THE ROLE OF LCPTP IN T CELL SIGNALLING AND ACTIVATION

By

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To my very supportive husband,

My "third supervisor"

Jean-Yves Masson

ABSTRACT

The aim of the project was to investigate the role of LCPTP (Leucocyte phosphotyrosine phosphatase) in T cell signalling and activation.

The investigation was divided into three parts. In the first part, biochemical studies were used to identify possible LCPTP substrates/interacting proteins. Antibodies specific for LCPTP were used to characterise potential interactions of endogenous LCPTP protein in cells. Recombinant LCPTP proteins, including wildtype and a substrate-trap protein were also generated. These were used to show that LCPTP interacts with members of the MAP kinase family, specifically ERK1 and ERK2, suggesting that these proteins are major LCPTP substrates.

In the second part, the effect of overexpressing LCPTP on T cell function was investigated. The Jurkat T cell line was transfected to produce stable cell lines expressing wildtype and mutant forms of LCPTP. LCPTP transfected cells were stimulated via the TCR and CD28 and monitored for cytokine production, surface marker expression and cell proliferation. It was shown that LCPTP inhibited IL-2 production and expression of the surface IL-2 receptor, CD25. LCPTP also protected cells from TCR induced cell death and promoted homotypic aggregation.

Following on from the observations made in the Jurkat T cell line, the final part of the project was aimed at looking at the effects of LCPTP in primary T cells. LCPTP adenovirus constructs were made and used to infect both naïve T cells as well as antigen differentiated Th1 and Th2 populations. Overexpressed LCPTP substrate-trap protein lead to increased levels of cellular phosphoERK. Increased levels of LCPTP resulted in changes in cytokine production in TCR stimulated cells relative to control cells. Cells overexpressing LCPTP had a higher basal level of proliferation.

The results obtained support a role for LCPTP in regulating the early stages of T cell activation by changing MAP kinase activity.

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ABBREVIATIONS

‘	minute
[ ]	concentration
°C	Degree Celcius
2YT	Yeast tryptone
Ab	Antibody
Act	Activated
Ag	Antigen
APC	Antigen presenting cell
ATCC	American Type Culture Collection
bds	Beads
Bluo-Gal	Bluo-Galactosidase
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium
$CaCl_2$	Calcium chloride
CD2	Cluster of differentiation 2
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
cDNA	complementary deoxyribonucleic acid
CLD	Chronic lymphoproliferative disorders
Cpm	counts per minute
ConA	Concanavalin A
CsCl	Cesium chloride
CTL	Cytolytic T cells
CTLA-4	Cytotoxic T lymphocyte antigen 4
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FcεRI	High affinity IgE receptor
FCS	Fetal calf serum
g	Gram
GST	Glutathione S-transferase

ABBREVIATIONS

HCl	Hydrochloric acid
H_2O	Water
hrs	Hours
ICOS	Inducible costimulator
IFN-γ	Interferon-gamma
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-13	Interleukin-13
IL-2Rα	Interleukin-2 receptor alpha
IL-2Rβ	Interleukin-2 receptor beta
IP	Immunoprecipitation
IPTG	Isopropyl β-D-thiogalactopyranoside
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
kDa	KiloDalton
KIM	Kinase interacting motif
LB	Luria Bertani
LFA	Leucocyte function-associated antigen
LPS	Lipopolysaccharide
M	Molar
MAPK	Mitogen activated protein kinase
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
min	Minute
μg	Microgram
mg	Milligram
ml	Millilitre
mM	Millimolar
m.o.i.	Multiplicity of infection
mRNA	Messenger ribonucleic acid
mv	Millivolt

ABBREVIATIONS

MW	Molecular weight
N/A	Non activated
NaCl	Sodium chloride
ng	Nanogram
NHL	Non-Hodgkin's lymphoma
nm	Nanometre
OD_x	Optical density at wavelength of x nm
OKT3	Anti-CD3 clone
^{32}P	Phosphorus 32
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
Pen	Penicillin
PHA	Phytohemaglutinin
PKA	Protein kinase A
PMA	Phorbol myristate acetate
PPM	Phosphoprotein magnesium dependant phosphatases
PPP	Phosphoprotein phosphatases
PTP	Phosphotyrosine phosphatase
PWM	Pokeweed mitogen
RNA	Ribonucleic acid
rpm	Revolutions per minute
S	Second
sec	Second
SDS	Sodium dodecyl sulphate
SMAC	Supramolecular acivation clusters
Strep	Streptomycin
TAE	Tris-acetate-EDTA
TCR	T cell receptor
TE	Tris-EDTA
Th	Helper T cell
TNF	Tumour necrosis factor
transf	transfection

ABBREVIATIONS

Tris	Tris(hydroxymethyl)aminomethane
Tween20	Polyoxyethylenesorbitan monolaurate
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
vol	Volume
v/v	Volume/volume
WB	Western blot
w/v	weight/volume

CHAPTER ONE: INTRODUCTION

1.1 The immune system

The immune system has evolved to enable the human body to survive in an environment in which it is constantly being exposed to a range of pathogens eg bacteria, viruses, allergens, protozoa. The immune system includes a number of specialised cells such as phagocytic cells, natural killer cells, B and T cells and antigen presenting cells, which act in coordination to eliminate the invading foreign agents. The study of the science of immunology dates back to 1796 when Edward Jenner successfully vaccinated people against smallpox (Jenner, 1798).

There are two main immune mechanisms implicated in the elimination of foreign antigens: the innate response and acquired response. The innate response, or natural immunity, includes physical barriers provided by skin and mucous membranes, as the first line of defence and physiological factors such as pH environment and temperature to limit microbial growth. Cells involved in innate immunity include phagocytic cells, neutrophils and macrophages. These recognise pathogen associated motif patterns (PAMPS) expressed on the surface of invading organisms via receptors including the Toll family and CD14 (Fearon and Locksley, 1996); (Madzhitov et al., 1997); (Hirschfeld et al., 1999); (Hemmi et al., 2000); (Akira et al., 2001). Natural killer cells recognise abnormal cells that have lost the MHC class I molecule ; often a result of a microbial infection or a malignant transformation. Natural killer cells eliminate target cells by releasing perforin and granzymes, which mediate cell lysis and activate apoptotic pathways (Abbas et al., 1994); (Delves and Roitt, 2000a). Soluble mediators, including cytokines, such as IL-12, released by cells involved in natural immunity, activate and direct the antigen-specific adaptive immune response (Vasselon and Detmers, 2002).

The acquired immunological response is antigen specific which distinguishes it from innate immunity. A first encounter with a foreign agent triggers the differentiation of effector cells as well as establishing immunological memory. Further encounters with the same foreign pathogen will stimulate the immune system to mount a quicker, more effective response leading to the elimination of the invading

agent. Acquired immunity is subdivided into two types dependent on the components of the response: humoral immunity and cellular immunity. Humoral immunity is mediated by proteins, termed antibodies, which are antigen specific. Antibodies are produced by B lymphocytes. Cellular immunity is mediated by T lymphocytes (Abbas et al., 1994).

B lymphocytes mature in the bone marrow and recognise native antigen exposed on the surface of invading organisms via specific receptors on the B cell surface. Upon antigen recognition, B cells clonally expand and some cells will differentiate to become plasma cells, which secrete high affinity antibodies to neutralise the antigen. The function and activity of B lymphocytes is dependent on the help of specialised T lymphocytes: helper T cells (Abbas et al., 1994).

T lymphocytes can be subdivided into helper T cells, cytotoxic T cells and regulatory T cells. T cells are derived from the bone marrow but undergo maturation in the thymus. In the thymus, T cells are subjected to both positive and negative selection, which means that cells with the potential for self reactivity are eliminated or rendered anergic (Sebzda et al., 1999); (Viret and Janeway, 1999); (Anderson et al., 1999); (Starr et al., 2003). T cells recognise peptide antigens bound non-covalently to proteins, which form part of the major histocompatibility complex (MHC). This MHC complex is subdivided into two classes: class I and II. Antigen peptides derived from extracellular proteins (bacterial or fungal products and allergens) bind to class II proteins in the endosomal compartment in cells. MHC class II proteins are mainly expressed on B lymphocytes, macrophages and on dendritic cells. Peptides bound to MHC class II proteins are presented to $CD4^+$ T lymphocytes (Delves and Roitt, 2000b). $CD4^+$ effector T cells, or helper T cells, can be divided into Th1 and Th2 subsets. Th1 cells secrete IL-2 and interferon-γ and promote inflammatory responses, for example, by activating macrophages. Th2 cells secrete IL-4, IL-5 and IL-13 and promote humoral immunity by stimulating B cell growth and differentiation leading to antibody production.

A third population of $CD4^+$ T cells characterised as $CD25^{+high}$ have been recently identified and they function as regulatory T cells. This specialised

population of T cells controls the activation of helper T cells by suppressing the proliferation and cytokine induction of T cells receiving weak TCR signals although permitting the immune response of T cells in contact with a strong TCR signal together with a co-stimulatory signal (see section 1.3 for more details) (Thornton and Shevach, 1998); (Jonuleit et al., 2001); (Dieckmann et al., 2001); (Baecher-Allan et al., 2001).

Endogenously synthesised peptides (for example, viral products, intracellular parasites, tumour antigens) bind to class I molecules in the cytosol. MHC class I molecules are expressed on the surface of the majority of nucleated cells and the peptide they present is recognised by $CD8^+$ cytotoxic T cells, which can destroy infected cells (Delves and Roitt, 2000b).

$CD8^+$ cells, or cytotoxic T cells (CTL), destroy target cells by one of a number of different mechanisms. These include the release of perforin and cytolysin causing cell lysis and ligation of Fas, which activates signalling pathways, inducing cell apoptosis (Harty et al., 2000); (Wong and Pamer, 2003). $CD8^+$ T cells also release cytokines, specifically, interferon-γ and tumour necrosis factor (TNF), which induce macrophage activation (Harty et al., 2000); (Wong et al., 2003). $CD8^+$ T cell lysis is antigen specific and requires cell contact (Harty et al., 2000); (Delves and Roitt, 2000b); (Wong et al., 2003).

Antigen presenting cells (APCs) include macrophages, dendritic cells (DCs) and B cells. These cells take up foreign agents and enzymatically process them into small peptides. These peptides are then presented on the surface of the APC in association with MHC II proteins (Abbas et al., 1994). Dendritic cells are regarded as the professional antigen presenting cells and are located in strategical sites such as in the skin and in the mucosal epithelia. Immature dendritic cells are highly adept at antigen capture and processing. Once they have captured antigen, immature DCs migrate to T cell rich areas in lymphoid tissue where they undergo maturation, upregulating a number of costimulatory and adhesion molecules. This enables the DCs to effectively activate both naive and memory T cells (Austyn et al., 1988);

(Hauss et al., 1995); (McLellan et al., 1996); (Banchereau and Steinman, 1998); (Woodhead et al., 2000).

1.2 T cell receptor signalling complex

The CD4 glycoprotein expressed on a subset of T cells, facilitates the interaction between the T cell and the APC and it is often referred to as a coreceptor. CD4 binds to the invariable domain of the MHC class II molecule helping to stabilise the interaction between the T cell and APC (Abbas et al., 1994); (Guermonprez et al., 2002). CD4 via its associated LCK kinase also plays a key role in initiating signal transduction in the T cell (Qian et al., 1993). Other molecules on the surface of the T cell, including CD2 and LFA-1, act as adhesion molecules and provide further interactions via ligands on the APC, which help to maintain a close contact between T cell and APC. The major T lymphocyte surface molecules and their respective ligands on the APC are shown in Figure 1 (Abbas et al., 1994).

