH2 domain (Fukui et al., 1991), which led to the original proposal that v-Src associates with PI3K through binding of a phosphorylated tyrosine in p85α to the Src-SH2 domain. (Cantley et al., 1991; Fukui et al., 1991). However, mutations in the v-Src SH3 domain also decrease the amount of PI3K binding to Src, and result in a partially transformed, fusiform morphology (Wages et al., 1992). In vitro, the v-Src SH3 domain, but not the SH2 domain, bound PI3K in lysates of CEFs and was able to form a complex with the amino-terminal region of the p85 subunit of PI3K (Liu et al., 1993). These results suggested that the v-Src SH3 domain may mediate the association with PI3K. Moreover, direct physical interaction between the p85 subunit of PI3K and the SH3 domains of several Src family members was demonstrated (Liu et al., 1993; Pleiman et al., 1993; Prasad et al., 1993; Prasad et al., 1993) and this association was shown to involve binding of the Src or Src family SH3 domain to one or two proline-rich sequences on p85 (Kapeller et al., 1994; Liu et al., 1993; Pleiman et al., 1994). In activated B cells, stimulation of PI3K activity correlated with binding of p85 to the SH3 domains of Fyn or Lyn, and this activation could be blocked by a peptide corresponding to the first proline rich motif in p85α (Pleiman et al., 1994). In contrast, successive rounds of anti-phosphotyrosine immunoprecipitations from cell extracts depleted the amount of PI3K activity able to bind v-Src (Fukui et al., 1991). More recently, there has been additional evidence that tyrosine phosphorylation of p85α is important for binding of PI3K to Src through the Src SH2 domain (Fukui et al., 1991; Haefner et al., 1995). This study demonstrated that all Src homology domains cooperate to positively regulate the binding of PI3K in response to activation of the oncprotein.
Clearly, the association of Src with PI3K is complex and involves multidomain interactions including contributions from both the SH2 and SH3 domains of Src. The exact mechanism of binding of p85α to Src remains unclear however, and the site or sites on p85α that are tyrosine phosphorylated by Src have not been determined.

5.4.1 Aims
To date, most studies of the interactions of p85α with Src have examined which regions of Src associate with P85α and the subsequent effect of the associated PI3K activity on the transforming ability of Src. In the light of the intramolecular interactions in p85α described previously, it is possible that the intermolecular interactions with Src may involve multiple regions of p85α. Using the panel of p85α deletion mutants and c-Src expressed in insect cells, this study attempted to map the domain or domains of p85α that can associate with Src. p85α serves as a substrate of Src and subsequently becomes tyrosine phosphorylated. The efficiency of this phosphorylation is examined and an attempt was made to delineate the domain containing the site of phosphorylation in p85α.

5.4.2 Results

5.4.2.1 Recombinant p85 proteins are substrates for Src family protein-tyrosine kinases
The list of Src substrates is long and diverse. Many proteins bind to and are phosphorylated by Src, and others have elevated levels of tyrosine phosphorylation in Src transformed cells, but have not been shown to interact directly with Src. The p85α regulatory subunit belongs to the former group. In order to show that p85α serves as a substrate for c-Src and therefore becomes phosphorylated, baculoviruses encoding both proteins were used to co-express p85α and c-Src in insect cells. Lysates from infected cells were subjected to SDS-PAGE and immunoblotted with an antiphosphotyrosine antibody (Figure 5.9A) (PY20 see section 2.6.2) or subjected to an in vitro protein kinase assay (Figure 5.9B). p85α was phosphorylated on tyrosine residues when co-expressed with Src.

5.4.2.1.1 A complex forms between the p85 proteins and Src
As shown above, p85α is a phosphorylation substrate for c-Src. However, in order to investigate whether p85α and c-Src formed a complex or whether p85α was simply an exogenous substrate for c-Src, lysates of Sf9 cells co-infected with recombinant baculovirus encoding c-Src and one of the p85 mutants, were incubated with an anti-c-
Src antibody and the immunocomplexes were collected using Protein A-Sepharose CL6B. All p85α mutants were able to be expressed in Sf9 cells when Src was co-expressed with them (Figure 5.10). p85α and all mutants were co-immunoprecipitated with Src by an antibody directed against the SH3 domain of Src, suggesting that the reported interactions between the SH3 domain of Src and the p85αP1 motif (Kapeller et al., 1994; Pleiman et al., 1994) do occur. However, p85α mutants in which the intramolecular p85α SH3-P1 interaction is disrupted (p85αΔP1a, p85αΔP1aP2 and p85αΔSH3) are co-immunoprecipitated with Src more efficiently than wild type p85α (Figure 5.10), suggesting that unlocking the intramolecular p85α interaction as described in the first part of Chapter 5, increases the potential for p85α to participate in intermolecular interactions. In vivo, the SH3 domains of Src and p85α may compete for p85α P1 binding.

Interestingly, p55γ and p49α were also able to co-immunoprecipitate with c-Src (Figure 5.10). p55γ is a naturally occurring isoform of p85α that lacks the amino terminal domains of p85α, including the SH3 domain, the first proline-rich motif and the BH domain. However, p55γ contains a proline-rich sequence identical to the p85αP2 motif. It has previously been shown that p85αP2 does not bind Src SH3 ((Kapeller et al., 1994)) (Figure 5.4(B)). In order to confirm that the observed binding of p55γ to c-Src did not involve the Src SH3 domain and p85αP2, GST fusions of the SH3 domains from c-Src (GST-c-Src-SH3), p85α (GST-p85αSH3), and Fyn (GST-fyn-SH3), were immobilised on glutathione Sepharose CL4B and incubated with lysates of Sf9 cells infected with baculovirus encoding p55γ. Neither GST-c-SrcSH3 or the other SH3 domains were able to co-precipitate p55γ (Figure 5.11(i) and Figure 5.11(ii)). p49α, which comprises just the two SH2 domains and the inter-SH2 domain, and has no proline rich regions, also co-precipitates with c-Src, although to a lesser extent suggesting that, in addition to the Src-SH3 p85αP1 interaction, Src must interact with another region of p85α. Furthermore, the interaction must be with either the SH2 domains or inter-SH2 domain in p85α, since these regions are common to both p49α and p55γ.

5.4.2.2 Phosphorylation of Src is required for efficient phosphorylation of the p85α proteins

Activated forms of Src are autophosphorylated on Tyr416 (Ferracini and Brugge, 1990), however, the exact role of this phosphorylated residue in the activation of Src is unclear. Phosphorylation of Tyr416 does not seem to be absolutely required for activation of Src, but may modulate substrate specificity (Brown and Cooper, 1996).
The effect of autophosphorylation on the association of Src with p85α was examined. In order to keep endogenous autophosphorylation to a minimum, c-Src was immunoprecipitated from lysates of Sf9 cells 1.5 days post-infection (Courtneidge et al., 1993). The immunoprecipitate was divided into two and half the sample was pre-autophosphorylated using non-radiolabelled ATP (as described in section 2.7.3). Unphosphorylated and pre-phosphorylated Src were incubated with lysates of Sf9 cells infected with baculoviruses encoding p85α or p85 mutants. Src was then immunoprecipitated using an anti Src antibody and the resultant immunocomplexes were subjected to an in vitro protein kinase assay and analysed by autoradiography of an SDS-PAGE gel (fig 5.12). If Src was not auto phosphorylated first, it was unable to phosphorylate p85α to a detectable level in the 20 minute duration of the kinase assay (Figure 5.12, left panel). In the absence of autophosphorylation, it has already been shown that Src associates with p85α, however, when Src was auto- phosphorylated first, it was able to phosphorylate p85α (Figure 5.12). Clearly, the preferred substrate for Src tyrosine kinase activity is Tyr 416 on Src itself, but autophosphorylation activates Src tyrosine kinase activity and increases its ability to phosphorylate exogenous substrates. Src is able to phosphorylate p85α even when not pre-phosphorylated in vitro (Figure 5.9), however when reaction time is limiting, pre-autophosphorylation increased the level of phosphorylation of p85α and all p85α mutants examined. The unlocked mutants of p85α were not significantly better substrates for Src than wild type p85α (Figure 5.12), although they were better able to bind Src than wild type p85α (Figure 5.10). This may suggest that binding of p85α and phosphorylation of p85α by Src are two separate events.

5.4.2.2.1 The association of PI3K with phosphorylated Src

The effect of autophosphorylation of c-Src on its ability to bind PI3K activity was also examined. c-Src was immunoprecipitated as previously described and split into two samples. One sample was pre-autophosphorylated and the other was not. Equal amounts of purified p85α/p110α or p49α/p110α complexes were added to the Src immunoprecipitates, unbound PI3K was washed away then bound PI3K was assayed for its ability to phosphorylate PI (Figure 5.13). More PI3K activity was associated with pre-autophosphorylated Src than non-prephosphorylated Src. This may be because phosphorylated Src binds better to PI3K, or because phosphorylated Src activates the same amount of bound PI3K compared to non-phosphorylated Src, or possibly both. In either case, activation of Src by autophosphorylation of Tyr 416 increases the level of Src-associated PI3K activity.
5.4.2.2.2 The p85α carboxy-terminal SH2 domain can bind the Src tyrosine kinase

It has previously been demonstrated that the Src SH3 domain binds p85αP1 (see Figure 5.4 and Figure 5.8A). However, p55γ and p49α lack the p85αP1 motif but were both able to co-precipitate with Src in insect cells (Figure 5.10) and serve as Src substrates (Figure 5.12). In order to further examine the region or regions in the carboxy terminus of p85α that are able to interact with c-Src, a number of amino-terminal p85α deletion mutants were expressed as GST-fusion proteins. These include p49α with the putative p110α binding site (residues 445-485) deleted (GST-p49αΔBS110), the amino terminal SH2 domain (GST-p85αN-SH2) and the carboxy terminal SH2 domain (GST-p85αC-SH2). These proteins were immobilized using glutathione Sepharose CL4B and incubated with Sf9 cell lysates containing c-Src. Alternatively, c-Src was immobilized using an anti c-Src antibody and incubated with the fusion proteins. The complexes were subjected to an in vitro protein kinase assay or examined for co-precipitation by SDS-PAGE (Figure 5.14).

It has been demonstrated that Src must be pre-autophosphorylated in order for p85 to serve as an efficient substrate for Src (see Figure 5.12). Immobilised Src was pre-autophosphorylated and was able to phosphorylate GST-p85αΔBS110 but not GST-p85αN-SH2 or GST-p85αC-SH2 (Figure 5.14A). Firstly, this suggests that phosphorylation of p85 by Src does not occur in either of the SH2 domains of p85α. Secondly, phosphorylation is likely to occur in the inter-SH2 domain but not in the amino acids corresponding to the p110 binding site. In order to examine the carboxy terminal region or regions of p85 that interact with Src, both unphosphorylated and pre-phosphorylated Src were incubated with the p85 mutants. The protein complexes were subjected to SDS-PAGE and analysed by Western blotting using an anti-GST antibody (Figure 5.14B). The p85 mutants were unable to bind unphosphorylated Src but GST-p85αC-SH2 and GST-p85αΔBSp110 could bind phosphorylated Src. A longer exposure of the western blot showed that the GST-p85αN-SH2 domain could also co-precipitate with phosphorylated Src, although to a much lesser extent than either GST-p85αΔBS110 or GST-p85αC-SH2. These experiments show that phosphorylated Src can recruit itself to p85α in a way that allows it to phosphorylate p85α more efficiently.

It has previously been shown that phosphorylated c-Src can associate with increased PI3K activity (section 5.4.2.2.1). This increased association may be due to an interaction between the p85αC-SH2 domain and the autophosphorylated Tyr416 of Src, thereby bringing the Src tyrosine kinase into close proximity with its substrate, a tyrosine residue within the inter-SH2 domain of p85α.
5.4.3 Discussion

The interactions between Src and PI3K are clearly complex. The data described in the first part of chapter 5 and in the literature (Kapeller et al., 1994) clearly demonstrate that the SH3 domain from c-Src has the capacity to bind the first proline-rich motif of p85α. Both the Src SH3 domain and full length Src bound to p85α more efficiently when the SH3 domain of p85α was deleted (Figure 5.8A, 5.10), suggesting that unlocking the intramolecular p85αSH3-P1 interaction increased the accessibility of the p85αP1 motif to the Src SH3 domain. To simplify interpretation, the c-Src used in this study was itself unlocked and therefore constitutively activated. It has been reported previously that SF9 cells contain no Csk, which is required to phosphorylate Tyr 527 and repress the Src kinase activity (Okada et al., 1991). All mutants of p85α were able to bind Src, although to different extents. As described above, p85αΔSH3 bound to Src more efficiently than p85α. It is difficult to interpret the effects of mutating proline residues in the p85αP1 and p85αP2 motifs on Src binding, as these mutations both unlock the endogenous p85αSH3P1 interaction, increasing Src binding, but also mutate the Src SH3 binding site thus presumably decreasing Src binding. It is the balance of these two effects which would result in the observed level of binding. The binding of Src SH3 to p85αP1 sequences in which these proline residues had been mutated was not measured directly, but it is likely that the Src SH3 domain had an appreciable affinity for mutated p85αP1. The Src SH3 binding site in Src itself is a poorly conserved proline-rich motif, thus the Src SH3 domain may be more promiscuous in its binding specificity compared to p85αSH3. If this were the case, mutating proline residues in p85αP1 would decrease the affinity of p85αSH3 for p85αP1 to a greater extent than Src SH3, leading to the observed increase in binding of Src SH3 to p85αΔP1a and p85αΔP1aP2 (Figure 5.10). Another possibility, is that the Src SH3 binding site overlaps with but is not identical to the p85αSH3 binding site therefore mutating the two proline residues in p85αP1 may not disrupt the Src SH3 binding site. Mutation of proline-rich residues in p85αP2 also apparently increased the amount of bound Src (Figure 5.10). It has previously been shown that the Src SH3 domain is unable to bind p85αP2 (see Figure 5.4.) and therefore it is possible that proline mutations in p85αP2 exert a long range effect on the binding between Src and p85αΔP2. Interestingly, p85αΔP2 had a slightly higher apparent molecular weight when analysed by HPSEC compared to p85αΔP1a (Table 6.2), supporting the idea that it has a less compact structure.

Src was able to bind all p85α mutants better when it was pre-autophosphorylated on tyrosine 416, suggesting that an SH2-domain mediated interaction may be involved.
p55γ and p49α bind Src less efficiently than the other p85α mutants. This may be due to the absence of p85αP1 in both these mutants which is the known Src binding site. In addition however, Src must also be able to interact with the carboxy terminus of p85α. The location of this Src binding site was further examined. When Src was pre-autophosphorylated, it was able to co-precipitate all mutants containing the carboxy terminal SH2 domain of p85α, but not the amino terminal SH2 domain. It therefore appears that Src binds to p85α via a dual interaction of SrcSH3-p85αP1, which firstly requires unlocking of the p85αSH3P1 interaction, presumably by a competition mechanism. Secondly, an interaction between Src-pTyr416-p85α-C-SH2 which requires pre-autophosphorylation of Tyr416 by Src. Such a dual interaction would stabilise the protein complex but the outcome of the formation of this complex is still not clear. One possible role of this dual interaction, would be to increase the activity of PI3K (Figure 5.13). Alternatively, this interaction may create binding sites for other intracellular proteins.

p85α is also a tyrosine phosphorylation substrate for Src. Both unphosphorylated and pre-autophosphorylated Src phosphorylate p85α, although p85α is more efficiently phosphorylated by pre-autophosphorylated Src over the 20 minute time period studied (Figure 5.12), suggesting that autophosphorylation of Src Tyr 416 increases the rate, rather than the extent, of this phosphorylation reaction. The rate of this reaction is probably increased by recruitment of p85α to Src via binding of its C-SH2 domain to Src tyr 416. The role of phosphorylation of Tyr 416 in the activation loop of Src is unclear. The analogous tyrosine residue is phosphorylated in many kinases. In some cases, for example in CDK2, phosphorylation of Thr 160 is required for activation of CDK kinase activity (Johnson et al., 1996). However, while Thr160 is phosphorylated during physiological activation of CDK2, Tyr416 is not phosphorylated to a significant extent when Src is activated during mitosis (Chackalaparampil and Shalloway, 1988) or following PDGF stimulation (Gould and Hunter, 1988). In comparison, Tyr 416 does become phosphorylated following platelet activation (Clark and Brugge, 1993). Mutation of Tyr416 dramatically suppresses tumorigenicity (Kmieciak and Shalloway, 1987; Snyder and Bishop, 1984) but only moderately affects in vitro kinase activity (Kmieciak and Shalloway, 1987; Piwnica Worms et al., 1987). The activation loop is not well resolved in either the Src or the Hck three-dimensional structures, suggesting that it is flexible when Src is in its locked, inactive state. In comparison, the activation loop was resolved in the three dimensional structure of inactive CDK2, and was seen to occlude the active site cleft. Autophosphorylation of Tyr416 therefore does not seem to be required for activation of Src kinase activity, and the mechanism behind the increased tumourigenicity of Src which retains the potential to become
autophosphorylated is unclear. It is possible that phosphorylation of this site may create a new binding site for the recruitment of SH2 and PTB domain containing proteins. In this study, the binding of Tyr416 to the carboxy terminal SH2 domain of p85α supports such a hypothesis. The binding of phospho-Tyr416 to an SH2-domain containing substrate may promote tumourigenicity by recruiting the substrate to the catalytic domain of Src.

The site on p85α that is tyrosine phosphorylated by Src has been mapped to a region of the interSH2 domain that is not within the putative p110α binding site. At present, it is not known how the phosphorylation of p85α by Src affects PI3K function. Pre-auto phosphorylation of Src resulted in co-immunoprecipitation of more lipid kinase activity with anti-Src antibodies compared to non-phosphorylated Src. This may be due to co-immunoprecipitation of more PI3K due to enhanced binding of PI3K to Src via the p85αCSH2-Src pTyr 416 interaction. Alternatively, the same amount of PI3K may be co-immunoprecipitated but phosphorylation of p85α in the inter-SH2 domain may activate lipid kinase activity. Phosphorylation of a tyrosine residue in the interSH2 domain of p85α may also create new binding sites for other SH2 or PTB domain containing proteins, and thus recruit other intracellular proteins to the activated Src/PI3K complex. It is difficult to resolve the effects on PI3K of Src binding and Src phosphorylation. However, mutants described in this study may help to resolve this; for example, p85αΔSH3 bound Src better compared to that of p85α but were both phosphorylated to the same extent.

To date, the nature of the signals that trigger unlocking of the intramolecular p85αSH3P1 interaction are unknown, although there is one report of an increase in exogenous SH3 domain binding to p85α following B cell receptor activation, and concomitant activation of PI3K (Pleiman et al., 1994). However, it is clear that unlocking this interaction permits binding of signalling molecules, such as Src, via their SH3 domains, and this binding may then be stabilized by further interactions between other domains in these multi-domain proteins, such as SH2 domains and phosphorylated tyrosine residues. This binding can, in the case of Src, trigger a chain of events including phosphorylation of p85α by Src and the creation of potential binding sites for other signalling molecules. It is not clear, however, whether these events affect the lipid kinase activity of PI3K, and thus activation of pathways leading to PI3K-induced mitogenesis, or whether these events represent a bifurcation of PI3K signalling pathways, with the SH3 domain mediated pathways eventually leading to the cytoskeletal rearrangements and endocytic events in which PI3K has been implicated.
Figure 5.1 Proteins from bovine brain extracts bound to recombinant GST-SH3 domains. Associated proteins were resolved by SDS-PAGE and visualized by silver staining. Lane 1, GST-p67-C-SH3, Lane 2, GST-p67-N-SH3, Lane 3, GST-p47-C-SH3, Lane 4, GST-n-Src-SH3, Lane 5, GST-c-Src-SH3, Lane 6, GST-PLCγ-SH3, Lane 7, GST-RasGAP-SH3, Lane 8, GST-p85α-SH3, Lane 9, GST
Figure 5.2 Analysis of the differential binding of GSTp85α mutants to rat brain and liver proteins by Silver stained SDS-PAGE. Lanes 1, 5 and 9, GST. Lanes 2, 6 and 10, GST-SH3. Lanes 3, 7, and 11, GST-SH3-BH. Lanes 4, 8 and 12, GST-p85α.
Figure 5.3 Binding of Grb2 and GST-SH3 domains of p85α and c-Src to immobilised proline-rich peptides. Green, Grb2; Black, p85α and Red, c-Src. (A) The first proline-rich sequence from p85α, P1 (B) The second proline rich sequence from p85α, P2.
Figure 5.4 (i) Binding of Grb2 and GST-SH3 domains from p85\(\alpha\) and c-Src to the amino-terminal dynamin proline-rich peptide.
Figure 5.5 Binding of GST-p85α proline mutants to Y751 peptide (see text). Black, p85α; Pink, GSTp85αΔP1a; Purple, GSTp85αΔP2; Green, GSTp85αΔP1aP2
Figure 5.6 Binding of p85α mutants to immobilised proline-rich peptides. (A) The first proline-rich sequence from p85α, P1. (B) The amino-terminal proline-rich peptide from dynamin. Black, p85α; Pink, GSTp85αΔP1a; Purple, GSTp85αΔP2; Green, GSTp85αΔP1aP2.
Figure 5.7 Model for locking and unlocking of p85α SH3-P1 interaction.