The T cell receptor (TCR) is composed of the α and β chains. This heterodimer recognises and binds to foreign peptide and the self MHC class II molecule. Associated non covalently with the TCR is the CD3 complex composed of the γ, δ and the ε chains and the zeta homodimer (Abbas et al., 1994). Each member of the CD3 complex possesses an extracellular domain, with glycosylation sites on the γ and δ chains, and an intracellular domain, which contains an immunoreceptor tyrosine-based activation motif (ITAM) (tyr-X-X-leu sequence found twice in a stretch of 17 amino acid residues) (Cambier, 1995). Each ζ chain has a very short extracellular domain and a long cytoplasmic tail, which contains three ITAMs. The ITAMS are important for initiating signal transduction in the T cell (Weissman, 1994).

Ligation of the TCR alone is insufficient to lead to full T cell activation. A second signal is provided by the binding of a costimulatory molecule to its ligand on the APC (Weiss, 1991). One of the most important costimulatory molecules for naïve T cell activation is CD28. Cognate ligands of CD28 include B7.1 (CD28) and B7.2 (CD86) (Aruffo and Seed, 1987); (June et al., 1990). The signal through CD28 is essential for optimal production of IL-2, expression of IL-2 receptor on the surface of

the cell and progression of the cell cycle (Jenkins et al., 1991); (Harding et al., 1992). The signalling pathway via CD28 enhances the TCR signal. Helper T cells are more dependent on the second signal for activation than cytotoxic T cells (Lenschow et al., 1996); (Whitmire and Ahmed, 2000). Another costimulatory molecule named ICOS (inducible costimulatory molecule) is upregulated 24–48 hrs following T cell activation and therefore, is not present on naive T cells (Hutloff et al., 1999). ICOS binds to ICOSL and its stimulation enhances the production of effector cytokines: IFN-γ, TNF-α, IL-4, IL-5 and IL-10. Hence ICOS plays a key role in memory and effector cell function (Hutloff et al., 1999); (Coyle and Gutierrez-Ramos, 2001); (Frauwirth and Thompson, 2002).

T cell proliferation is regulated by signals from molecules, which provide a negative signal into cells. CTLA-4 (cytotoxic T lymphocyte antigen 4) binds to the same ligands as CD28 but with a higher affinity (Brunet et al., 1987). Like ICOS, CTLA-4 is induced during activation but upon ligation it inhibits T cell proliferation and IL-2 synthesis (Thompson and Allison, 1997). It is likely that a balance between the activation and inhibition signals given by the costimulatory molecules sets the threshold for lymphocyte activation (Frauwirth and Thompson, 2002).

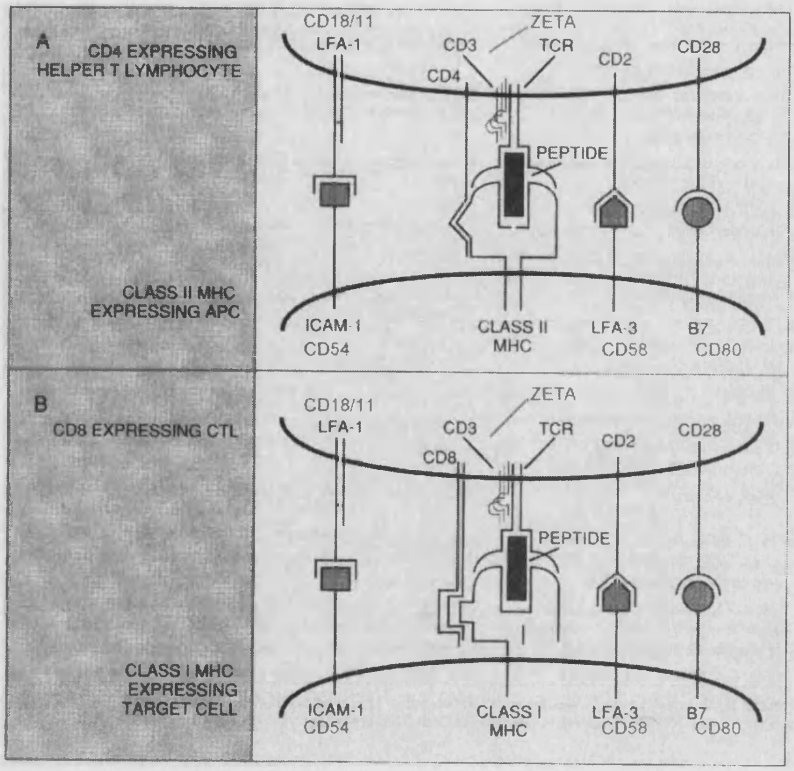


Figure 1.1: T lymphocyte surface molecules and their ligands involved in antigen recognition and T cell activation

Figure from Abbas et al., 1994. A. Interactions between a $CD4^+$ T cell and an antigen-presenting (APC). B. Interactions between a $CD8^+$ cytolytic T lymphocyte (CTL) and an antigen-presenting (APC) (Abbas et al., 1994).

1.3 The T cell receptor signalling pathway

1.3.1 Initiation of T cell signalling

Following contact with an APC, T cells can respond functionally in three ways: 1) by undergoing apoptosis 2) by proliferating or 3) by becoming anergic. The outcome will depend on the context in which the T cell is stimulated. If a T cell encounters a strong signal, in the absence of costimulation, apoptosis will occur. Alternatively, TCR ligation in the absence of costimulation can drive cells into a state of anergy. Anergy is a state in which cells become functionally unresponsive to further stimulation with the same antigen and may be one mechanism by which cells become self tolerant (Abbas et al., 1994); (Lechler et al., 2001); (Walker and Abbas, 2002); (Nel, 2002); (Nel and Slaughter, 2002); (Schwartz, 2003). TCR ligation and

appropriate costimulation results in transcription of the IL-2 gene. IL-2 acts as an autocrine growth factor driving G1 to S cell cycle progression and T cell proliferation.

Following T cell activation, one of the earliest events detectable includes an increase of protein tyrosine phosphorylation. This is mediated by increased activity of kinases. The phosphorylation of a tyrosine residue on a protein can have two major consequences. Firstly, phosphorylated proteins containing specific tyrosine phosphate binding sites provide binding sites for other proteins via their SH2 domains. For example, TCR zeta phosphorylation results in the recruitment and binding of zeta associated protein (ZAP-70) via its two tandem SH2 domains and this, in turn, provides further binding sites allowing recruitment of other signalling molecules to the complex (Koch et al., 1991). Secondly, numerous enzymes can be activated via tyrosine phosphorylation and perpetuate the signal; eg ZAP-70, once bound to zeta, becomes tyrosine phosphorylated at residues 492-493 by Lck and as a result of this, its kinase activity increases (Mege et al., 1996).

1.3.4 The molecules and structures involved in TCR signalling pathways

1.3.4.1 Kinases

Binding of an antigen to the T cell receptor leads to activation of kinases, which induces a rapid increase in protein tyrosine phosphorylation. This initiates signalling pathways which control the activation, proliferation and differentiation of the T cell. Four classes of nonreceptor protein tyrosine kinases have been identified: Src (Lck, Fyn), Syk/Zap-70, Tec (Itk, Rlk) and the Csk family. Apart from possessing a catalytic domain, the kinase protein structures include additional conserved domains which enables them to associate with specific targets and to localise to distinct parts of the cell (Chow and Veillette, 1995); (Chu et al., 1998); (Latour and Veillette, 2001); (Lewis et al., 2001).

1.3.4.2 Phosphatases

The activity of kinases in cells is regulated by the activity of phosphatases. The phosphotyrosine phosphatases implicated in the immunological signalling are categorised in two groups: transmembrane phosphatases and cytoplasmic

phosphatases. Details on the main phosphatases, which play a role in T cell signalling are given in section 1.4.

1.3.4.3 Adaptors

Adaptors can be phosphorylated on key residues, which act as docking sites for other proteins and they act to provide a scaffold enabling the assembly of large signalling complexes. They do not have any enzymatic or transcriptional activity but their role in protein-protein or lipid-protein interaction is important for directing signalling pathways (Leo et al., 2002). Two groups of adaptors are found in cells: transmembrane adaptor proteins (LAT, TRIM, SIT and PAG/Cbg) and cytosolic adaptor proteins (Grb2, Gads and SLP-76) (Cantrell, 2002); (Leo et al., 2002).

1.3.4.4 Lipid raft microdomains

The lipid raft microdomains are regions in the plasma membrane with a unique protein and lipid composition. The concentration of signalling proteins in membrane rafts is higher than elsewhere on the cell membrane and it is proposed that they play an important role in amplifying signalling pathways (Ahmed et al., 1997); (Zajchowski and Robbins, 2002). Upon TCR activation, TCR complexes become concentrated into lipid rafts leading to activation of signalling cascades (Montixi et al., 1998); (Janes et al., 2000); (Viola, 2001).

1.3.5 Immunological synapse

The immunological synapse is a physical structure formed when a T cell receptor interacts with an MHC class II-peptide complex initiating an immunological response (Creusot et al., 2001); (Bromley et al., 2001). This physiological interaction can be divided mainly into four stages: T cell polarisation, T cell:APC adhesion, T cell signalling complex formation (see section 1.3.6 for more details), leading finally to sustained signalling required for activating transcription factors required for cell proliferation and differentiation (Grakoui et al., 1999). Following chemokine exposure, T cell polarisation occurs by migration of the TCR to the site of contact with the MHC class II-peptide complex (Kupfer and Singer, 1988). This migration is orchestrated by the rearrangement of cytosketetal proteins, including actin and tubulin (Lanzavecchia, 1997); (Viola and Lanzavecchia, 1999); (Dustin and Cooper, 2000);

(Peterson et al., 2001). The adhesion site can be subdivided into two areas named the supramolecular acivation clusters (SMACs). The central core SMAC is enriched in TCR and CD28 molecules and the peripheral SMAC contains mainly large adhesion molecules like LFA-1 and ICAM-1 and smaller receptors like CD2 and CD58 which maintain a close contact between T cell and APC (Monks et al., 1998); (Bromley et al., 2001); (Dustin, 2002). The formation of an immunological synapse occurs within minutes following the first contact between T cell and antigen-presenting cell and its duration may last for hours, leading to secretion of diverse cytokines and differentiation of the T cell into specialised effector subsets (see section 1.3.7) (Iezzi et al., 1998).

1.3.6 Activation of T cell signalling pathways

The reorganisation of the surface molecules during the immunological synapse induces changes in the cellular status quo. The CD45 molecule, a transmembrane phosphotyrosine phosphatase of 180-220 kDa, situated near the TCR, dephosphorylates the autoinhibitory site of two major T cell Src kinases: Lck and Fyn (Shiroo et al., 1992); (McFarland et al., 1993). Lck is non-covalently associated with the cytoplasmic tail of the CD4 molecule (Glaichenhaus et al., 1991) and Fyn is non covalently associated with the CD3 ε chain (Osman et al., 1995). Both kinases are maintained in an inactive state by Csk kinase which phosphorylates a tyrosine residue at the C-terminal end of the protein inhibiting kinase activity (Bergman et al., 1992); (MacAuley et al., 1993). Dephosphorylation by CD45 of this inhibitory site enables autophosphorylation of the Src kinases, increasing their kinase activity (Biffen et al., 1994); (Stone et al., 1997); (Thomas and Brown, 1999).

Once dephosphorylated by CD45, Lck and Fyn can then phosphorylate the tyrosine residues within the ITAMs of the ζ and the CD3 molecules (Alberola-Ila et al., 1997); (Qian and Weiss, 1997) (see Figure 2). This leads to the recruitment of ZAP-70 and Syk kinases (Chu et al., 1998). A fraction of the phosphorylated zeta chain is located in the lipid rafts and this puts ZAP-70 in close proximity to one of its substrates, LAT (linker of T cell activation) (Kosugi et al., 1999). ZAP-70 phosphorylates LAT and this allows the binding of other adaptor proteins including Grb2, Cbl and SLP-76 and the proteins Sos and Vav which are a guanine nucleotide

exchange factor. Each of these becomes phosphorylated enabling the activation of the Ras-Raf pathway and subsequently the phosphorylation and increased activity of the mitogen-activated protein (MAP) kinases, ERK1-ERK2, permitting the induction of AP-1 and NFAT transcription factors (Weiss and Littman, 1994); (Cantrell, 1996); (Zhang et al., 1998); (Finco et al., 1998); (Zhang et al., 1999). Another action mediated by LAT phosphorylation is the recruitment of PLCγ-1 to the plasma membrane and hence access to its substrates. Phosphorylation and activation of PLCγ-1 allows the formation of the secondary messengers, inositol triphosphosphate (IP_3) and diacylglycerol (DAG). These, in turn, drive the elevation of intracellular calcium and the regulation of serine kinases including PKC ending in the activation of NF-κB and NFAT transcription factors (Sieh et al., 1994); (Winkler et al., 1993); (van Leeuwe and Samelson, 1999); (Kane et al., 2000). Other ZAP-70 substrates include SLP-76 and Vav which form a complex and initiate activation of the Rac/Cdc42 pathway leading to the activation of the c-jun kinases (JNK) and the induction/activation of the AP-1 transcription factor (Coso et al., 1995); (Crespo et al., 1996); (Teramoto et al., 1996). Depending on the signals received by the T cell, different combinations of transcription factors are induced. This promotes differential gene transcription which determines cell fate (Rao et al., 1997); (Foletta et al., 1998); (Powell et al., 1998); (Medena and Borst, 1999).

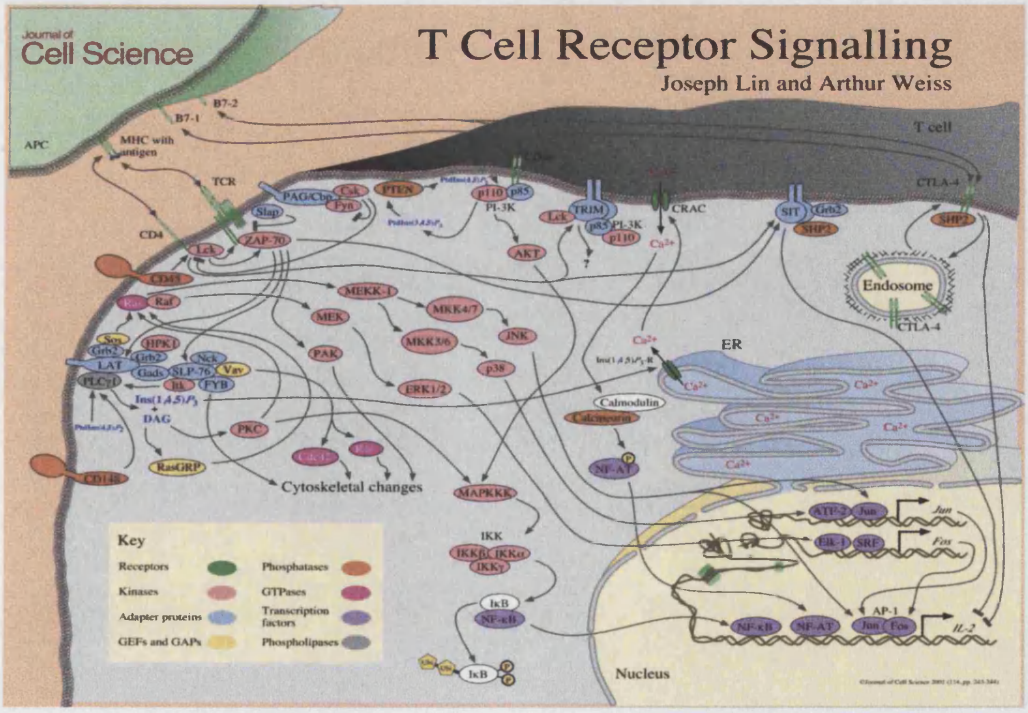


Figure 1.2: T cell signalling pathways

Figure from Lin and Weiss, 2001. See text for more details (Lin and Weiss, 2001).

1.3.7 T cell fate: Th1 and Th2 subsets

In the late eighties, Mosmann and Coffman characterised two different functional subsets of Th cell, which regulated distinct immune responses. Following activation, naive helper T cells clonally expand and produce IL-2. They can further differentiate into Th1 or Th2 cells, each subset expressing a distinct pattern of genes and they can be distinguished by cytokines they express (Mosmann et al., 1986); (Mosmann and Coffman, 1989); (O'Garra and Arai, 2000). Differentiation into Th1 or Th2 cells is dependent on a number of factors, including the nature and the concentration of antigen, the affinity of the interaction between the TCR and the APC, the type of APC as well as strength and nature of co-stimulation signals received. For example, ligation of LFA-1 on the T cell by its reciprocal ligand, ICAM-1 on an APC has been shown to inhibit Th2 development (Constant et al., 1995); (Constant and Bottomly, 1997); (Salomon and Bluestone, 1998); (Luksch et al., 1999). Badou et al. (2001) suggested that a weak TCR signal triggers Th2 differentiation while stronger TCR stimulation induces MAP kinases controlling Th1 differentiation (Badou et al., 2001). Th1 and Th2 cytokines each inhibit the production of the reciprocal subset's cytokines (Mosmann et al., 1986); (O'Garra, 1998); (O'Garra and Arai, 2000).

1.3.7.1 Th1

Th1 cells are implicated in the elimination of intracellular pathogens (bacteria, parasites, yeasts, viruses). Th1 cells produce IL-2 and interferon-γ. These cytokines are important for macrophage activation and the growth and maintenance of natural killer cells and $CD8^+$ cells. Th1 cells also regulate the production of complement-fixing antibodies of the IgG2a isotype, characteristic of cellular immunity (Mosmann et al., 1986); (O'Garra, 1998); (O'Garra and Arai, 2000). IL-12 enhances Th1 commitment by inducing the activation of the transcription factor STAT4 (Trinchieri, 1995); (Murphy et al., 1999); (Murphy and Reiner, 2002). Another transcription factor selectively induced in Th1 cells is T-bet (Szabo et al., 2000). The introduction of T-bet into differentiated Th2 cells has been shown to revert the phenotype to that of

a Th1 cell (Szabo et al., 2000). The MAP kinase p38 pathway is selectively engaged in Th1 cells (Rincon et al., 1998).

1.3.7.2 Th2

Th2 cells are known to have a role in the humoral immune response by producing IL-4, IL-5, IL-9, IL-10 and IL-13 cytokines which regulate B cell proliferation as well as antibody class-switching, activation of mast cells and eosinophils. Th2 cells are involved in the elimination of helminths and other extracellular parasites (Mosmann et al., 1986); (Sher and Coffman, 1992); (Urban et al., 1992). The presence of IL-4 during the differentiation process will favour the Th2 differentiation by activating the STAT6 transcription factor (Paul and Seder, 1994); (Swain et al., 1990). Other transcription factors reported to direct the Th2 subset include Gata-3 and c-Maf. Similar to T-bet in Th2 cells, the introduction of Gata-3 into Th1 cells mediates a reversal of the phenotype to that of a Th2 cell (Ouyang et al., 2000); (Lee et al., 2000); (Ho et al., 1996); (Zheng and Flavell, 1997); (Feber, 1999); (Murphy and Reiner, 2002).

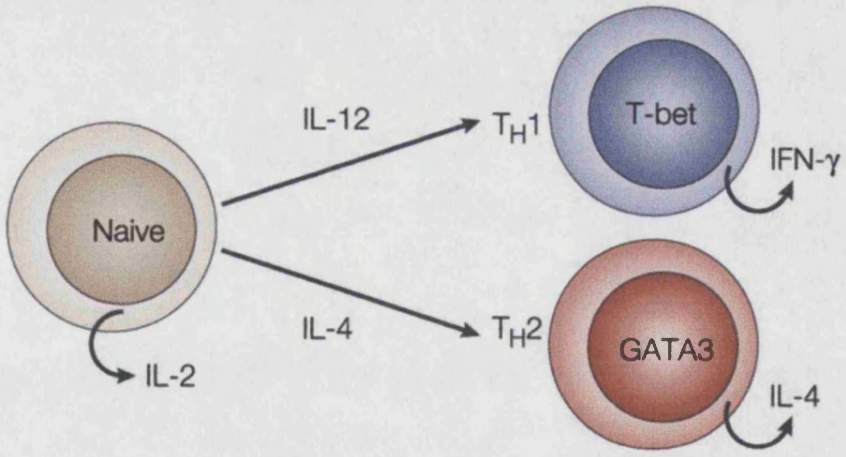


Figure 1.3: T cell fate: Th1 and Th2 differentiation.
Figure from (Murphy and Reiner, 2002). See text for more details.

1.4 Protein phosphatases

Cell signalling pathways, which mediate a variety of cellular functions such as cell proliferation, differentiation and metabolism are regulated by the addition or the

removal of a phosphate group, which is achieved by the coordinated actions of kinases and phosphatases. The role of protein kinases in cells have been well documented. The important role of the phosphatases in signalling pathways has been appreciated relatively recently with the first phosphatase being cloned in 1990. Phosphatases play both positive and negative roles in T cell signalling, switching on as well as terminating kinase activity (Mustelin et al., 2002).

1.4.1 Different families of phosphatases

There are two kinds of phosphatases in cells; they are categorised by their substrate specificity. Firstly, the phosphoserine/threonine phosphatase group, which can be subdivided into phosphoprotein phosphatases (PPP) and phosphoprotein magnesium dependent phosphatases (PPM) and secondly, the phosphotyrosine phosphatase (PTP) group. The first group of phosphatases act by removing a phosphate group from serine and/or threonine residues only. They are implicated in several processes, including stress activated signalling pathways, mitosis and cell cycle progression. The phosphotyrosine phosphatase family of phosphatases remove phosphate groups from tyrosine residues. The PTPs can be further subdivided into three classes: the specific PTP, the dual specificity phosphatases and the low molecular weight PTP.

1.4.2 Phosphotyrosine phosphatases

The specific PTP or standard PTP, are restricted by their ability to remove a phosphate group from tyrosine residues only. The dual specificity phosphatases hydrolyse preferentially phosphotyrosine residues but can also remove phosphate groups from serine/threonine residues at a rate 40 to 500 fold slower than for tyrosine residues (Zhang, 1995). The low molecular weight PTP proteins are distinguished from the first two classes of PTPs by possession of a short specific motif CX_3R instead of the [I/V]HCXAGXXR[S/T]G phosphotyrosine activation site (where X represents a random amino acid in the single-letter amino acid code) (Streuli et al., 1990); (Guan and Dixon, 1991); (Barford et al., 1994).

1.4.2.1 Standard phosphotyrosine phosphatase

Standard PTP can be further subdivided into two families based on their location in the cell: the transmembrane PTP and the cytoplasmic PTP. The transmembrane PTP are also called receptor-like PTP. Transmembrane phosphatases contain an extracellular domain of variable length and composition, a single transmembrane sequence and usually two duplicated cytoplasmic PTP domains (Guan and Dixon, 1991). The reason why these PTP have two distinct PTP domains is still unclear (Streuli et al., 1990). There is a consensus sequence (HCSAGVGR) in the 1st catalytic domain of transmembrane PTP, which has enzymatic activity. Pulido et al (1995) have proposed a protein-protein interaction function, for the second domain (Pulido et al., 1995). The extracellular domains have the potential to serve as a receptor for a specific ligand.