The proposed model suggests that the SH3 domain of p85α is bound to its own P1 motif. Mutation of P1 unlocks the SH3 domain, which is then able to bind the exogenous substrates such as dynamin. Unlocking the SH3-P1 interaction would also allow exogenous SH3 domains, such as those from the Src family tyrosine kinases, to bind p85α-P1.
Figure 5.8 Co-precipitation of \( \text{p85}\alpha/\text{p110}\alpha \) and \( \text{p85}\alpha\Delta\text{SH3}/\text{p110}\alpha \) by exogenous SH3 domains. \( \text{GSTp85}\alpha\text{SH3} \) and \( \text{GSTc-Src-SH3} \) were immobilized on glutathione sepharose beads and incubated with SF9 cell lysates containing recombinant \( \text{p85}\alpha/\text{p110}\alpha \) or \( \text{p85}\alpha\Delta\text{SH3}/\text{p110}\alpha \). The resulting complexes were resolved on SDS-PAGE (A) or assayed for PI3K activity (B).
Figure 5.9 Phosphorylation of p85α by c-Src *in vivo* and *in vitro*. (A) p85α and c-Src were co-expressed in Sf9 cells and analysed by SDS PAGE followed by Western blotting using an anti-phosphotyrosine antibody. Lysate of Sf9 cells expressing c-Src alone (Lane 1), or co-expressing p85α. (B) Lysates prepared from Sf9 cells infected with recombinant baculovirus expressing c-Src alone (Lane 1) or c-Src co-expressed with p85α (Lane 2), were immunoprecipitated with anti c-src antibodies and the immunocomplexes were subjected to a protein kinase assay *in vitro*. 
Figure 5.10 Association of p85α mutants with c-Src in vitro.

p85α mutants were co-expressed with c-Src in Sf9 cells. Lysates were immunoprecipitated with an anti c-Src antibody and the resulting immune complexes were analysed by SDS-PAGE. Western blotting was performed with an anti p85α antibody directed against the carboxy terminal SH2 domain and with an anti p55γ antibody to detect p55γ.
Figure 5.11 The SH3 domains of p85<i>a</i>, c-Src and Fyn do not co-precipitate p55<i>γ</i> in vitro. GST fusion proteins of the SH3 domains of p85<i>a</i>, c-Src and Fyn were immobilized on glutathione sepharose and incubated with a lysate containing baculo-expressed p55<i>γ</i>. The bound proteins were analysed by (A) Coomassie-Blue stained SDS-PAGE and (B) Western Blotting with an antibody directed against p55<i>γ</i>.
Figure 5.12 Phosphorylation of c-Src is required for the efficient phosphorylation of p85α mutants. Sf9 cell lysates containing c-Src were immunoprecipitated with an anti-c-Src antibody. The immune complex was split into two and one half was pre-phosphorylated by incubation with non-radioactive ATP. Both unphosphorylated and pre-phosphorylated immobilized Src was then incubated with Sf9 cell lysates expressing the p85α mutants. The resultant protein complexes were subjected to protein kinase assays, resolved by SDS-PAGE and analysed by autoradiography.
Figure 5.13 PI3K activity associated with unphosphorylated and pre-phosphorylated c-Src. Sf9 cell lysates containing c-Src were immunoprecipitated with an anti-c-Src antibody. The immune complex was split into two and one half was pre-phosphorylated by incubation with non-radioactive ATP. Both unphosphorylated and pre-phosphorylated immobilized Src was then incubated with Sf9 cell lysates expressing either p85α/p110α or p49α/p110α. The immune complexes were subjected to PI3K assays. Associated PI3K activity of p85α/p110α with unphosphorylated Src (Lane 1) and pre-phosphorylated Src (Lane 2). Associated PI3K activity of p49α/p110α with unphosphorylated Src (Lane 3) and pre-phosphorylated Src (Lane 4).
Figure 5.14 (A) SF9 cell lysate containing c-Src was immunoprecipitated with an anti-c-Src antibody and was pre-phosphorylated by incubation with non-radioactive ATP. The immobilised c-Src was then incubated with the carboxy terminal GSTp85α mutants. The resultant protein complexes were subjected to protein kinase assays, resolved on SDS-PAGE and analysed by autoradiography. Lane 1, p49αΔBS110; Lane 2, GSTp85αN-SH2; Lane 3, GSTp85αC-SH2; Lane 4, c-Src alone. (B) c-Src containing SF9 cell lysate was immunoprecipitated with an anti-c-Src antibody. The immune complex was split into two and one half was pre-phosphorylated by incubation with non-radioactive ATP. Both unphosphorylated and pre-phosphorylated immobilised Src was then incubated with purified GSTp85α mutants and the resulting protein complexes were analysed by SDS-PAGE and Western blotting with an affinity purified anti-GST antibody.
Chapter 6: DIMERIZATION OF p85α

6.1 Introduction

The participation of PI3K in numerous signalling pathways and biological responses has generated interest in understanding the mechanism by which PI3K activity is regulated. Not only is diversity generated by the different classes of PI3K but also by the diversity within Class IA PI3Ks (section 1.4.7.3). The number of different p85 adaptor subunits suggests there are likely to be different mechanisms of signalling through PI3K.

The first adaptors to be identified for the Class IA catalytic subunits were p85α and p85β (Otsu et al., 1991). Both proteins contain two SH2 domains separated by an inter SH2 domain, through which the adaptor subunit interacts with p110 (Dhand et al., 1994). The p85 subunit also contains a BH domain, as discussed previously. It is flanked by proline-rich sequences that are potential targets of SH3 domains, including the p85α SH3 domain itself (Chapter 5) (Kapeller et al., 1994; Liu et al., 1993). The p85α BH domain interacts with Cdc42Hs and Rac but not with Rho (Li et al., 1995; Tolias et al., 1995; Zheng et al., 1994). G protein binding leads to the activation of the lipid kinase of the p110 subunit (Zheng et al., 1994), but the physiological significance of these results has yet to be established. The ability of the PI3K BH domain to interact with these small G proteins does not correlate with activation of GTP hydrolysis and it has not been possible to show that the p85 BH domain acts as a GAP for the G proteins with which it binds.

p55γ is the bovine homologue of p55PIK, which was first isolated during the screening of a mouse cDNA expression library for binding to recombinant IRS-1 (Pons et al., 1995). A rat homologue of p55γ has also been described (Inukai et al., 1996). p55γ contains two SH2 domains and an inter-SH2 domain, including 35 amino acids that correspond to the minimal p110 binding site in p85α (Dhand et al., 1994). The aminoterminal SH2 and carboxy-terminal SH2 domains are 89 and 81% identical to p85α, respectively, and 83% and 74% identical to p85β. However, p55γ lacks several protein-binding domains found in the amino-terminal portion of p85α and p85β, including the SH3 domain, the first proline-rich motif, and the BH region. They are replaced by a unique 34-amino acid sequence. p85α, p85β and p55γ are encoded by different genes but spliced variants of the p85α gene product have also been identified.
recently (Inukai et al., 1996; Inukai et al., 1997). These include p50α and p55α (also known as p85/AS53). A sequence comparison shows p55α and p50α are homologous to p55γ, except for the 34 amino acid sequence, which is replaced by a unique 6-amino acid sequence except that in p50α (section 1.4.7.3.1).

The significance of this adaptor diversity is unclear, since they apparently do not display selectivity in binding class1A catalytic subunits. In addition, little is known about the specificity of these p85 isoforms for RTK binding. Some adaptor isoforms have a restricted tissue specific distribution, thus it is possible that such structural modifications may be of regulatory significance.

6.1.1 Aims

The data described in the previous chapter suggests the p85αSH3 domain is bound to its own P1 motif. Mutagenesis studies were carried out to investigate whether this interaction was intermolecular or intramolecular. The effect of this interaction on the oligomerisation status of full length p85α was examined. A panel of p85 deletion mutants, including the naturally occurring bovine isoforms p55γ and p49α, which is the bovine homologue of p50α but lacks the 6 residue amino terminal extension and the P2 motif, were expressed as recombinant proteins in bacteria (E.coli), insect (Sf9) and mammalian (COS7) cells. The structure and function of the intact p85α and various combinations of its domains were examined using a several biochemical and biophysical techniques. Together, these results begin to elucidate, at a structural level, the mechanism by which PI3K maybe activated and regulated downstream of transmembrane receptors.

6.2 Results

6.2.1 HP-SEC of Recombinant p85α, p85β, p55γ and p49α

For the initial comparative study, the recombinant p85 isoforms, p85α, p85β, the naturally occurring isoform p55γ and an amino-terminal p85 deletion mutant, p49α, (Table 6.1) were produced using the baculovirus expression system. The proteins were purified using phosphotyrosine affinity chromatography as described in section 2.4.1.2. Each protein had a molecular weight that would be predicted from its amino acid sequence (Table 6.1) when examined by reducing SDS-PAGE (Figure 3.11). In
comparison, the apparent molecular weight of these proteins when examined by high performance size exclusion chromatography (HP-SEC) under native conditions did not always agree with that predicted from their amino acid sequence. The apparent molecular weights of p85α and p85β by HP-SEC were 162±14 kDa and 151±12 kDa respectively, suggesting that p85α and p85β are dimeric under native conditions (Figure 6.1 and Table 6.1). The shorter isoforms, p55γ and p49α, had an apparent size of 82±4 kDa and 69±7 kDa respectively, which is larger than, but not double their predicted monomeric molecular weights of 53 kDa and 50 kDa respectively. These initial results suggested that full length p85 had the potential to dimerize in vitro. It was unclear, however, whether p55γ and p49α were forming dimers or not.

HP-SEC measures the molecular volume or Stoke’s radius of a protein, rather than the actual molecular weight. There are, three mechanisms by which a protein can have a higher apparent molecular weight than would be predicted from its amino acid sequence. Firstly, it could form a dimer or other oligomers, and this would seem to be the case for p85α and p85β, as their apparent molecular weights by HP-SEC are double their predicted molecular weights. Secondly, an equilibrium could exist between two oligomeric states, such as an equilibrium between monomer and dimer. If the rate at which the monomer and dimer interconvert is less than the time the proteins take to move through the column, an average molecular weight depending on the proportions of monomer and dimer in the equilibrium will be seen. Thirdly, the shape of a protein influences its retention time by HP-SEC. Because the proteins used as standards are idealised compact, globular proteins, a protein which is less compact, due to perhaps partial unfolding or an elongated shape, will not have its molecular weight correctly estimated by comparison to the standard proteins used to calibrate the column.

6.2.2 HP-SEC of p85α, p55γ and p49α COS7 transfectants

In order to ensure that the apparent molecular weights by HP-SEC of these proteins were not artefacts of being highly purified and concentrated, we studied their apparent molecular weights at low concentration in a cell lysate. The cDNAs encoding p85α, p55γ and p49α were transiently transfected into COS7 cells as described in section 2.2.2.3. The cells were harvested 48hrs after transfection and lysed in hypotonic lysis buffer. The supernatants containing the expressed proteins were subjected to HP-SEC and the resulting fractions analysed by SDS-PAGE and Western blotting with appropriate monoclonal antibodies (Figure 6.2).
p85α was detected in fractions 36, 37 and 38, which corresponded to a molecular weight range of 93-176 kDa (Figure 6.2A and Table 6.1). Whilst this is a broad range, the majority of the p85α elutes at a time that corresponds to a molecular weight greater than that of p85α (83 kDa) and there is a population at a dimeric molecular weight (176 kDa) which is in agreement with the molecular weight of the homogeneous, purified recombinant protein. Thus p85α is also apparently dimeric at low concentration, and when in a solution with a high total protein concentration, which would be expected to compete for any non-specific interactions that may cause oligomerisation of highly purified p85α.