The cytoplasmic family proteins contain a single catalytic domain (Guan and Dixon, 1991). They have a consensus sequence common to all cytoplasmic PTP: HCSAGIGR (Streuli et al., 1990). In some PTP, other domains for protein-protein interactions, such as SH2 domains, can be present. All PTPs possess a unique characterised motif for the active site in the catalytic domain containing essential cysteine and arginine residues: [I/V]HCXAGXXR[S/T] (Streuli et al., 1990); (Guan and Dixon, 1991); (Barford et al., 1994). The PTP family has the characteristic of requiring another amino acid to close the active site cleft during the catalysis. This is an aspartic acid residue, which is located approximately 30 to 40 amino acids upstream from the signature motif (Zhang et al., 1994); (Hengge et al., 1995); (Fauman and Saper, 1996). The PTP do not require any ion or cofactor for their function but they are inhibited by vanadate and by hydrogen peroxide (H_2O_2) (Heffetz et al., 1990); (Secrist et al., 1993). Vanadate will bind to the enzymatic centre acting, as a phosphate analogue and inhibits the release of substrate from the phosphatase (Gresser and Tracey, 1990). H_2O_2 oxidises the thiolate anion of a cysteine residue in the PTP-reactive center, blocking the formation of a phosphorylcysteine intermediate, which is a critical step in dephosphorylation (Jin et al., 1998).

1.4.2.2 Dephosphorylation mechanism

The conserved cysteine residue, which sits at the bottom of a deep catalytic pocket in the phosphatase is essential for the hydrolysis of the phosphate group on the tyrosine. This cysteine is positioned in such manner that it can make a nucleophilic attack on the phosphorus atom, creating a covalent thiol phosphate bond between the phosphotyrosine of the substrate and the cysteine (Guan and Dixon, 1991); (Barford et al., 1994); (Stuckey et al., 1994). The cysteine attack is followed by a transfer of charges, mediated by the upstream invariant aspartic acid. The arginine acts to position the phosphate residue in the pocket (Zhang, 1998). Thus, the substrate binding to the PTP allows a conformational change of a surface loop, creating a phosphotyrosine recognition pocket and inducing a catalytically competent form of the enzyme (Jia et al., 1995). The depth of the pocket controls the phosphatase specificity (Pannifer et al., 1998)

1.5 T cell signalling: the role of phosphatases

1.5.1 Transmembrane phosphotyrosine phosphatases

1.5.1.1 CD45

The role of CD45 in early T cell signalling has been outlined in section 1.3.5. CD45 is expressed on the surface of all nucleated hematopoietic cells and represents up to 5-10% of the cell surface membrane proteins (Thomas, 1989). The extracellular domain of CD45 is very long and can vary by alternative splicing of exons 4, 5 and 6 (Thomas, 1989). The cytoplasmic domain of CD45 contains two PTP domains. The domain closest to the membrane accounts for most of the PTP enzymatic activity but it is possible that the two domains have independent activities, which could be differentially regulated (Neel, 1997). Cells lacking the CD45 molecule fail to respond to stimulation by antigen or mitogenic antibodies (Pingel and Thomas, 1989).

The best described role for CD45 is its activation of Src-family kinases (Lck and Fyn) (Shiroo et al., 1992); (McFarland et al., 1993). The kinases will then activate different components of the signalling pathway, leading to the phosphorylation of TCR ζ, CD3-ε, Slp-76, Vav, PLCγ1, Cbl and HS1, all of which are positive regulators of T cell signalling (Biffen et al., 1994); (Stone et al., 1997); (Thomas and Brown, 1999).

Other substrates reported for CD45 include TCR ζ (Furukawa et al., 1994). It would be predicted that dephosphorylation of TCR ζ ITAMs would have a negative influence on signalling which is an opposite effect of the previous actions described above for CD45 in T cell signalling. It has been shown that when the MHC molecules present peptide to T cells, the CD45 protein is excluded from the T cell/APC contact zone within which TCRs polarise and thus CD45 is unable to complete the dephosphorylation of the TCR ζ ITAMs (Thomas, 1994); (Thomas and Brown, 1999); (He et al., 2002). Under these conditions, Lck and Fyn gain exclusive access and are able to fully phosphorylate ITAMs on CD3 and zeta. Termination of TCR signalling could be achieved by CD45 regaining proximal contact with zeta thereby returning it to an unphosphorylated form (Furukawa et al., 1994); (Alberola-Ila et al., 1997); (Qian and Weiss, 1997); (Mustelin et al., 1999). A recent report states that the Jak kinases could also be a physiological substrate for CD45, abrogating the Jak-Stat pathway necessary for cytokine production (Irie-Sasaki et al., 2001).

CD45 is located at the same chromosomal location as LCPTP (see section 1.6), another PTP, suggesting that there could have been a duplication of an ancestral gene encoding the PTP domain to generate two distinct phosphatases (Adachi et al., 1994a).

1.5.1.2 CD148

The PTPase CD148 is also called HPTPη/DEP-1 (density-enhanced protein tyrosine phosphatase-1). It is found in all hematopoietic lineages but is expressed more abundantly on monocytes, B cells, NK cells and memory T lymphocytes with lower levels on $CD4^+$ T cells (Tangye et al., 1998). The protein has a molecular weight of 180-250 kDa and has one catalytic phosphatase domain in the intracellular region (Schraven et al., 1997). Crosslinking of CD148 induces tyrosine phosphorylation of PLC-γ1, which has also been reported for CD45. However, unlike CD45, crosslinking of CD148 has been shown to induce an increase of $[Ca^{2+}]_i$ (de la Fuente-Garcia et al., 1998). Overexpression of CD148 in Jurkat T cells, resulted in inhibition of ZAP-70 tyrosine phosphorylation (Tangye et al., 1998). CD148 would

thus appear to be a PTPase involved in down-regulation of T-cell activation. A recent study using an inducible system expressing CD148 protein, reports that CD148 inhibits many events following TCR activation, such as CD69 upregulation, ERK phosphorylation and calcium mobilisation by the specific inhibition of LAT and PLC-γ1 phosphorylation (Baker et al., 2001).

1.5.2 Cytoplasmic phosphotyrosine phosphatases

1.5.2.1 PEP

PEP phosphatase is expressed only in hematopoietic cells where it is reported to associate with Csk (Matthews et al., 1992); (Cloutier and Veillette, 1996). This phosphatase is a member of the PEST PTPases which contain an N-terminal PTPase domain and a C-terminal with several proline, glutamic acid, serine and threonine-rich motifs (Mustelin et al., 2002). The interaction of PEP with Csk is interesting because Csk leads to a negative regulation of the Lck kinase, which is phosphorylated by Csk on the inhibitory activation site (Bergman et al., 1992) suppressing TCR signalling (Chow et al., 1993). Gjorloff-Wingren (1999) and Cloutier (1996) suggested that PEP could have a role in the dephosphorylation of the Src or Syk kinases family members (Gjorloff-Wingren et al., 1999); (Cloutier and Veillette, 1999). This phosphatase is mainly localised in the cellular membrane, in the cytosol and a tiny amount is found in the nucleus. Deletions in PEP and confocal microscopy data suggest that the C-terminal domain is necessary for cellular membrane associations and the N-terminal domain is necessary for the presence of PEP in the nucleus (Davidson et al., 1997); (Gjorloff-Wingren et al., 1999).

1.5.2.2 LYP

LYP PTP is expressed predominantly in lymphoid cells (both immature and mature B and T cells) and has a molecular weight of 85-105kDa (Cohen et al., 1999). The name LYP is derived from lymphoid phosphatase. The gene is located on the human chromosome 1p13, a common site for chromosomal rearrangement in hematopoietic cancers (Lion et al., 1992). LYP protein shares 70% homology with PEP. When resting peripheral T cells were stimulated with anti-CD3 or PHA, Cohen et al (1999) observed a significant increase in the expression of LYP protein (Cohen et al., 1999). The messenger RNA of LYP undergoes an alternative splicing at the C-

terminal end and produces two forms of LYP: LYP1 and LYP2. LYP2 is mainly detected in fetal liver tissue and has only one SH3 domain (this domain possibly interacts with proline rich proteins) compared to four SH3 domains in LYP1 expressed in both the early and the late stage of T cell development. The creation of C-terminal changes by alternative RNA splicing appears to be a widely used mechanism for generating functional diversity in phosphatases (Reddy and Swarup, 1995); (Cohen et al., 1999). Overexpression of LYP showed a constitutive interaction with c-Cbl, an adaptor protein (Cohen et al., 1999). c-Cbl is heavily tyrosine phosphorylated after T cell stimulation (Ota and Samelson, 1997) and has been shown to associate with the kinases, Syk and ZAP-70, negatively regulating their functions (Lupher et al., 1999). When phosphatases are inhibited by vanadate, c-Cbl becomes hyperphosphorylated (Ota and Samelson, 1997). Cohen et al. (1999) suggested that LYP could maintain c-Cbl in a basally dephosphorylated state and could function to negatively regulate c-Cbl and associated proteins in the TCR signalling pathway (Cohen et al., 1999). Another negative function has recently been discovered for LYP. Hill and collaborators (2002) showed in 293T cells that LYP coprecipitated with the adaptor molecule, Grb2 under conditions when both proteins were overexpressed (Hill et al., 2002). Overexpression of wild-type LYP or a substrate-trap (D195A) mutant inhibits anti-CD3/anti-CD28 transcriptional activity in Jurkat T cells confirming the negative regulatory role of LYP in T cell signalling (Hill et al., 2002).

1.5.2.3 SHP-1

The name SHP-1 is derived from SH2 domain containing protein tyrosine phosphatase. SHP-1 is mainly expressed in cells of hematopoietic origin (Mustelin et al., 1999). The enzyme binds through its two SH2 domains to receptors that have an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM). Studies in motheaten mice which have a loss-of-function mutation in the SHP-1 protein, have shown SHP-1 to be a negative regulator of signalling through the TCR. These mice have enhanced and prolonged tyrosine phosphorylation of TCR zeta and CD3 and also prolonged MAP kinase activation following TCR stimulation (Pani et al., 1996). However, the IL-2 response to TCR stimulation is unchanged in motheaten mice (Lorenz et al., 1996). In HTLM-1 transformed T cells, SHP-1 dephosphorylates the

75kDa subunit of the interleukin-2 receptor and the associated tyrosine kinases JAK1 and JAK3. By this action, SHP-1 downregulates the IL-2 induced signalling response in T cells (Migone et al., 1998). Lorenz et al (1996) found that SHP-1 can inactivate Lck and Fyn kinases and Plas et al (1996) suggested that ZAP-70 is a direct target for the phosphatase (Lorenz et al., 1996); (Plas et al., 1996); (Brockdorff et al., 1999). Furthermore, SHP-1 dephosphorylates c-Src from Jurkat T cells indicating that SHP-1 is implicated in the activation of this kinase (Somani et al., 1997). Gjörloff-Wingren et al. (2000) have shown that SHP-1 is evenly distributed in the cytosol of Jurkat T cells. Activation of Jurkat T cells by anti-CD3 antibody did not change SHP-1 location (Gjörloff-Wingren et al., 2000).