In contrast, p55γ and p49α were detected in fractions which corresponded to molecular weight ranges of 40-61 kDa and 26-49 kDa respectively in this system (Figure 6.2B, 6.2C and Table 6.1). However, the highest molecular weight population for both p55γ and p49α are not greater than their predicted monomeric molecular weights of 55 kDa and 49 kDa respectively. This suggests that these proteins are monomeric within the context of a cellular environment, and the slightly higher than predicted molecular weight observed for the recombinant proteins was due to destabilization of the native structure outside the cellular environment, leading to these proteins being less compact than average. Interestingly, p55γ and p49α also seem to bind other intracellular proteins to form high molecular weight (>300 kDa) complexes. However, the nature of these complexes was not investigated further. In addition, the Western blot of p55γ suggested that there are two p55γ variants in COS7 cells. Reprobing this blot with anti-phosphotyrosine antibodies did not reveal the presence of any tyrosine phosphorylation in this protein, thus it is unlikely that the higher molecular weight band is a phosphorylated form of p55γ (data not shown). The cause of this higher molecular weight form is, unknown but it may be due to differential splicing of p55γ mRNA, or another type of post translational modification.

6.2.3 HP-SEC of p85 deletion mutants

The observation that p85α and p85β were dimeric under native conditions, but that p55γ and p49α were not, was intriguing. Thus, further deletion mutants were constructed in order to map the domains of p85α that were involved in this dimerization.

HP-SEC was also carried out in buffers containing either 5mM DTT or 8M urea in order to determine the nature of the interaction through which p85 dimerizes. The apparent molecular weights of p85α and p55γ were unaltered in the presence of 5mM
DTT (Table 6.1), suggesting that dimerization of p85α is not due to the presence of a disulphide bond. In contrast, p85α had an apparent molecular weight of 85±5 kDa in the presence of 8M urea. 8M urea disrupts non-covalent, but not covalent bonds, therefore the dimerization of p85α must be via a non-covalent interaction. The apparent molecular weight of p49α was unaffected by 8M urea (Table 6.1), further suggesting that it is monomeric under native conditions.

When the SH3 domain of p85 was deleted, the resulting protein (p85αASH3) had an apparent molecular weight (139±7 kDa) approximately double that predicted from its amino acid sequence (74 kDa) (Table 6.1). This suggests that removal of the SH3 domain does not disrupt the dimer interface to a significant extent. Similarly, deletion of the BH domain (p85αABH) also did not disrupt dimerization, as its molecular weight by HP-SEC (145±11 kDa) was still approximately double that observed by SDS-PAGE (64 kDa). In contrast, deletion of both the SH3 and BH domains, as is the case for both p55γ and p49α, has already been shown to result in a monomeric protein. Therefore, the dimerization interface resides in the amino-terminal half of the molecule, either in the SH3 or BH domains or the intervening regions.

When the amino terminal half of p85 was expressed on its own, either with or without one of the SH2 domains (p85αSH3-BH-SH2 and p85α SH3-BH)(Table 6.1), their apparent molecular weights by HP-SEC (101±10 kDa and 53±4 kDa respectively) were still approximately double those of SDS-PAGE (50 kDa and 38 kDa respectively). This confirms that the amino-terminal half of p85α contains all the amino acid residues required for dimerization.

A construct comprised of just the SH3 domain and the following 20 amino acids, the P1 motif, (p85αSH3-P1, Table 6.1), had an apparent molecular weight (23±2 kDa) approximately double that predicted (12 kDa), suggesting it is too dimeric. In contrast, the SH3 domain was unable to dimerize when expressed alone, as it had a similar apparent molecular weight by HP-SEC to that predicted from its amino acid sequence (13±1 kDa and 9.7 kDa respectively). However, p85αSH3-P1 does not contain the entire dimerization interface as neither deletion of the SH3 domain nor mutation of two proline (P96 and P99) residues in the P1 motif (p85αΔP1a, Table 6.2) converted p85α to a monomeric species. Additionally, the apparent molecular weight of the BH domain alone (32±2 kDa) was approximately 1.5 times that predicted (22 kDa). The BH domain may therefore also contribute to the dimerization interface.
6.3.4 HP-SEC of p85 proteins containing mutations in the p85αP1 motif

The studies described above map the dimerization interface in p85α to the amino terminal half of the molecule. There seems to be more than one domain involved in this dimerization interface as deletion of either the SH3 or BH domains does not result in monomeric p85α, but neither of these domains can dimerize completely on their own. The only element common to all dimerizing mutants of p85α was the P1 motif, suggesting that the p85α SH3-P1 interaction described in Chapter 5 may be intermolecular, and participate in dimerization. In order to address this question, mutants of p85α in which proline residues in the P1 and P2 motifs were mutated to alanine were subjected to HP-SEC. The apparent molecular weights of these mutants were essentially the same as that for wildtype p85α (170 kDa), suggesting they are also dimeric. As already described, the apparent molecular weight of p85αΔSH3 was approximately 150 kDa which is consistent with it being a dimer of a 74 kDa protein (Table 6.2). These results demonstrate that mutations in either the proline-rich motifs or the SH3 domain of p85α has no effect on its oligomerisation status.

It was possible however, that mutation of P96 and P99 to alanine was not sufficient to disrupt the endogenous p85αSH3-P1 interaction, therefore an arginine residue (R90) and two additional proline residues (P91 and P92) were mutated to alanine and expressed as a mutant of SH3P1 as GST-fusion proteins in E.coli. The apparent molecular weights of p85αSH3, p85αSH3-P1 and mutants of p85αSH3-P1 were compared using analytical size-exclusion chromatography. The two mutants of p85αSH3-P1 contained either two (P96 and P99; p85αSH3ΔP1a) or three (P91, P92 and R90 p85αSH3ΔP1b) point mutations (Table 6.3). As expected, p85αSH3 eluted with an apparent molecular mass (13.4 kDa) similar to that expected from its amino acid sequence (9.7 kDa). In contrast, SH3-P1 had an apparent molecular mass (23 kDa) approximately double that predicted from its amino acid sequence (12 kDa), and therefore is dimeric. Mutation of P96 and P99 in the proline-rich motif (p85αSH3ΔP1a) gave rise to two protein peaks. The first population had an apparent molecular weight of 14 kDa, which was consistent with the predicted monomeric molecular weight (11 kDa). The second population had an apparent molecular weight of 24 kDa and was therefore dimeric. The apparent molecular weight of p85αSH3ΔP1b (12.5 kDa) in which an arginine and two proline residues are mutated to alanine is similar to that of p85αSH3, suggesting that this mutant is monomeric. It is therefore apparent that mutation of greater than two proline residues is necessary to completely disrupt the endogenous SH3-P1 interaction. The binding of these proteins
to exogenous p85αP1 peptide was analysed using the biosensor. As previously
described, p85αSH3 bound to p85αP1 (Figure 5.4 and 6.3), however p85αSH3P1
did not bind exogenous p85αP1, suggesting the SH3-P1 interaction is locked.
p85αSH3P1b was able to bind exogenous p85αP1 to the same extent as p85αSH3
(Figure 6.3), suggesting that the p85αSH3P1 interaction was completely disrupted.
p85αΔSH3P1a was able to bind p85αP1 to a greater extent than p85αSH3P1, but less
than p85αSH3, which is consistent with a partial unlocking of the p85αSH3P1
interaction and the two peaks observed by HP-SEC. As previously described (section
5.2.3), mutation of P96 and P99 to alanine in full length p85α (p85αΔP1a and
p85αΔP1aP2) resulted in these mutants binding more efficiently to exogenous proline-
rich peptides compared to p85α. However, p85αΔP1a and p85αΔP1aP2 are fully
dimeric under native conditions as determined by HP-SEC (Table 6.2). These results
have three implications. Firstly, it seems unlikely that p85αP1 comprises the entire
p85α dimerization interface, as mutations shown to partially unlock the p85αSH3P1
interaction fail to result in a monomeric p85α population. Secondly, the p85αSH3-P1
interaction is unlikely to contribute to dimerization as neither deletion of the SH3
domain or mutation of proline residues in the p85α motif, both of which have been
shown to unlock the p85α SH3-P1 interaction, result in monomeric p85α. Thirdly,
dimerization of p85αSH3P1, which has been previously reported (Chen and Schreiber,
1994), is unlikely to occur via the same interface as dimerization of p85α, since
mutations that disrupted the dimeric interface in SH3P1 did not result in a monomeric
population of p85α.

The dimerization interface of p85α was therefore mapped to the amino-terminal half of
the molecule, with at least the SH3 domain, the BH domain or the P1 motif contributing
residues to the interface. Further work is required to determine the relative amount of
binding affinity contributed by the different domains within the amino terminal half of
p85α. The dimeric interface is unlikely to involve the p85αSH3P1 interaction as
discussed above, therefore this interaction must be intramolecular.

6.2.5 Alternative techniques to confirm HP-SEC data

6.2.5.1 Co-immunoprecipitations from COS7 cells transfected with
p85α mutants

To confirm that p85α could dimerize in a cellular context, a myc epitope tagged version
of p85α was transfected into COS7 cells, with or without an untagged version of
p85αΔBH. This is a deletion mutant of p85α that is still able to dimerize in vitro (Table 6.1). Both proteins were expressed in COS7 cells, as demonstrated by
immunoprecipitation from COS7 cell lysates from cells transfected with a single cDNA
The difference in molecular weight between these two forms of p85α allowed them to be resolved by SDS-PAGE.

When both cDNA’s were co-transfected, a monoclonal antibody against the myc tag was able to immunoprecipitate both the myc-tagged p85α and co-precipitate untagged p85αΔBH, suggesting they can form mixed dimers 

in vivo (Figure 6.4A lane 1). The same experiment was carried out with COS7 cells co-transfected with myc-tagged p85α and untagged p49α. Again both proteins were expressed (Figure 6.4A lane 2 and Figure 6.4B. lane 2). However, an antibody against the myc tag was unable to co-precipitate p49α, suggesting that p85α and p49α do not have the ability to form mixed dimers 

in vivo (Figure 6.4B lane 1). Therefore, mutants of p85α that have the ability to dimerize 

in vitro can form mixed heterodimers 

in vivo, where as monomeric forms of p85α cannot.

### 6.2.5.2 Determination of Molecular Weights by Sedimentation Equilibrium

Sedimentation equilibrium analytical ultracentrifugation (SE-AUC) was carried out in collaboration with Mr M. Bottomley of the Department of Biochemistry University College London and used to confirm that p85α was dimeric under native conditions. SE-AUC measures the degree to which a molecule sediments under gravitational force, an effect which is proportional to its molecular weight. SE-AUC has the advantage that the sedimentation equilibrium properties of a molecule are unaffected by its shape, and thus are a direct measure of molecular weight. SE-AUC can also detect self-association of molecules as a lack of adherence to the Lamm equation, which describes the distribution of non-associating ideal solute particles in a gravitational field. The occurrence of self association is manifested as a non-random distribution of residuals to the fit of the experimental data to a derivative of the Lamm equation. SE-AUC of purified, recombinant p85α-SH3P1, p85α-SH3-BH and p85α demonstrated that these proteins have the ability to self-associate and exist as an equilibrium between monomeric and dimeric forms under the conditions used (data not shown). In contrast, p49α displayed no tendency to self-associate, had an apparent molecular weight of 49 kDa by SE-AUC and therefore was monomeric (M.Bottomley, unpublished results).