1.5.2.4 SHP-2

The SHP-2 phosphatase is closely related to SHP-1 protein and is expressed ubiquitously in all cell types (Mustelin et al., 1999). In contrast to its homologue SHP-1, SHP-2 appears to have a positive role in the regulation of TCR signalling following receptor ligation (Mustelin et al., 1999). Phosphorylation of SHP-2 in its C-terminal end enhances the recruitment of Grb2 to the membrane and the translocation of Sos and activation of Ras (Bennett et al., 1994); (Li et al., 1994). Tailor et al. (1996) have shown that after stimulation, SHP-2 becomes tyrosine phosphorylated and then associates with Grb2, c-Cbl and PI3K (Tailor et al., 1996). In addition, a 110 kDa protein homologue of Dos (Daughter-of-Sevenless), coprecipitates with SHP-2 and the dephosphorylation of this protein is involved in the regulation of the MAP kinase signalling pathway (Adachi et al., 1997). SHP-2 activity has been reported to increase MAP kinase activation in T cells following observations that overexpression of a SHP-2 substrate-trap results in inhibition of TCR-stimulated ERK-2 phosphorylation (Frearson and Alexander, 1998). A negative role for SHP-2 has also been suggested by virtue of its association with the inhibitory CTLA-4 receptor in mice (Marengere et al., 1996). Like SHP-1, experiments using confocal microscopy have shown that SHP-2 is equally distributed throughout the cytosol in transfected Jurkat T cells and stimulation with anti-CD3 did not change its location (Gjörloff-Wingren et al., 2000).

1.6 The role of phosphotyrosine phosphatase LCPTP in T cell signalling

The nomenclature regarding this hematopoietic restricted phosphatase differs in the literature. After an explanation of the differences between the two proteins, the term LCPTP will be used throughout the manuscript.

HePTP (accession number M64322) also known as LCPTP (accession number D11327) was first identified in 1992. The name HePTP comes from hematopoietic phosphotyrosine phosphatase and the name LCPTP is derived from leucocyte phosphotyrosine phosphatase (Zanke et al., 1992); (Adachi et al., 1992b). Zanke's group used a tyrosine phosphatase consensus sequence-specific degenerate oligonucleotide in a PCR using cDNA from pokeweed mitogen (PWM)-stimulated human peripheral T lymphocytes to find HePTP (Zanke et al., 1992). They characterised the protein and found that HePTP includes only one phosphatase domain. Murine HePTP mRNA transcription was shown to be increased up to 10-15 fold, following anti-CD3, PHA, LPS or Con A stimulation. Increased HePTP mRNA was noticeable at 24 hrs with a maximal increase at 72 hrs post-stimulation while the protein level increased only moderately. *In vivo* expression was seen in BALB/c murine T and B lymphocytes but not in murine non-hematopoietic tissues (brain, liver, kidney and ovary) (Zanke et al., 1992). Seven months later, Adachi et al., published the chromosomal mapping of LCPTP from the PCR product B-PTP-4, which was obtained from total RNA of pre-B cells NALM-6 (Adachi et al., 1992a) from a PEER cDNA library. Their observations corroborate Zanke's findings with the addition to identifying the chromosomal locus occupied by LCPTP: 1q32.1 where a deletion is observed in non-Hodgkin's lymphomas (NHL) and chronic lymphoproliferative disorders (CLD) (Mitelman et al., 1990). They also found that LCPTP has a consensus sequence for a nontransmembrane PTP and that the human mRNA is expressed in two transcriptional sizes: 4.0 kilobases and 2.9 kilobases (Adachi et al., 1992a). The main difference between the two proteins is the location of the presumptive translation initiation codon. LCPTP has 21 more amino acids in the N-terminal end compared to HePTP caused by a missing cytosine in the HePTP sequence. Other differences are located within the coding sequence at (R-234 Q L T,

LCPTP)-(R G V T, HePTP) and (T-336 D E, LCPTP)-(T A E, HePTP) (Adachi et al., 1992a); (Swieter et al., 1995); (Mustelin et al., 1998).

Zanke et al. (1994) looked at the expression of LCPTP in diverse murine and human cell lines. LCPTP was found to be expressed at high levels in myeloid (HL-60, MO7E, OCI AML 1 through 4), B and T lymphoid (Jurkat, CTLL, Molt-4, Molt-3, P30/OKUBO, Raji, HPB-ALL) and myeloma (OCI My-4, OCI My-5) cells (Zanke et al., 1994). By fluorescent in situ chromosomal hybridisation (FISH), the gene was mapped to chromosome 1q32.1 (Adachi et al., 1992a). The analysis of patients with myelodysplastic syndrome (myeloid hypoplasia and monocytosis) showed triplication of the LCPTP gene (Zanke et al., 1994). The overexpression of LCPTP in NIH 3T3 fibroblasts caused altered morphology, disorganised growth, anchorage independent colony formation and subtle differences in the pattern of tyrosine phosphoproteins. It was concluded that unbalanced LCPTP activity may contribute to non-regulated growth and establishment of clonal dominance of cells and it was suggested that LCPTP plays a role in the regulation of cell proliferation, survival or differentiation (Zanke et al., 1994).

The rat homolog of LCPTP was discovered by Swieter et al. (1995). It shares 92% identity with the human sequence. They showed a tyrosine phosphorylation of LCPTP upon FcεRI aggregation in the RBL-2H3 cell lines. This post-translational modification seems to be calcium dependent and it was suggested that LCPTP played a role in IgE receptor aggregation (Swieter et al., 1995). By immunofluorescence microscopy, in RBL mast cells, LCPTP was shown to be localised into discrete compartments in the cytoplasm, was absent from the nucleus and could not be detected associated with the cell surface membrane (Swieter et al., 1995). It was shown in a more recent study that LCPTP was localised in the cytoplasm, with a clear enrichment near the plasma membrane. This location did not seem to change upon T cell activation (Gjörloff-Wingren et al., 2000). LCPTP has been labelled as an early response gene. This follows studies in which the IL-2-dependent cell line ILT-Mat (Adachi et al., 1994b) or the IL-3-dependent pro-B cell BAF-B03 F7 clones expressing IL-2Rβ and IL-2Rα (Adachi et al., 1995) were stimulated with IL-2. A rapid increase in LCPTP mRNA and LCPTP protein, peaking at 6 and 8 hours

respectively was seen after IL-2 stimulation (Adachi et al., 1994b); (Adachi et al., 1995). In the IL-3-dependent cell lines, the increase in LCPTP mRNA was abolished by the presence of tyrosine kinase inhibitors and promoted by the overexpression of activated-Lck or activated-Raf-1 kinases suggesting an involvement of these kinases in the LCPTP mRNA induction (Adachi et al., 1995).

Recent studies have implicated the phosphatase as playing a role in TCR signalling (Saxena et al., 1998); (Saxena et al., 1999a); (Oh-hora et al., 1999); (Saxena et al., 1999b); (Pettiford and Herbst, 2000). IL-2 gene transcription activity was measured following cotransfection of the reporter gene NFAT/AP-1 luciferase (derived from the 5' IL-2 gene promotor) and another plasmid containing HA-LCPTP wild-type in J-TAg and JCaM1 cell lines. It was found that when LCPTP is transiently overexpressed in anti-CD3 activated cells, the activity of the reporter NFAT/AP-1 luciferase was greatly diminished. In contrast, a catalytically inactive mutant (C270S) had no effect (Saxena et al., 1998). When the two proteins, LCPTP and MAPK ERK2 were transfected simultaneously, a reduction of MAPK kinase activity was observed, but the same experiment with JNK kinase overexpression did not change the kinase activity (Saxena et al., 1998). Thus a specific role for LCPTP in dephosphorylating the MAPK and not the JNK members was identified. LCPTP and MAPK family members: ERK1, ERK2 and p38 were overexpressed in Jurkat and JCaM1 cell lines and a constitutive association, mediated via the N-terminus of LCPTP, was reported between the wild-type and the catalytically inactive mutant LCPTP and MAPK family members (Saxena et al., 1999a). A ratio of 1:1 between the phosphatase and the kinase was put forward for the dephosphorylation action of the recombinant LCPTP (Saxena et al., 1999a). Oh-hora et al. (1999) demonstrated the same constitutive association between LCPTP and the MAPK family members ERK1, ERK2 and p38, in the 293 T cell line along with the importance of the N-terminal extremity of LCPTP for the binding, named KIM motif (kinase interacting motif: GLQERRGSNVSLTLDM) (Pulido et al., 1998); (Oh-hora et al., 1999). Two other phosphatases, which also possess KIM motifs, are STEP and PTP-SL. STEP and PTP-SL are found primarily in neuronal cells and have been shown to be associated with ERK1 and ERK2, when using overexpressed proteins (Pulido et al.,

1998). In addition, other phosphatases: PTP-ER (Karim and Rubin, 1999), MKP-3 (Zhou et al., 2001) and the yeast Ptp3 (Zhan and K.L., 1999) contain the KIM motif.

Further studies by Saxena et al. have demonstrated a potential crosstalk between the cAMP system and the MAPK cascade (Saxena et al., 1999b). Treatment of radiolabelled Jurkat T cells with ^{32}P, with a membrane-permeable cAMP analogue (8-CTP-cAMP) induced serine phosphorylation on overexpressed LCPTP, implicating activity downstream of cAMP kinase action. An *in vitro* experiment in which PKA, a kinase activated by cAMP, and LCPTP proteins were added together, showed that PKA can phosphorylate Ser23 on LCPTP, within the KIM motif. GST pull-downs of this phosphorylated form of LCPTP have a reduced association with overexpressed MAPK family members. Treatment of a Jurkat T cell line with cAMP caused a small activation of the MAPK family members sufficient to induce further events such as c-fos transcription (Saxena et al., 1999b). Pettiford and Herbst (2000) showed that in the human chronic myelogenous leukemic cell line, K562, ERK2 is the only substrate for LCPTP. ERK2 interacted with the catalytically inactive phosphatase but not with the wild-type protein. The substrate, ERK2, needed to be phosphorylated for the interaction to occur and the weak interaction between LCPTP and p38 only occurred under mild immunoprecipitation conditions (Pettiford and Herbst, 2000).

A genetically modified mouse lacking the LCPTP gene has been generated (Gronda et al., 2001). The mild phenotype observed included an enhanced activation of ERK1 and ERK2 following PMA or anti-CD3 stimulation. All other physiological measurements made failed to show any differences compared to the control. This included looking at the distribution of hematopoietic lineages in bone marrow and peripheral blood, *in vitro* proliferation of bone marrow progenitors, IL-2 production, Th2 differentiation and surface expression of activation markers on lymphoid cells (Gronda et al., 2001). It was concluded that LCPTP is a regulator of the MAPK family members but its deletion in the mouse is compensated by an alternative signalling protein, or proteins (Gronda et al., 2001).

Saxena's model is illustrated in Figure 4. This data is based on use of recombinant LCPTP protein.

Table 1.1 summarises data available on the phosphatases implicated in T cell signalling with regard to their substrates, localisation in the cell and the role they play in regulating TCR signalling.

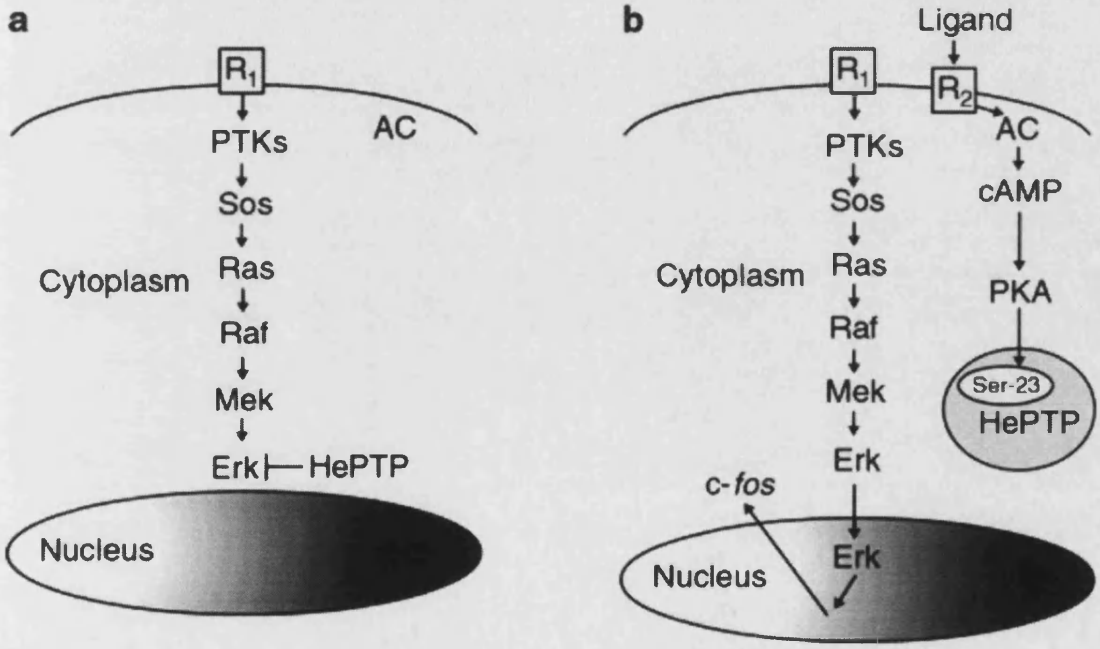


Figure 1.4: Model for LCPTP (HePTP) in T cells signalling

Figure from Saxena et al., 1999. "A. In the absence of external stimuli, the classical pathway of MAP kinase activation (originating at a receptor, R1) through Ras, Raf and Mek has a low basal activity, which is counteracted by HePTP. B. Stimulation of receptors (R2) that couple to adenylate cyclase (AC) turn on the new pathway that involves phosphorylation of HePTP at Ser 23 by PKA, leading to dissociation of ERK from HePTP. This release from inhibition results in MAP-kinase activation and subsequent c-fos induction." (Saxena et al., 1999a).

Table 1.1: Main phosphatases implicated in T cell signalling

Phosphatase	Proposed substrate	Localisation	Action in TCR signalling
CD45	Lck, Fyn, Zeta (?)	Membrane	Positive
CD148	ZAP-70 (?)	Membrane	Negative
PEP	Src, Syk kinases	Cytosol, nucleus	Negative (?)
LYP	c-Cbl	Cytosol	Negative (?)
SHP-1	ZAP-70, Lck, Fyn, 75kDa protein	Cytosol	Negative
SHP-2	Grb2, c-Cbl, PI3K, 110 kDa protein (?)	Cytosol	Positive
LCPTP	MAPK	Cytosol	Negative

AIMS

The major aims of this study are:

⇒ To investigate the role of LCPTP in T cell signalling and activation.

⇒ To propose a model for the involvement of LCPTP in T cell signalling and activation.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Molecular Biology

2.1.1 Bacterial strains

Escherichia coli strain DH5α (*sup*E 44 Δ*lac*U169 *lacz*ΔM15 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1) was used as general transforming bacteria for plasmid expansion (Grant et al., 1990). *Escherichia coli* strain DH10B Admid294, containing a low copy plasmid with a full-length adenoviral genome, lacZatttTn7::E1 and an inefficient E3 gene ($E1^-E3^-$), supplemented with a mini-Tn7 element plasmid (Richards et al., 2000) was used for the adenoviral expression system. Cells were cultured in Luria Bertani medium (LB - 10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 5 g/l NaCl) or on solid medium (LB + 1.5% (w/v) agar) with specific antibiotic added for each plasmid used at a concentration of 100 μg/ml for ampicillin or kanamycin, 15 μg/ml tetracycline, 20 μg/ml chloramphenicol, 300 μg/ml Bluo-Gal (Life Technologies, UK) and 40 μg/ml isopropylthio-β-galactoside (IPTG) (Sigma, UK). Cells were stored at –80°C in 30% (v/v) glycerol in LB.

2.1.2 Preparation of competent DH10B Admid294 cells

Single colonies of DH10B Admid294 were grown in LB supplemented with tetracycline and chloramphenicol for 18 hours at 37°C in a shaking incubator. A total of 200 ml of 2YT (16 g/l bacto-tryptone, 10 g/l bacto-yeast extract, 5 g/l NaCl) was cultured and grown until an absorbance of $A_{590} = 0.375$ was obtained. Cells were then centrifuged in a Sorvall rotor SS-34, for 12 min at 5000 rpm at 4°C. Cells were resuspended in cold 60mM $CaCl_2$/15% glycerol. Cells were centrifuged for 10 min at 3000 rpm at 4°C, resuspended in 60mM $CaCl_2$/15% glycerol and incubated on ice for 90 min. Cells were centrifuged for 10 min at 2500 rpm at 4°C and cell pellets were resuspended in 4 ml of 60mM $CaCl_2$/15% glycerol, aliquoted in 0.150 ml volumes into eppendorf tubes and stored at -80°C.

2.1.3 Transformation of DNA into competent cells

Competent DH5α cells were obtained following the protocol described in Sambrook et al. (1990) (Sambrook et al., 1989). 50 ng DNA was mixed with 0.2 ml of the

competent cell suspension. After 30 min incubation on ice, cells were heat-shocked for 45 sec at 42°C and immediately transferred back onto ice. After 2 min, 0.8 ml LB was added and samples were incubated for 1 h at 37°C. 0.2 ml aliquot was spread on LB agar plates 1.5% (w/v) agar) containing the appropriate antibiotic. Plates were incubated at 37°C overnight, or until colonies were observed.

2.1.4 Preparation of Plasmid DNA

A 5 ml (Mini-prep kit) or 400 ml (Maxi-prep kit) culture of DH5α containing the plasmid of interest was grown up overnight with aeration in a 37°C shaking incubator. Cells were harvested by centrifugation for 3 min at 13 000 rpm (Mini-prep) (Eppendorf minicentrifuge) or 15 min at 6 000 rpm (Maxi-prep)(Sorvall centrifuge). Plasmid DNA was isolated using the Qiagen plasmid purification kit (Mini-prep or Maxi kit), according to the manufacturer's protocol. The DNA pellets obtained were air-dried, resuspended in 0.1 ml or 0.5 ml TE buffer (10 mM Tris-HCl pH. 8.0, 1 mM EDTA) and stored at -20°C.

2.1.5 Cloning of LCPTP from a cDNA library

The human LCPTP gene (accession number: D11327) was amplified by PCR from an H9 library. The H9 cDNA library was donated by M. Suda of GlaxoNippon, Japan. The cDNA was generated from poly (A+) RNA extracted from a mixture of non-activated and 12-O-tetradecanoylphorbol-13-acetate-activated H9 T-cells and synthesised using an oligo(dT) primer and random primers. The primers used were LCPTP-1 (CGCGTGGATCCATGGTCCAGCCCATGGGGGGCGCTC) and LCPTP-2 (CGCGTGGATCCGCAGGGGCTGGGTTCCTCAGGCAGC) bearing *BamHI* restriction sites (underlined). This yielded an 1120-bp fragment flanked with a *BamHI* site of each side.

2.1.6 Cloning of LCPTP into pcDNA3.1 plasmid

The PCR product LCPTP was digested with *BamHI* and then subcloned into the pcDNA3.1 (Invitrogen, UK) containing N-terminal cmyc-tag (CAGCC ATG GAG CAA AAG CTC ATT TCT GAA GAG GAC TTG TTG) kindly provided by Dr Janet Smith (GlaxoSmithKline, UK).

2.1.7 Site directed mutagenesis

Site directed mutagenesis was performed using a QuickChange Site-Directed Mutagenesis Kit following the manufacturer's protocol (Stratagene, UK) to generate the substrate-trap mutant phosphatase (called Trap) D257A-C291A and the null mutant phosphatase (called Null) R297M. The primers used were LCPTP-9 (CGGCTGGCCAGCCCATCAGACACC) and LCPTP-10 (GGTGTCTGATGGGCTGGCCAGGCCG) for D257A and LCPTP-11 (CTATCGTAGTCCACTCCAGTGCAGGGATTGG) and LCPTP-12 (CCAATCCCTGCACTGGAGTGGACTACGATAG) for C291S, LCPTP-15 (CAGTGCAGGGATTGGCATGACGGGCTGCTTCATC) and LCPTP-16 (GATGAAGCAGCCCGTCATGCCAATCCCTGCACTG) for R297M. Amplification of these plasmids was done using the transformation and DNA isolation protocols described above (sections 2.1.3 and 2.1.4).

2.1.8 Cloning of LCPTP into pGFP plasmid

The green fluorescent vector (GFP) construct was obtained from Clontech (GenBank accession #: U19280, UK). Cloning of LCPTP into the GFP vector was performed using constructs from plasmids pcDNA 3.1 myc-wt-LCPTP; pcDNA 3.1 myc-C291S-D257A-LCPTP; pcDNA 3.1 myc-R297M-LCPTP provided by Dr Angela Boyhan (GlaxoSmithKline, UK). GFP vector was linearised by cutting in the multi-cloning site with *BamHI* (New England Biolab, UK). The different LCPTP genes (wt, C291S-D257A and R297M) were cut from the pcDNA 3.1 cmyc plasmid using *BamHI* enzyme (New England Biolab, UK). The LCPTP inserts were religated into the GFP vector to create: GFP-LCPTP wt, GFP-LCPTP C291S/D257A and GFP-LCPTP R297M constructs.

2.1.9 Cloning of LCPTP into pCR259 plasmid

The plasmid pCD259 was obtained from Dr Nigel Sharp (GlaxoSmithKline, UK). Cloning of LCPTP into the pCR259 vector was performed using constructs from plasmids pcDNA 3.1 myc-wt-LCPTP; pcDNA 3.1 myc-C291S-D257A-LCPTP provided by Dr Angela Boyhan (GlaxoSmithKline, UK). pCR259 vector was linearised by cutting in the multi-cloning site with *NheI* and *NotI* (New England Biolab, UK). The 5'polymerase Klenow (New England Biolab, UK) was used to fill

in ends. The different LCPTP genes (wt and C291S-D257A) were cut from the pcDNA 3.1 cmyc plasmid using *BamHI* enzyme (New England Biolab, UK) and the ends were filled by the 5'polymerase Klenow (New England Biolab, UK). The LCPTP inserts were ligated into the pCR259 vector to create: pCR259-LCPTP wt and pCR259-LCPTP C291S/D257A.

2.1.10 Insertion of pCR259-LCPTP into the homing vector pCR249, selection of pCR249-LCPTP

Transformation of pCR259-LCPTP was performed as described in section 2.1.3 in DH10B Admid294 and plated on LB medium supplemented with ampicillin, chloramphenicol, tetracycline, Bluo-Gal and IPTG. After 18 hours, white colonies were picked and grown in LB broth supplemented with chloramphenicol. Mini-preps were performed following the Qiagen protocol (section 2.1.4). Once the DNA was obtained, transformation into DH5α was performed and cells were plated on LB medium supplemented with chloramphenicol, Bluo-Gal and IPTG. After a further 18 hours, white colonies were picked and transferred to replicate plates each containing one of the different antibiotics (ampicillin, tetracyclin or chloramphenicol). Cells growing on LB with chloramphenicol only were selected for bulk preparation.

2.1.11 Agarose gel electrophoresis

Gels containing 0.7-1.2% (w/v) agarose in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) were prepared using the Bio-Rad Wide Mini-Sub cell system. 1/6 volume agarose gel loading buffer (6x) (50 mM Tris-HCl (pH 8.0), 50% (v/v) glycerol, 0.2% (w/v) bromophenol blue) was added to DNA samples and gels were run in TAE buffer at 100 mV. Following electrophoresis, DNA was visualised under short-wave UV light.