### 6.2.6 HP-SEC of recombinant p85α/p110α complex

The experiments described above clearly demonstrate that p85α exists as a dimer, both 

in vitro and in vivo. However, p85α is generally in complex with a class 1A catalytic
subunit \textit{in vivo}. It is not known whether p85α ever exists \textit{in vivo} without an associated catalytic subunit. The effect of bound p110α on the ability of p85α to dimerize was therefore examined.

p85α was co-expressed in the baculovirus expression system with an engineered form of p110α, in which an epitope (glu) tag was incorporated at the carboxy-terminus to aid purification. The apparent molecular weight of high-purity recombinant p110α/glutag/p85α complex was also examined under native conditions by HP-SEC and found to be approximately 200-220 kDa (Figure 6.5 A), in agreement with previous reports (Carpenter et al., 1990; Shibasaki et al., 1991; Woscholski et al., 1994). However, a small higher-molecular weight shoulder was also observed which corresponded to an apparent molecular weight of approximately 300 kDa. SDS-PAGE of fractions across this peak revealed that the shoulder comprised PI3K and not a contaminant protein (data not shown) and the shoulder had PI3K activity (Figure 6.5A).

In contrast, when a diphosphorylated peptide derived form the p85αSH2 domain binding sequence in the PDGFβ receptor was added to the enzyme complex in an equimolar amount, the apparent molecular weight of the p110α/p85α was shown to increase to between 260-440 kDa, depending on protein concentration (Figure 6.5 B). Furthermore, lipid kinase assays of the fractions from HP-SEC demonstrated the existence of two enzymatically active populations. The first population was consistent with a protein complex having an apparent molecular weight of 200 kDa, whilst the other active population correlated with the higher molecular weight species. It appears that at least two populations of p110α/p85α complex exist in equilibrium in the presence of diphosphopeptide. The potential of p85α to dimerize is therefore only realised in the presence of p110α when a peptide that may mimic an activated receptor is added.

p49α and p110α were also co-expressed using the baculovirus expression system and affinity purified via the carboxy-terminal epitope tag on p110α. As before, the enzyme complex was subjected to HP-SEC (Figure 6.6 A) and had an apparent molecular weight of 160 kDa. This is consistent with a 1:1 complex of p49α and p110α proteins. Lipid kinase assays across the fractions correlated with a protein of the same size.

In contrast to p85α/p110α, addition of a diphosphorylated peptide to a complex of p110α and p49α, an isoform of p85 that does not have the potential to dimerize, does not increase the apparent molecular weight of this complex by HP-SEC (Figure 6.6 B).
Peptide binding may induce a conformational change in this complex, as the peak shape changes upon addition of peptide, however it is not the same change as observed when the same peptide is added to a complex of p110α/p85α (Figure 6.5B). The lipid kinase activity again mirrors the shape of the peak as detected by UV absorbance at 220nm. There are also some smaller weight species which may be either breakdown products of the enzyme complex or impurities in the protein preparation, however only one peak is enzymatically active.

The lack of apparent dimerization of p49α/p110α suggests that dimerization of p85α/p110α is not simply an artefact of being cross-linked by a diphosphorylated peptide because the same SH2 domains are in p49α/p110α and addition of diphosphorylated peptide does not result in apparent dimerization. The observed increase in molecular weight is therefore specific for p85α, the form of p85 already shown to dimerize. The increase in molecular weight of p85α/p110α complex is unlikely to be due to a non-specific effect of diphosphopeptide on the SH3 and BH domains that are present in p85α but not p49α, as addition of the same peptide to p85α alone does not cause the same shift in apparent molecular weight (Figure 6.6C). p85α alone is a constitutive dimer, and therefore does not require binding of the diphosphopeptide to induce dimerization.

The restriction of this observed increase in apparent molecular weight to the p110α/p85α complex suggests that this phenomenon is more likely to be due to the existence of an equilibrium between PI3K monomers and dimers, rather than a conformational change induced by peptide binding. Furthermore, it suggests that the inducible dimerization of p85α/p110α occurs through the same binding surface as the constitutive dimerization of p85α.
6.3 Discussion

In this study, a number of techniques have been used to demonstrate that p85α has the potential to dimerize both \emph{in vitro} and \emph{in vivo}. A series of p85 deletion mutants were used to define the interaction surface for dimerization, which was shown to be in the amino-terminal region of p85α, which includes the SH3 domain, the first proline-rich motif and the BH domain. It seems that both the SH3 and BH domains of p85α are involved in dimerization, however neither was able to fully dimerize as isolated domains (Table 6.1). Dimerization was shown to involve a non-covalent interaction between p85α molecules, as this interaction could be disrupted by 8M urea but not 5mM DTT. It was not possible to define further which regions of the amino-terminus of p85α were involved in binding, thus it would seem that this interaction surface involves residues which may be distributed widely in the primary structure of p85α, but may be positioned close together in three-dimensional space and therefore cooperate to form a single binding surface, in which no single domain contains detectable binding affinity.

Although the three-dimensional structures of the p85α SH3 domain (Booker \emph{et al.}, 1993; Koyama \emph{et al.}, 1993; Liang \emph{et al.}, 1996), show no evidence of dimerization, there is evidence that other SH3 domains have the potential to form dimers. Eps8 is a protein involved in signal transduction (Fazioli \emph{et al.}, 1993) whose function is not yet well understood, although it has been suggested to have a role in cell proliferation (Fazioli \emph{et al.}, 1993). Despite the absence of a functional SH2 domain, Eps8 associates with activated EGF receptor and is a substrate for this and other receptor tyrosine kinases (Castagnino \emph{et al.}, 1995). Native Eps8, a 97 kDa protein, contains a single SH3 domain, several proline-rich regions and a polylysine sequence (Fazioli \emph{et al.}, 1993). Unlike most SH3 domains, the optimal ligand for the Eps8-SH3 domain is not a proline-rich motif, but a sequence containing consecutive Asp and Tyr residues, with a single proline residue two or three amino acids either amino or carboxy side of this motif (Kishan \emph{et al.}, 1997). The recent three-dimensional structure of the Eps8 SH3 domain (Kishan \emph{et al.}, 1997) showed that Eps8-SH3 is a \textquoteleft strand-exchanged\textquoteright dimer, in which two different polypeptide chains form part of the SH3 domain fold. The prototypic SH3 domain fold can be considered as two clasped hands, in which each hand represents a small β-sheet. In the Eps8-SH3 domain, however, two sets of hands form a dimer in such a way that \textquoteleft hands\textquoteright from the different polypeptide chains are clasped together. The interface formed by the palms of the clasped hands is virtually identical in both the prototypic SH3 domain monomer and the Eps8 strand-exchanged SH3 domain dimer. In the prototypic SH3 domain fold, this interface is the
hydrophobic core of the protein. In the intertwined dimer, the two palm-to-palm interfaces form an extensive dimer interface. The n-Src loop, which in other SH3 domains folds back on to itself to form a hairpin loop, extends into the neighbouring SH3 domain in Eps8. A hairpin conformation of this loop is therefore sterically prohibited and, as a result, strands following the n-Src loop, are swapped between two SH3 domains.

One structural consequence of this mode of dimerization of Eps8 SH3 domain is that part of the classical polyproline type II (PPII) helix binding groove of each monomer is included in the dimer interface, and therefore has reduced accessibility to exogenous ligands, and may explain its unusual ligand binding specificity. Closer analysis of other SH3 domains show that the n-Src loop is a conformationally flexible region, which may open up to allow strand swap with another SH3 domain in a coordinated fashion to form a strand-exchanged dimer as in the case of Eps8-SH3 dimer. The dimerization of Eps8-SH3 appears to be a reversible process, as both monomer and dimer peaks were observed when Eps8-SH3 was analysed by SEC. Monomeric and dimeric forms of Eps8 may therefore exist in equilibrium. Eps8 was also shown to exist as a dimer in vivo when transfected into COS7 cells. Dimerization leads to occlusion of the ligand binding groove on the Eps8 SH3 domain, which may provide a potential regulatory mechanism for Eps8. Activation of Eps8 may lead to monomerization and thus increased binding of SH3 domains to exogenous ligands.

Another example of a dimerization of two SH3 domains is the heterodimerization of the hematopoietic cell-specific signalling molecule, Vav, and the ubiquitous adaptor protein, Grb2 (Ye and Baltimore, 1994). Using a yeast two-hybrid approach, the amino-terminal Vav SH3 domain was shown to bind to the carboxy-terminal Grb2 SH3 domain. The study was unable to demonstrate binding to structures any shorter than an intact SH3 domain. Both Vav and Grb2 SH3 domains contain proline rich sequences, but heterodimerization did not seem to involve an SH3 domain-proline-rich sequence interaction, as the proline-containing sequences in smaller fragments of the Vav and Grb2 SH3 domains displayed no SH3 domain binding.

The BH domain has a slightly higher apparent molecular weight than predicted by HP-SEC (Table 6.1), but it is not clear whether this represents dimerization. There is, however, indirect evidence that the BH domain may have the potential to dimerize, as crystals of the BH domain of p85α revealed a dimer in the asymmetric unit (Muschacchio et al., 1996). This interaction was presumed to be weak, as there was no evidence of dimer formation during purification of the domain. A very weak interaction may only
be apparent at extremely high concentrations, such as in a protein crystal. Crystals of
the BH domain contained two monomers per asymmetric unit, related by a proper two-
fold non-crystallographic symmetry axis. The dimerization interface was hydrophobic,
with the side chain of Met 176 from one monomer inserting into a small exposed pocket
formed by leu 161, ile 177, and val 181 from the other monomer. The small size of this
interface suggests this dimerization is a low affinity interaction and explains why dimer
formation was not detected during purification.

One region common to all the dimerizing forms of p85α is the first proline-rich motif,
P1. It has been widely accepted that in addition to a structural role, proline residues
have an important role in protein-protein interactions. The possibility that two type II
polyproline helices formed by the P1 motifs in the dimer contribute to the dimer
interface cannot be excluded. This issue was not addressed in this study, but it would
be interesting to analyse the apparent molecular weight of P1 alone in order to determine
whether it has the potential to dimerize. The results described in section 6.2.4
however, show that mutations in the P1 motif of full length P85α do not disrupt
dimerization (Table 6.2) but that the same mutations in p85αSH3-P1 disrupt
dimerization incompletely (Table 6.3). Thus, it is more likely that each of the three
regions of the amino-terminus of p85α, the SH3 domain, the P1 motif and the BH
domain co-operate to form a dimerization interface of sufficient affinity in full length
p85α to be detectable at the concentrations used in this study. The affinities of the
individual domains or regions, however seem to be too low for dimerization to be
detected at the concentrations used.