2.1.12 DNA concentration determination

DNA concentrations were determined by measuring the optical density (OD) at 260 nm, using a quartz cuvette and a Pharmacia Ultrospec 2000 spectrophotometer. Calculations were based on the assumption that an OD_{260}= 1 (measured in a cuvette with a 1 cm pathlength) is equivalent to 50 μg/ml dsDNA.

2.1.13 Solvent extraction of DNA

Samples were mixed thoroughly with an equal volume phenol/chloroform (50:50) and the two phases (organic and aqueous) separated by low speed centrifugation at 5000 rpm for 5 minutes. The aqueous phase was retained for further processing.

2.1.14 Ethanol precipitation of DNA

DNA samples were diluted 1/10 with 3 M sodium acetate (pH 5.2) followed by the addition of 2.5 volumes ethanol (ice-cold). After 15 min at -80°C, or 2 hr at -20°C, precipitated DNA was pelleted by centrifugation (13 000 rpm, 15 min, 4°C). DNA pellets were washed with 70% (v/v) ethanol (ice-cold), air dried and resuspended in TE buffer.

2.1.15 Production of transfection stock of adenovirus in HEK293 cells

A total amount of 2.5 ng of pCR249-LCPTP was digested with *PacI* restriction enzyme, purified with phenol/chloroform (as described in section 2.1.13) and precipitated with ethanol (described in section 2.1.14). The digested DNA was mixed with lipofectamine (Sigma, UK) and added to HEK293 cells growing at 80% confluency in 6 well plates. After 24 hours incubation, the transfection mix was removed and replaced with DMEM medium supplemented with 2% FCS and incubated for 5-7 days. Cells were harvested and freeze/thawed three times before centrifuging at 2000 rpm for 15 min at 4°C to remove cellular debris. Glycerol was added at a final concentration of 10% and the transfection stock was then aliquoted and stored at -80°C or used for master seed stock infection.

2.1.16 Production of master seed stock of adenovirus in HEK293 cells

Five T175 flasks of HEK293 cells grown to 75% confluency were taken, medium removed and replaced with 2 ml of transfection stock adenovirus diluted in 10 ml total of DMEM/2% FCS. Flasks were incubated at 37°C for 5 days or until cell lysis was apparent. Cells and medium were then harvested and freeze/thawed twice before centrifugation at 2000 rpm for 15 min at 4°C. Clarified medium was aliquoted and stored at -80°C or used for seed stock infection.

2.1.17 Titration of adenovirus

Fibroblast HER911 cells were plated at a concentration of 1 x 10^4 cells/well in flat bottomed 96 well plates in 50 µl of DMEM/2% FCS/Pen/Strep. Log and half-log dilutions of virus (range from $10^{-4.5}$ to 10^{-12}) were prepared in DMEM/2% FCS. A total of 50 µl of each virus dilution was added to the wells; 7 wells were plated per dilution. Plates were incubated for 7 days at 37°C. After incubation, cell growth was visualised in each well using a light microscope set on low magnification. Wells in which cell lysis had occurred were scored. The titration of the virus measured in m.o.i. (multiplicity of infection) was calculated as half the TCID50 (dilution of virus at which there is a 50% probability of an infectious particle being present). The TCID50 value was calculated as Log TCID50 = Highest dilution giving 100% lysis formation + (difference in log between dilution * 0.5) – (difference in log between dilution) * (total number of wells with lysis formation (including last row in which cell lysis occurred in wells))/number of wells per row.

2.1.18 Production of seed stock of adenovirus in HEK293 cells

Twenty T175 flasks of HEK293 cells grown at 75% confluency were taken, medium was removed and replaced with master seed stock adenovirus at a m.o.i. of 0.01 diluted in 10 ml total of DMEM/2% FCS per flask. Flasks were incubated at 37°C for 5 days or until cell lysis was apparent. Cells and medium were then harvested and freeze/thawed twice before centrifugation at 2000 rpm for 15 min at 4°C. Clarified medium was aliquoted and stored at -80°C or used for batch stock infection.

2.1.19 Production of batch stock of adenovirus in HEK293 cells

Forty T175 flasks of HEK293 cells grown at 75% confluency were taken, medium removed and replaced with master seed stock adenovirus at a m.o.i. of 10 diluted in 10 ml total of DMEM/2% FCS per flask. Flasks were incubated at 37°C for 3 days or until cell lysis was apparent. Cells and medium were then harvested and freeze/thawed twice before centrifugation at 2000 rpm for 15 min at 4°C. Clarified medium was aliquoted and stored at -80°C or purified using caesium chloride density gradients.

2.1.20 Caesium chloride purification; step gradient

A solution of CsCl 1.4 (265 g CsCl + 4.35 ml 1 M Tris-HCl pH 7.9 + 430.65 ml H_2O) was layered in SW28 ultra-clear tubes (Beckman, UK) followed by a solution of CsCl 1.2 (134 g CsCl + 4.6 ml 1 M Tris-HCl pH 7.9 + 455.4 ml H_2O). The virus suspension was layered on top of the caesium chloride. A total of 6 tubes were centrifuged using a Beckman centrifuge rotor SW28 at 23 000 rpm for 90 min at 4°C, with the brake set at 0. After centrifugation, the concentrated band of intact virus was removed with an 18G syringe needle and virus was mixed with an equal volume of 10 mM Tris-HCl pH 7.9, ready to transfer onto the continuous gradient.

2.1.21 Caesium chloride purification; continuous gradient

Using a mixer gradient former (Bethesda Research Laboratories, USA), a gradient was poured using CsCl 1.4 solution and CsCl 1.2 solution. The virus Tris-HCl mix was then layered on top of the gradient. Tubes were centrifuged for 16-20 hrs at 23000 rpm at 4°C, with the brake set at 0. The concentrated band of virus was extracted using an 18G syringe needle. The virus stock was added to a dialysis cassette (Pierce, USA) and dialysed three times against a solution of 10 mM Tris-HCl pH 8.0, 2 mM $MgCl_2$, 5% sucrose. The virus was stored at -80°C.

Table of antibodies used

Antibody	Source	Type	Application	Dilution/amount
Mouse IgG	Sigma	Polyclonal	Cell activation	10 μg/ml
Mouse	Sigma	Polyclonal	Western blot	1 :100000
Rabbit	Sigma	Polyclonal	Western blot	1 :10000
DR	Becton Dickinson	Polyclonal	Cell separation	100 μl/2.5 x 10^7 cells
CD3 human (OKT3)	A.T.C.C.	Monoclonal	Cell activation	10 μg/ml
CD2-FITC	Becton Dickinson	Polyclonal	FACS	1 :1000
CD3 mouse	Pharmingen	Monoclonal	Cell activation	1 μg/ml
CD3-FITC	Becton Dickinson	Polyclonal	FACS	1 :1000
CD3-FITC	Becton Dickinson	Polyclonal	FACS	1 :1000
CD8	Becton Dickinson	Polyclonal	Cell separation	50 μl/2.5 x 10^7 cells
CD11a-FITC	Becton Dickinson	Polyclonal	FACS	1 :1000
CD14	Becton Dickinson	Polyclonal	Cell separation	100 μl/2.5 x 10^7 cells
CD18-FITC	Becton Dickinson	Polyclonal	FACS	1 :1000
CD20	Becton Dickinson	Polyclonal	Cell separation	100 μl/2.5 x 10^7 cells
CD25-PE	Becton Dickinson	Polyclonal	FACS	1 :1000
CD28 human	In house	Monoclonal	Cell activation	10 μg/ml
CD28 mouse	Pharmingen	Monoclonal	Cell activation	1 μg/ml
CD28-PE	Becton Dickinson	Polyclonal	FACS	1 :1000
CD56	Becton Dickinson	Polyclonal	Cell separation	100 μl/2.5 x 10^7 cells
CD62L-PE	Becton Dickinson	Polyclonal	FACS	1 :1000
CD69-FITC	Becton Dickinson	Polyclonal	FACS	1 :1000
CD152-PE	Becton Dickinson	Polyclonal	FACS	1 :1000
CD154-FITC	Becton Dickinson	Polyclonal	FACS	1 :1000
cmyc	Santa Cruz	Polyclonal	Western blot, IP	1 :500
LCPTP	In house	Polyclonal	Western blot, IP	1 :1000
Phospho-tyrosine	Upstate Biotech	Monoclonal	Western blot	1 :2000
Active MAPK	Promega	Polyclonal	Western blot	1 :1000
Phospho-LCK	Upstate Biotech	Polyclonal	Western blot	1 :1000
IL-4	R&D System	Polyclonal	Cell activation	5 μg/ml, 10 ng/ml
IL-12	R&D System	Polyclonal	Cell activation	5 μg/ml
IFN-γ	R&D System	Polyclonal	Cell activation	1 μg/ml
Fas-PE	Becton Dickinson	Polyclonal	FACS	1 :1000
FasL-FITC	Becton Dickinson	Polyclonal	FACS	1 :1000

2.1.22 Table of vectors/plasmids used

Vector	Insert	Tag	Constructed by
pcDNA3.1cmyc	-	cmyc	Dr. Janet Smith
pcDNA3.1 cmyc	LCPTP wt	cmyc	Nikol Heinrichs
pcDNA3.1 cmyc	LCPTP Trap	cmyc	Nikol Heinrichs
pcDNA3.1 cmyc	LCPTP Null	cmyc	Nikol Heinrichs
pMBP	LCPTP wt	Maltose	Dr Angela Boyhan
pGEX	-	GST	Pharmacia
pGEX	LCPTP wt	GST	Dr Angela Boyhan
pGEX	LCPTP Trap	GST	Dr Angela Boyhan
pGFP	-	GFP	Clontech
pGFP	LCPTP wt	GFP	Isabelle Brodeur
pGFP	LCPTP Trap	GFP	Isabelle Brodeur
pGFP	LCPTP Null	GFP	Isabelle Brodeur
pCR259	-	-	Cynthia Richard
pCR259	LCPTP wt	-	Isabelle Brodeur
pCR259	LCPTP Trap	-	Isabelle Brodeur
pCR259	LCPTP anti-sense	-	Isabelle Brodeur
pCR249	-	-	Cynthia Richard
pCR249	LCPTP wt	-	Isabelle Brodeur
pCR249	LCPTP Trap	-	Isabelle Brodeur
pCR249	LCPTP anti-sense	-	Isabelle Brodeur
pCR249	Gata3	-	Dr Nigel Sharp

2.2 Protein chemistry techniques

2.2.1 Isolation of GST-fused protein on beads

E. coli DH5α containing the plasmid GST-LCPTP wild-type or substrate-trap (kindly given by Dr Angela Boyhan, GlaxoSmithKline, UK) was inoculated in 5 ml LB broth containing ampicillin at 100 ng/ml for 18 hours. The 5 ml culture was inoculated into 500 ml of LB/Ampicillin broth and incubated at 37°C with aeration until an OD_{600} = 0.75 was obtained. GST-LCPTP production was induced by the addition of 0.1 mM IPTG for 4 hours at 30°C. After growth expansion, cells were harvested by

centrifugation at 7500 rpm for 7 min at 4°C (Sorvall, SS-34). Pellets were resuspended in buffer A (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1mM DTT, 1 mM PMSF). Cells were sonicated on ice and centrifuged at 15 000 rpm for 1 hour at 4°C (Sorvall, SS-34). Supernatant was mixed end-over-end with Glutathione Sepharose 4B beads (Amersham Pharmacia Biotech, UK). Beads were recovered by centrifugation at 3000 rpm for 5 min at 4°C and washed with buffer A. Beads coupled with GST-LCPTP fusion proteins were stored at 4°C in PBS/0.05% sodium azide. In order to generate polyclonal antisera against LCPTP, recombinant GST-LCPTP protein was eluted from the beads using 5 mM glutathione in buffer A pH 8.0.