In the presence of the catalytic subunit, p110α, p85α does not appear to self-associate
at the concentrations examined. The recombinant complex had an apparent molecular
weight of approximately 200 kDa by HP-SEC consistent with a 1:1 complex of 85kDa
and 110kDa subunits. If p85α can dimerize in the absence, but not in the presence of
p110α, there must be a region in p110α that prevents it, implying that the amino-
terminai domains of p85α interact with p110α as well as the inter-SH2 domain (Dhand
et al., 1994). To date there is no known evidence for the interaction of either the SH3
domain or the BH domain with the catalytic subunit, however it is possible that in the
context of the full length p85α, these domains interact weakly with the catalytic
domain. Indeed, the three dimensional structures of Src and Hck clearly show an
interaction between the SH3 domain and the small lobe of the catalytic domain.
Alternatively, the p85 subunit may undergo a conformational change in the presence of
p110 that prevents it from realising its potential to dimerize. Little is known about the
three dimensional structure of the p110 subunit of PI 3K and these questions may not
be fully answered until the three dimensional structure of PI3K is solved.
PI3K activation is apparently required for mitogenic responses to some growth factors and oncogene products. The binding to receptors or substrates containing phosphorylated YXXM (pTyr-Xaa-Xaa-Met) motifs has been reported to activate the lipid kinase activity of PI3K both in vitro and in vivo (Backer et al., 1992). Activation of PI3K in vitro can be mimicked by synthetic phosphopeptides that contain the YXXM motif, and this phosphopeptide-mediated activation correlated with conformational changes in the p85 subunit (Backer et al., 1992; Carpenter et al., 1993; Panayotou et al., 1992). Thus, it was proposed that regulation of PI3K catalytic subunit, p110, required a conformational change in the regulatory subunit, p85, which was driven by SH2 domain binding to phosphorylated YXXM motifs. The highly purified recombinant PI3K used in this study was induced to dimerize by a YXXM motif containing diphosphopeptide. A 2-3 fold activation of its lipid kinase activity was observed which is in agreement with reported studies (data not shown) (Backer et al., 1992; Carpenter et al., 1993). Because dimerization is inducible by a phosphopeptide that mimics a stimulated receptor, it is possible to speculate that dimerization may be the first event in PI3K mediated signalling.

The existence of some forms of PI3K, (e.g. p55γ or p50α and p110α), that do not have the potential to dimerize may indicate a mechanism for divergent signalling. PI3Ks mediate signalling through the insulin receptor and IRS 1 has been shown to preferentially utilize forms of the PI3K regulatory subunit which do not have the potential to dimerize (Inukai et al., 1996). It is unknown what implication the lack of dimerization has for PI3K signalling, but clearly this represents another regulatory mechanism that may mediate specificity in signal transduction pathways.

Competition experiments with different dimerizing forms of p85α did not demonstrate the formation of mixed heterodimers in vitro (data not shown), but mixed heterodimers could form when different p85α mutants were co-expressed and therefore co-translated (Figure 6.4), suggesting that the dimerization affinity of p85α alone is relatively high. Recently, there is growing evidence to suggest that p85 may have a separate role which excludes p110. One report has shown that p85 is involved in the cellular apoptotic response to oxidative damage and that this function of p85 in the cell death pathway does not involve PI3K (Yin et al., 1998). p85 is also upregulated by p53 and involved in p53-mediated apoptosis following H2O2 administration. The activity of PI3K was unaffected by oxidative stress indicating that p85 can also function independently of PI3K. Finally a recent study investigating the role of PI3K in V12 Ras transformation suggests that in addition to the interaction of Ras with p110, other
signals mediated by p85 are also required for Ras transformation (Rodriguez Viciana et al., 1997)\(^1\) (section 4.3). It is unknown whether p85 ever exists *in vivo* without an associated catalytic subunit, but if it did, it would presumably be dimeric. It is possible to speculate that a pool of p85 dimers exists in the cell that is available to bind p110 as soon as it is translated as since p110 is not very stable without its regulatory subunit (Yu et al., 1998).

Growth factors and cytokines exert their effects by binding to cell surface receptors, which are often activated by ligand-induced dimerization or oligomerisation (section 1.1). Moreover, the elucidation of several intracellular signal transduction pathways has revealed that the activities of several components of these pathways are also regulated by dimerization. For instance, certain cytoplasmic signal transduction molecules dimerize after activation, and the active form of transcription factors are often dimers. It appears that dimerization is an extensively used mechanism to regulate the activity of signal transduction molecules.
Table 6.1 Summary of data from analytical high-performance size-exclusion chromatography (HP-SEC) for p85α, p85β and p85 mutants. p85 mutants are represented pictorially and their amino acid specifications are listed in Table 3.2. The apparent molecular weight (in kDa) is calculated from a comparison of the retention time of each protein with a plot of log (molecular weight) versus retention time for a range of standard proteins.

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<th>Molecular Weight by HP-SEC in 8M urea (kDa)</th>
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Figure 6.1 HP-SEC of naturally occurring p85α isoforms. Size-exclusion chromatography of recombinant p85α (-----), p85β (-----), p55γ (------) or p49α (-----). The retention times of the molecular weight standards (kDa) are indicated, as is the void volume (Vo). The Superose-12 column was equilibrated and run in 20mM Tris pH 8.0 containing 150mM NaCl at 0.3 ml/min.
Figure 6.2 HP-SEC of cytosolic extracts of transfected COS7 cells. Cytosolic extracts prepared from unstimulated COS7 cells, transfected with p85α (panel A), p55γ (panel B) or p49α (panel C), were subjected to size-exclusion chromatography. Aliquots of each fraction were analysed by western blotting with an appropriate mouse monoclonal antibody (lower strip of each panel A, B and C). Absorbance at 220nm (---) and the corresponding elution volumes of the molecular weight standards (kDa) are indicated, as is the void volume (Vo). The Superose-12 column was equilibrated and run in 20 mM Tris pH 8.0 containing 150 mM NaCl and 10 mM 2-mercaptoethanol at 0.3 ml/min. Unfractionated cytosolic extracts were analysed in parallel (lysate) with the relevant antibody.
Table 6.2 Summary of data from analytical high-performance size-exclusion chromatography (HP-SEC) for p85α and p85 mutants with point mutations in their proline-rich motifs. p85 mutants are represented pictorially and their amino acid specifications are listed in table 3.2. The apparent molecular weight (in kDa) is calculated from a comparison of the retention time of each protein with a plot of log (molecular weight) versus retention time for a range of standard proteins.

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<td>74</td>
<td>139±7</td>
</tr>
</tbody>
</table>
Table 6.3 Summary of data from analytical high-performance size-exclusion chromatography (HP-SEC) for p85αSH3-P1 and p85αSH3-P1 mutants with point mutations in their proline-rich motifs. The amino acid specifications for p85αSH3-P1 and p85αSH3-P1 mutants are listed in table 3.2. The apparent molecular weight (in kDa) is calculated from a comparison of the retention time of each protein with a plot of log (molecular weight) versus retention time for a range of standard proteins.

<table>
<thead>
<tr>
<th></th>
<th>Predicted Molecular Weight (kDa)</th>
<th>Molecular Weight by HP-SEC under native conditions (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p85α SH3-P1</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>p85α SH3ΔP1a</td>
<td>12</td>
<td>24 and 14</td>
</tr>
<tr>
<td>p85α SH3ΔP1b</td>
<td>12</td>
<td>12.5</td>
</tr>
<tr>
<td>p85α SH3</td>
<td>9.7</td>
<td>13.4</td>
</tr>
</tbody>
</table>
Figure 6.3 Binding of p85αSH3-P1 mutants to immobilized p85α P1. p85αSH3-P1, (Red), p85αSH3ΔP1a, (Green) p85αSH3ΔP1b, (Blue) and (p85αSH3) Black.
Figure 6.4  p85α from transfected mammalian cells co-precipitates with p85αΔBH, but not with p49α. Lysates were prepared from untreated COS7 cells transfected with p85α, p85αΔBH, or both (panel A), and p49α or both p85α and p49α (panel B). To demonstrate co-precipitation, myc-tagged p85α was immunoprecipitated with an anti-myc tag monoclonal antibody (9E10, panels A and B) and immunoblotted for p85α or p49α using mouse monoclonal antibodies
Figure 6.5 HP-SEC and corresponding lipid kinase activity profile of the recombinant p110α/p85α complexes in the presence or absence of di-tyrosine phosphorylated peptide derived from the PDGFβ receptor. The elution profile of recombinant p110α/p85α in the presence (panel B) or absence (panel A) of diphosphorylated PDGF receptor peptide was analysed by HP-SEC. Aliquots of each fraction were assayed for lipid kinase activity (— —). Absorbance (— —) and relative elution times of the molecular weight standards (kDa) are indicated, as is the void volume (Vo). The Superose-12 column was equilibrated and run in 20 mM Tris pH 8.0 containing 150 mM NaCl and 10 mM 2-mercaptoethanol at 0.3 ml/min.
Figure 6.6 HP-SEC and corresponding lipid kinase activity profile of recombinant p110α/p49α and p85α in the presence or absence of di-tyrosine phosphorylated peptide derived from the PDGFRβ receptor. The elution profile of recombinant p110α/p49α in the presence (panel B) or absence (panel A) of diphosphorylated PDGF receptor peptide was analysed by HP-SEC. Aliquots of each fraction were assayed for lipid kinase activity (— — —). Absorbance at 220nm ( — — — ) and relative elution times of the molecular weight standards (kDa) are indicated, as is the void volume (Vo). The elution profile of recombinant p85α (panel C) in the presence ( — — — ) or absence ( — — — ) of diphosphorylated PDGF receptor peptide was also analysed by absorbance at 220nm by HP-SEC. The Superox-12 column was equilibrated and run in 20 mM Tris pH 8.0 containing 150 mM NaCl and 10 mM 2-mercaptoethanol at 0.3 ml/min.
Chapter 7: GENERAL DISCUSSION

The PI3K family of enzymes has been implicated in a large array of biological responses including mitogenesis, cytoskeletal re-organisation, cellular migration and differentiation and protection from apoptosis. Members of this family of enzymes exhibit different substrate specificities, and one subclass, the class 1A enzymes, possess adaptor subunits which contain a variety of regulatory domains. The multidomain structure of the p85 adaptor subunits suggest this subclass of enzymes have numerous binding partners, and are therefore likely to be subject to complex modes of regulation.

Some components of signalling pathways involving the class 1A PI3Ks are known. Upon ligand stimulation, growth factor receptors become autophosphorylated at specific tyrosine containing sequence motifs, which bind directly to the SH2 domains of p85, and activate the lipid kinase activity of PI3K by translocating it within proximity of its substrates, which reside in the cell membrane (reviewed in Panayotou and Waterfield, 1993). Activation of PI3K by the insulin receptor involves an alternative recruitment mechanism, in which PI3K binds to IRS-1, a tyrosine phosphorylation substrate of the insulin receptor that acts as an SH2 domain docking protein (reviewed in Yenush and White, 1997). In response to these extracellular stimuli, PI3K phosphorylates inositol lipids at the D3 position to form the second messengers PtdIns(3,4)P2 and PtdIns(3,4,5)P3. Recently, some of the downstream components of signalling pathways involving PtdIns(3,4,5)P3 have been identified. PKB or AKT is a serine-threonine kinase which contains an amino-terminal PH domain which binds PtdIns(3,4,5)P3, leading to partial activation of its kinase activity. Full activation of AKT requires phosphorylation on two serine residues by two serine/threonine kinases, 3-phosphoinositide-dependent protein kinase 1 (PDK1) and PDK2 (Alessi et al., 1997; Stokoe et al., 1997). PDK1 also contains an amino-terminal PH domain and is activated by PtdIns(3,4,5)P3. AKT influences cell metabolism by phosphorylating GSK3 and phosphofructokinase, as well as transmitting a potent survival signal. In part, this is via phosphorylation and inactivation of Bad, a Bcl-2 family member that is involved in the induction of apoptosis (Datta et al., 1997; del Peso et al., 1997). PtdIns(3,4,5)P3 has also been shown to regulate p70S6k (Alessi et al., 1998; Pullen et al., 1998). Phosphorylation of p70S6k by proline kinases in the autoinhibitory domain, then by a PtdIns(3,4,5)P3-dependent kinase, induces a conformational change that allows further phosphorylation by PDK1, resulting in a full activation of its serine/threonine kinase activity (reviewed in Downward, 1998). The activation of
p70\textsuperscript{s6k} leads to increased translation of messenger RNAs that encode essential components of the protein synthetic apparatus (Brown and Schreiber, 1996).

Not all signalling processes involving PI3K follow this PtdIns(3,4,5)P\textsubscript{3} generating pathway. It is known that p110 can associate with, and be activated by, GTP-bound Ras (Kodaki \textit{et al.}, 1994; Rodríguez Viciana \textit{et al.}, 1994). Alternatively, PI3K can also be regulated by diverse mechanisms which involve interactions between various signalling proteins and the p85 regulatory subunit. For example, the p85\textalpha SH3 domain has been shown to interact with the cytoskeletal proteins N-WASP (this thesis), and bind to and activate the GTPase activity of dynamin (Gout \textit{et al.}, 1993). The p85 proline-rich sequences interact with the SH3 domains of the Src family tyrosine kinases, which results in the activation of PI3K (Pleiman \textit{et al.}, 1994). In addition, the BH domain of p85 has been shown to interact with GTP bound Cdc42 and Rac1, which also results in a modest stimulation of PI3K activity (Zheng \textit{et al.}, 1994). At present it is unclear which of these proteins is an upstream regulator or a downstream effector of PI3K. It seems unlikely that all these proteins associate with PI3K at the same time, but it is not yet known whether the binding of one protein to PI3K regulates its ability to interact with another exogenous binding partner.

Clearly, p85\textalpha has the potential to participate in a number of different signalling pathways which may not always directly involve the lipid kinase activity of p110 and the production of PtdIns(3,4,5)P\textsubscript{3} as described above. This is not the first example of signalling pathways involving multidomain kinases without requiring their intrinsic kinase activity. As described in section 1.3.3.1.4, the targeted gene-disruption of the \textit{c-Src} gene in mice causes only one major defect, even though Src is involved in many signalling pathways. These mice have severe osteopetrosis due to an intrinsic defect in their osteoclasts, which are the cells that reabsorb bone (Lowe \textit{et al.}, 1993). Ectopic expression of wildtype Src in Src-/- mice reverses this phenotype, while expression of a kinase-defective version of Src reverses a subset of defects in Src-/- osteoclasts, demonstrating that there are some Src functions that do not absolutely require its kinase activity (Scwartzberg \textit{et al.}, 1998). Similarly, there is some evidence for p85\textalpha dependent pathways that do not absolutely require the lipid kinase activity of p110. For example, it has recently been shown that in response to oxidative stress, p85\textalpha expression is upregulated in a p53 dependent manner. The role of p85\textalpha in p53-mediated apoptosis does not require PI3K lipid kinase activity (Yin \textit{et al.}, 1998). This may suggest that p85 can also function independently of PI3K, and that it uses different pathways from that of p110 to transmit the signals that protect the cell from apoptotic death. Overexpression of p85\textalpha or Δp85 (a dominant negative form of p85\textalpha in which the p110 binding site is deleted) have been shown to reduce the ability of constitutively
top paragraph

Explain how this means p85 can function separately from p110. Is p85 or p110 just reducing binding to phosphotyrosines in the cells (even when they are unstimulated). (Synergy between p85 and p110)
active Ras to transform Swiss 3T3 cells. In addition, the ability of Ras to activate PI3K was reduced by overexpression of either form of p85α (Rodriguez Viciana et al., 1997). Ras has been shown to bind directly to p110α (Kodaki et al., 1994; Rodriguez Viciana et al., 1994) but its ability to activate PI3K activity clearly requires a p85-mediated mechanism, suggesting that p85 can function separately from p110 in this instance.

This thesis has investigated some aspects of binding and possible regulation of PI3K via its adaptor subunit, p85. Binding partners of the BH domain of p85α were examined in human cell lines and bovine tissue and an 80-90 kDa protein was identified. Although p85 was originally thought to be a GAP for Rho family proteins (because its BH domain has sequence homology to a number of known GAPs including RhoGAP, BCR, chimaerin and p190, section 1.3.4.4.2), there is no indication that p85 or its BH domain is capable of influencing the GTPase activity of a known small G protein. It is clear, however, that the BH domain binds specifically to GTP-bound Cdc42 and Rac1 (Bokoch et al., 1996; Zheng et al., 1994), suggesting that the function of the BH domain is to bind a subset of small G proteins. Binding of Cdc42 to p85 has been shown to modestly up regulate PI3K activity (Zheng et al., 1994) but it is not clear whether PI3K lies upstream or downstream of Rac1, Cdc42 or the 80-90 kDa binding protein, or what the outcome of this binding event is in signalling pathways in vivo. There is growing evidence that PI3K, Rac1 and Cdc42 function together in signalling pathways that mediate cell motility (Keely et al., 1997), but whether these pathways specifically involve direct interaction of the BH domain of p85α with Rac1 or Cdc42 is not yet known.

The SH3 domain of p85α was shown to bind proline-rich sequences from dynamin and N-WASP. Interestingly, it was also shown to bind its own P1 motif. Studies described in this thesis demonstrated that dynamin and the Src SH3 domain bound p85α poorly because the p85αSH3 domain was bound to its own P1 motif. The deletion of the p85αSH3 domain increased the binding of p85αP1 to exogenous SH3 domain-containing proteins. Similarly mutagenesis of the P1 motif increased the ability of the p85αSH3 domain to bind to proteins containing proline-rich sequences. The interaction of p85αSH3 and p85αP1 was apparently, therefore, intramolecular. It was hypothesised that this intramolecular interaction may constitute a regulatory mechanism.

Similar mutagenesis of the pseudo-polyproline helix in Src resulted in activation of the tyrosine kinase activity, suggesting that this intramolecular interaction inhibits the catalytic activity (Gonfloni et al., 1997). A regulatory mechanism whereby a normally
inaccessible SH3 domain must be unmasked or unlocked before it can interact with its
target seems to be an emerging theme for several signalling molecules. Intramolecular
interactions in multidomain proteins often form the basis of the regulation of these
molecules. Since proline-rich sequences are often found in SH3 domain-containing
proteins, intramolecular interactions may be a common feature that prevent SH3
domains from forming physiologically active complexes when not required. For
example, intramolecular interactions regulate the Src family tyrosine
kinases (reviewed in Superti-Furga and Gonfloni, 1997, and section 1.3.3.1.3). Phosphorylation of a carboxy-terminal tyrosine residue results in an intramolecular
association with the SH2 domain. An intramolecular interaction also occurs between
the SH3 domain and the pseudo polyproline type II helix. Together, these interactions
regulate Src catalytic activity by maintaining it in an inactive conformation in which the
catalytic cleft is sterically hindered.

Intramolecular interdomain interactions are also important for the regulation of the Tec
family of tyrosine kinases. Btk is a Tec family kinase with an amino-terminal PH
domain, a proline-rich sequence (the Tec homology, or TH region), and an SH3, an
SH2 and a kinase domain. Unlike the Src family tyrosine kinases, members of the Tec
family are not regulated by carboxy-terminal phosphorylation. Instead, the SH3
domain interacts with the TH region, which may restrict access of the SH3 domain to
potential binding partners (Andreotti et al., 1997). The data described in this thesis
suggests that PI3K also has the potential to be regulated by an intramolecular SH3-
domain proline-rich sequence interaction. Although there is no evidence that the p85
SH2 domains bind an intramolecular phosphotyrosine, it has been demonstrated that
PtdIns(3,4,5)P3 interacts with p85α SH2 domains and modulates the association of
PI3K with activated RTKs and other tyrosine phosphorylated proteins (Rameh et al.,
1995). This may be a variation on the same regulatory theme in which the product of a
kinase forms a negative feedback on enzymatic activity. Unlike the Src and Tec family
 tyrosine kinases, the catalytic domain of the class IA PI3Ks is located in a different
polypeptide chain to the regulatory SH2 and SH3 domains. p110 also has an intrinsic
Mn2+-dependent serine/threonine kinase activity that can phosphorylate a specific serine
residue (Ser 608), in the p85 subunit, resulting in inhibition of PI3K activity (Carpenter
et al., 1993; Dhand et al., 1994). PI3K therefore possesses some regulatory
mechanisms, for example intramolecular SH3 domain/proline rich sequence
interactions, in common with other multidomain kinases, but has additional layers of
complexity in the regulation of its kinase activity.

When the p85αSH3-P1 interaction is locked, p85αSH3 and p85αP1 mediated
signalling pathways are inhibited. To activate these pathways, this interaction clearly
needs to be unlocked, but at present it is unknown what events or mechanisms are involved in unlocking p85αSH3-P1 interactions. There are three possibilities. Firstly, upstream components of PI3K signalling pathways may regulate this interaction. This is a common regulatory theme among enzymes involved in signal transduction. For example, an upstream event such as PDGF receptor activation has been shown to recruit p85 to the membrane by binding of both its SH2 domains to pTyr containing motifs. This bidentate association has been shown to cause a conformational change in the p85 subunit of PI3K that upregulates the catalytic activity of p110 (Panayotou et al., 1992; Shoelson et al., 1993), suggesting the SH2 domain binding has a dual role in regulating both enzyme translocation and activity. The conformational change induced by the SH2 domains binding to activated receptors may also function to unlock the intramolecular SH3-P1 interaction. This mechanism would permit a co-ordinated regulation of lipid kinase activity and pathways involving the binding of exogenous SH3 domain-containing or proline-rich motif containing proteins. Another mechanism by which receptor activation disrupts an intramolecular SH3-P1 interaction has been described recently for Src. Stimulation of the PDGF-R in fibroblasts leads to phosphorylation of Tyr 136 in Src (Broome and Hunter, 1997). Normally, Tyr 136 interacts with the SH2-kinase domain linker and is therefore inaccessible to exogenous tyrosine kinases, suggesting that PDGF-induced activation of Src precedes Tyr 136 phosphorylation. Src binding to the activated PDGF-R may therefore induce a conformational change that exposes Tyr 136 and subsequent phosphorylation of Tyr 136 may help stabilise the open, activated conformation of Src. Tyr 136 is in the Src SH3 domain, and phosphorylation of this residue has been shown to modulate Src SH3 domain binding to exogenous proline-rich peptides (Broome and Hunter, 1997). Modelling studies of p85α have shown that a tyrosine residue equivalent to Tyr 136 in Src is also conserved in the p85αSH3 domain and lies in the SH3 ligand binding surface (Marketa Zevelibil, personal communication). Thus, the p85αSH3 domain has the potential to undergo phosphorylation following PDGF stimulation which would also unlock the intramolecular p85αSH3-P1 interaction.

A second mechanism that may unlock the p85αSH3-P1 interaction is direct competition by exogenous SH3 domain or P1 motif containing proteins. SH3 domains have been shown to have widely differing affinities for different proline-rich sequences, thus high affinity p85αSH3 domain or p85αP1 motif binding proteins would be able to compete efficiently with the endogenous SH3-P1 interaction for binding. The best example of this mechanism is again from the Src family tyrosine kinases. The HIV protein was shown to activate Hck by binding to its SH3 domain (Moarefi et al., 1997). A proline-rich sequence in Nef had a higher affinity for the Hck SH3 domain than the pseudo-
polyproline helix in Hck and so was able to directly displace the intramolecular Hck SH3-pseudopolyproline helix interaction.

A third possibility is that binding of other upstream effectors that are not directly involved in PtdIns(3,4,5)P3 formation may induce a conformational change that could lead to the unlocking of this intramolecular p85αSH3-P1 interaction. An analogy for this mechanism is the regulation of PH domain containing proteins by phospholipids. For example, the activation of AKT kinase activity is in part due to the binding of PtdIns(3,4,5)P3 to its PH domain, which elicits a conformational change in AKT that permits the constitutively active PDK1 to phosphorylate AKT. The PH domain of AKT must therefore be bound to the serine residue that is the phosphorylation substrate for PDK1, and unlocking of this intramolecular PH domain/serine residue interaction permits phosphorylation by the PDK1 kinase domain (Alessi et al., 1997; Stephens et al., 1998).

The events which follow the unlocking of the p85αSH3-P1 interaction are not yet known, but the identities of the binding partners of the p85αSH3 domain and the p85αP1 motif may give clues to downstream events. SH3 domains have been shown to direct subcellular localisation (Bar Sagi et al., 1993) and interact with cytoskeletal proteins. Studies in this thesis have reported the binding of the p85αSH3 domain to dynamin and N-WASP, two proteins that are involved in the regulation of the cytoskeleton. Dynamin is a large GTPase that functions in the endocytic pathway (reviewed in Urrutia et al., 1997) and N-WASP is an actin-depolymerising protein (Miki et al., 1996). The binding of the Src family kinase SH3 domains to p85αP1 and the role of Src in focal adhesion complex formation and integrin signalling (reviewed in Brown and Cooper, 1996) also suggest a role for p85α amino terminal domain interactions in cytoskeletal rearrangements. In addition, the interaction of the p85α BH domain with both GTP-bound Rac and Cdc42 (Bokoch et al., 1996; Zheng et al., 1994) and initial scrape loading studies described in this thesis suggest this domain may also be involved in pathways leading to the reorganisation of the actin cytoskeleton. Indeed, the involvement of PI3K in the cytoskeletal regulation has been widely reported (reviewed in Carpenter and Cantley, 1996), but the mechanism by which it fulfils this role is not yet clear. It is possible that the role of PI3K in these pathways does not require its lipid kinase activity (Rodriguez Viciana et al., 1997), but instead may be mediated by the SH3 and/or the BH domains. This may represent a bifurcation in the class IA PI3K signalling pathway where PtdIns(3,4,5)P3 mediated pathways regulate mitogenic and survival signals, while SH3 domains, BH domains and/or proline-rich motifs mediate cytoskeletal regulation. In addition, p85 isoforms that contain SH3 and
BH domains can participate in pathways involving the cytoskeleton while p85 isoforms that lack these domains cannot. The differences in the amino-terminal region observed among the regulatory subunit isoforms may represent an additional level of PI3K regulation.

Another binding partner for p85α identified in this thesis was p85α itself. The domains shown to be involved in this interaction were the SH3 domain, P1 motif and the BH domain. These three regions act co-operatively to form the binding surface that mediates this intermolecular dimerisation. The p85αSH3-P1 interaction does not appear to contribute to the dimerisation interface, further suggesting it is an intramolecular interaction. The data described in this thesis has demonstrated that dimerisation may be induced by a phosphopeptide that binds the SH2 domains of PI3K and mimics an activated PDGF receptor, suggesting that dimerisation may be part of the mechanism of activation of PI3K by binding to RTKs. However, the consequence of dimerisation on PI3K mediated signalling pathways is still unknown. The dimerisation interface involves the same regions of p85α that contribute to several other interactions, thus it is possible that dimerisation could influence the locking and unlocking of the p85αSH3-P1 interaction, or the ability of p85αSH3, p85αP1 or p85αBH to bind their exogenous substrates. Further studies are required to determine the effects of binding of PI3K to an activated RTK on SH3 and BH domain mediated signals.

The ability of certain isoforms of p85 to dimerise whilst others cannot, may represent a form of differential regulation among the class Iα PI3Ks. Furthermore, the p85 isoforms not able to dimerise lack both the SH3 and BH domains and therefore cannot participate in pathways involving these domains. The outcome of the ability of various class Iα PI3Ks to dimerize or not is not yet known. Ability to dimerise may influence which receptors can bind the different p85 isoforms. For example, dimerising forms of PI3K may be preferentially recruited to receptors containing two p85αSH2 domain-binding motifs. Alternatively, dimerisation may affect downstream binding of other effectors, for example by creating a novel binding surface on two p110 molecules.

The ability of signalling molecules to dimerise often plays a role in the regulation of these proteins. For example, ligand-induced dimerisation plays an essential role in the activation of RTKs, leading to autophosphorylation and an increase in tyrosine kinase activity (Ullrich and Schlessinger, 1990). Homo-dimerisation of AKT is induced by the binding of its PH domain to PtdIns(3,4)P2 and results in increased kinase activity (Franke et al., 1997). Homo-oligomerisation is also involved in the regulation of the serine/threonine kinase, Raf (Farrar et al., 1996). Dimerisation may therefore be a
general mechanism of regulation of serine/threonine kinases, and clearly requires further study. In contrast, it has been shown that ligand induced-dimerisation of a receptor tyrosine phosphatase CD45, inhibits its function (Majeti et al., 1998), thus dimerisation may be capable of either positive or negative regulation of enzymatic activity.

The regulation of large multidomain enzymes such as class 1a PI3Ks is clearly complex and can be mediated by dimerisation or SH2, SH3 and BH domain or proline-rich motif interactions. PI3K can interact with a large number of effectors, including other large multidomain proteins, such as Src (reviewed in Brown and Cooper, 1996). The data presented in this thesis has shown that, in addition to the previously reported association between the Src SH3 domain and the p85αP1 motif, Src was also able to interact with the carboxy-terminal SH2 domain of p85α. It has already been shown that the Src SH2 domain can also associate with PI3K activity in Src transformed cells (Haefner et al., 1995). These two multidomain proteins therefore interact with each other at multiple sites, suggesting tight regulation of these two kinases by each other, however the ultimate result of this complex interaction is not yet known. It has been demonstrated that this interaction results in an increase in PI3K activity, but it is also possible that PI3K may activate Src in certain pathways. It has been shown that the binding specificity of the Src SH2 domain and the substrate specificity of its catalytic domain are similar, suggesting that binding may upregulate Src kinase activity (Songyang, 1995). Thus, phosphorylated targets of Src family members may function both as upstream activators and as downstream effectors, making the distinction between the two functions ambiguous.

The data described in this thesis has begun to dissect the components involved in the interaction of these two large multidomain proteins. p85αP1 has been shown to bind to the Src SH3 domain, p85αC-SH2 has been shown to bind to the autophosphorylated tyrosine residue in Src and the p85α inter-SH2 domain has been shown to be a tyrosine phosphorylation substrate for Src. Clearly the interactions are complex. It is unknown at present what downstream effects are mediated by the binding and phosphorylation of PI3K and Src, and which signalling pathways these interactions are involved in. Deciphering these pathways will clearly be of future interest.

Src and PI3K are both involved in a large number of signalling pathways in the cell which makes it difficult to distinguish which biological responses are directly due to their activation. Similarly, Ras has been shown to be involved in multiple signal transduction pathways, and also interacts with PI3K via the p110 subunit. Three of the most important proteins in signal transduction therefore have the ability to bind to and
regulate each other. It is difficult to envisage how these three proteins could be coordinately regulated, however there is some evidence that these interactions do not all happen at the same time. For example, Ras undergoes a biphasic activation which leads to the sequential stimulation of divergent downstream signalling pathways (Foschi et al., 1997). The first phase of Ras activation stimulates one subset of Ras effectors, the ERK/MAPKs. The desensitisation of Ras is followed by a second phase of Ras activation which then stimulates the PI3K mediated pathway. Temporal separation of different signalling events may therefore allow PI3K to interact with its various binding partners at different times following RTK stimulation.

Thus, the PI3K family of enzymes participate in multiple signalling pathways and are implicated in a large number of biological functions in response to a wide range of extracellular stimuli. The structural and functional diversity of the enzymes suggests that the mechanisms by which they are regulated are complex. The studies described in this thesis have begun to dissect just a few of the interactions mediated by the p85 regulatory subunit of the class 1A PI3Ks. In doing so, these studies have shown how regulatory mechanisms have been conserved between different signalling proteins, yet have been adapted to fit the special regulatory needs of each protein. Clearly, there are layers of complexity surrounding the regulation of the PI3K family of enzymes which would account for their ability to interact with many signalling proteins and be involved in a diverse array of biological processes. Much remains to be learned about the exact roles that PI3K signalling complexes play in mediating these processes.
Chapter 8: BIBLIOGRAPHY


ERRATUM

Page 76 3rd paragraph: TOR family kinases

Page 77 3rd paragraph: It therefore appears that PI3K activation can be downstream of Ras. Ras brings the p110 subunit to the membrane. There is no intrinsic activation. In the case of p120/p101, Ras activates on its own but 10X less than βγ on it's own. These experiments rely on the presentation of Ras in lipid vesicles.

Page 96 3rd paragraph: Sodium Orthovanadate was added to both transfer buffer and blocking buffer. The purpose of this was to remove the background of the developed autoradiographs.

Page 97 1st paragraph: Reaction products loaded onto thin layer Silica Gel-60 plates were developed in a mixture of chloroform, methanol, and 4M ammonia (9:7:4) to separate radio labelled phospholipids from hot ATP.

Page 106 1st paragraph: "this association of Rac with PI3K in vivo was shown to be regulated by PDGF or insulin" It is not clear whether this interaction is physical. It is correct then to say the association is more conceptual.

Page 128: The activity of PI3K when P1 and the SH3 domain were removed from the p85 subunit, was not analysed due to the lack of time. There was no difference between the intrinsic activation of p85/p110 and p49/p110 by diphosphopeptide.

Page 153 2nd paragraph: AKT phosphorylation sites are threonine 308 and serine 473 and not 2 serines.

Page 158 2nd paragraph: Regulation of AKT is thought to be due to its membrane localisation which brings the enzyme closer to its substrate.