2.2.2 Isolation of MBP-LCPTP beads

E. coli transformed with plasmid containing the MBP-LCPTP gene (kindly provided by Dr Angela Boyhan, GlaxoSmithKline, UK) were induced with 0.1 mM IPTG as described in section 2.3.1. Cells were lysed by sonication (section 2.3.1) and cell lysate was incubated with amylose resin (Amersham Pharmacia Biotech, UK) packed in a 10 ml Bio-Rad column. The column was washed with buffer A to remove unbound material and MBP-LCPTP protein was eluted from the beads with 10 mM maltose in buffer A. Beads were stored at 4°C in 10mM Tris-HCL pH 7.6/0.05% sodium azide.

2.2.3 Purification of LCPTP antibody

Serum from rabbits immunised with GST-LCPTP protein was diluted 1:5 with 10 mM Tris-HCL pH 7.6 and loaded onto a column containing 1.4 ml MBP-LCPTP-amylose resin (New England BioLab, UK) at a flow rate of 1 ml/min. The column was washed with 10 mM Tris-HCl pH 7.6 to remove unbound material, and the antibody was eluted with 100 mM Glycine pH 2.5 and collected into fractions. Fractions containing antibody were neutralised with 1/10 volume 1 M Tris-HCl pH 8. Antibody was concentrated using a Centricon column (Amicon, USA), following the manufacturer's instructions.

2.2.4 Coupling LCPTP Ab to Aminolink gel

Aminolink coupling gel (Pierce, USA) was washed in PBS and purified antibody was added at a concentration of 1 mg antibody/0.5 ml gel. 5 M sodium borohydrate was added and the gel and antibody were mixed end-over-end for 18 hours, at 4 °C. The antibody coupled beads were isolated from the supernatant by centrifugation (13 000 rpm, 30 sec). The concentration of antibody bound to the beads was determined by measuring the absorbance of the protein remaining in the supernatant at 280 nM using a Pharmacia Ultrospec 2000 spectrophotometer. The amount of antibody bound = [solution Ab start] – [solution Ab end])/[solution Ab start]. Beads were washed in Tris-HCl pH 7.4 to block any remaining free amino groups and mixed for a further 30 min. Finally, the beads were washed with 1 M NaCl and stored in PBS/ 0.05% sodium azide.

2.2.5 Evaluation of antibody concentration

The absorbance of antibody solutions at 280 nm was measured using a Pharmacia Ultrospec 2000 spectrophotometer. Antibody concentration was calculated using the equation: OD_{280nm}= 1.4 : 1mg Ab/ml.

2.2.6 Evaluation of protein concentration (Bradford)

The Bradford assay ((Bradford, 1976)) was used to determine the concentration of the protein solution of interest. Samples were diluted to a volume of 0.8 ml in H_2O and mixed with 0.2 ml of protein assay dye reagent concentrate (Bio-Rad, UK). The colour was allowed to develop for 5 min at 20°C. The absorbance at 595 nm was measured, using a Pharmacia Ultrospec 2000 spectrophotometer, and unknown protein concentrations read against a standard curve obtained with known concentrations of BSA.

2.2.7 Cell activation

To activate the Jurkat cells for immunoprecipitations or Western blotting, cells were resuspended in 30 mM Hepes-buffered RPMI and prewarmed at 37°C for 10 min. Anti-Mouse IgG (Sigma, UK) was added at a final concentration of 10 μg/ml and incubated with the cells for 2 min (time 0). The anti-CD3 antibody (Clone OKT3, A.T.C.C.) was added at a final concentration of 10 μg/ml and reactions stopped at

suitable intervals by addition of SDS-sample loading buffer [125 mM Tris pH 6.8, 4 % (w/v) SDS, 20 % (w/v) glycerol, 0.2 M DTT, 0.013 % bromophenol blue (Sigma, UK)]. For immunoprecipitations, samples were snap frozen in liquid nitrogen instead of adding SDS-sample loading buffer.

2.2.8 Immunoprecipitations

Jurkat T cells (1 x 10^8) were activated as described above for 1 min in the presence of anti-CD3. Cells were snap frozen in liquid nitrogen and then lysed in 1 % n-octyl glucoside, 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM iodacetamide, 10 mM sodium orthovanadate, 2 mM EDTA, 0.076 TIU/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin and 10 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) (Sigma, UK). The lysate was separated from insoluble material by centrifugation for 5 min at 13000 rpm. LCPTP protein was immunoprecipitated using 3 μg anti-LCPTP antibody coupled to Aminolink Plus Coupling Gel (Pierce, UK) per sample. Following a 1 hour incubation at room temperature with constant mixing, the immunoprecipitates were washed with lysis buffer five times. Bound proteins were eluted by heating the Coupling gel in the presence of SDS-sample loading buffer for 5 min at 100°C. To control for nonspecific binding, lysate was incubated with Coupling gel in the absence of antibody. The MAPK members (ERK1, ERK2 and p38) were immunoprecipitated by mixing 3 μg of monoclonal antibody to ERK1 (Affinity Research Products, UK), ERK2 (Upstate Biotechnology, USA) or p38 (Upstate Biotechnology, USA)) with 40 μl 10% suspension of Protein A Sepharose (Sigma, UK) added to the cell lysate. Protein complexes were separated by 4-20% gradient SDS PAGE and proteins identified by Western blotting (See section 2.3.9).

2.2.9 Total cell lysates

Jurkat T cells or LCPTP stable cell lines (1 x 10^6) were taken and snap frozen in liquid nitrogen and then lysed in 1 % n-octyl glucoside, 10 mM Tris pH 8.0, 50 mM NaCl, 10 mM iodacetamide, 10 mM sodium orthovanadate, 2 mM EDTA, 0.076 TIU/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin and 10 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) (Sigma, UK). The lysate was separated from insoluble material by centrifugation for 5 min at 13 000 rpm. The

lysate was mixed with SDS sample loading buffer and heated for 5 min at 100°C. Samples were separated on a 4-20 % gradient SDS-PAGE (Novex-Pharmacia, UK).

2.2.10 GST pull-downs

Pull-downs with recombinant proteins were done by mixing either 3 μg of the GST fused protein (LCPTP wild-type or substrate-trap (C291S, D257A)) or the GST protein alone, coupled to Glutathione Sepharose 4B beads with the cell lysate. The pull-downs were performed and processed as described for the immunoprecipitations.

2.2.11 Western blots

Proteins were transferred from gels to PVDF (Millipore, UK) membrane using a semi-dry blotter (Amcos, Netherlands). Gels and 3MM filter paper were soaked in transfer buffer (5.8 g Tris, 2.95 g Glycine, 3.7 ml SDS 10%, 200 ml methanol), PVDF membrane was activated by immersion in methanol. To blot for cmyc or LCPTP, membranes were blocked in 3 % milk in PBS (Sigma, UK) and probed with cmyc rabbit polyclonal antibody (Santa Cruz, USA) or LCPTP rabbit polyclonal antibody (GlaxoSmithKline, UK), followed by goat anti-rabbit horseradish peroxidase-conjugated antibody (Sigma, UK) and then exposure to enhanced chemiluminescence (ECL) reagents (Amersham, UK). Phosphotyrosine proteins were detected using the 4G10 mouse mAb (Upstate Biotechnology, USA) followed by goat anti-mouse horseradish peroxidase-conjugated antibody (Sigma, UK). Active MAPK proteins were detected using the rabbit polyclonal anti-active MAPK antibody (Promega, UK) followed by goat anti-rabbit horseradish peroxidase-conjugated antibody (Sigma, UK) using I-Block buffer (0.2% I-Block (Tropix, USA), 0.1% Tween-20 (Sigma, UK) in PBS). Phosphorylated-Lck proteins were detected using the rabbit polyclonal anti-phospho-Lck antibody (Upstate Biotechnology, USA) followed by goat anti-rabbit horseradish peroxidase-conjugated antibody (Sigma, UK) using 3% milk in PBS. To verify that equal amounts of protein were loaded per track, gels were stained with Coomassie blue (0.1% Coomassie blue (R-250 Fluka, UK), 45% methanol, 45% acetic acid) and then destained in 20% methanol, 7% acetic acid solution. Semi-quantitation of differences in band intensities weas measured by densitometry using a Bio-Rad GS-710 calibrated imaging densitometer (Bio-Rad, UK).

2.2.12 Protein Sequencing

Immunoprecipitations using 1 x 10^9 cells were performed as described in section 2.3.7 with an additional step. A preclean step was performed: aminolink coupling gel alone, not bound to any antibody was mixed with the cell lysate for an hour. For 1 x 10^9 cells, approximately 15 μg of antibody was used for the immunoprecipitation. Proteins were separated on a 4-20% gradient SDS-PAGE (Novex, UK) and stained with Colloidal Blue (Novex, UK) following the manufacturer's instructions. Sequencing was carried out by the Cell Map Incubator Project group (GlaxoSmithKline, UK) with equipment using tandem on line mass spectrometry techniques (Bio-Rad, UK).

2.2.13 Intracellular cAMP measurement

1 x 10^5 cells were stimulated in the presence, or absence of 0.05 mM forskolin for 7 minutes (following (Kvanta et al., 1990) material and methods). After each treatment, cells were incubated for 2 minutes with anti-mouse IgG (10μg/ml final concentration) followed by the addition of anti-CD3 (10μg/ml final concentration) and incubated for 1 minute at 37°C. cAMP measurements were done using a cAMP enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech, UK) following the manufacturer's instructions.

2.3 Cell biology

2.3.1 Cell lines

The Jurkat T E6-1 cell line (A.T.C.C.) was maintained in RPMI 1640 medium (Life Technologies, UK) supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technologies, UK). Stable cell lines containing cmyc-LCPTP were maintained in the same medium supplemented with 1 mg/ml geneticin (Sigma, UK). Fibroblast lines HEK293 and HER911 (A.T.C.C.) were maintained in DMEM medium (Life Technologies, UK) supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technologies, UK).

2.3.2 Cell count

Viable cell counts were obtained using Trypan blue (Gibco-BRL, UK) exclusion to discriminate between live and dead cells. Cells were counted using a hematocytometer.

2.3.3 Transient transfections

2 x 10^7 Jurkat cells were resuspended in 0.3 ml RPMI 1640 medium (Life Technologies, UK), supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies, UK), in 0.4 cm electrocuvette (Bio-Rad, UK) and incubated with 30 μg of vector pcDNA 3.1 (Invitrogen, UK) containing cmyc tag or plasmids pcDNA 3.1 cmyc-LCPTP WT or pcDNA 3.1 cmyc-LCPTP C291S-D257A for 5 min. Cells were electroporated at 260 V, 960 μF, ∞ Ohms (Bio-Rad Gene Pulser, UK), washed, resuspended in complete RPMI medium and incubated at 37°C. 48 hours post-transfection, LCPTP immunoprecipitations were carried out.

2.3.4 Establishment of stable cell lines

The same protocol as that used for the transient transfections (section 2.3.3) was followed with the exception that 48 hours post-transfection, the medium was replaced with selective medium (RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 μg/ml gentamycin (Sigma, UK). Cells surviving gentamicin selection were cloned by limiting dilution in round bottomed 96 well plates (Costar, UK).

2.4 Cellular Immunology

2.4.1 Preparation of human $CD4^+$ cells

Defibrinated blood was obtained from healthy human volunteers and PBMC (peripheral blood mononuclear cells) were isolated by density centrifugation using Lymphoprep (Life Technologies, Scotland). $CD4^+$ T cells were prepared by immunomagnetic negative selection as follows. Cells were incubated with saturating concentrations of anti-CD14, anti-DR, anti-CD20, anti-CD8 and anti-CD56 antibodies (Becton Dickinson, UK) for 30 min at room temperature. Cells were washed twice and sheep anti-mouse IgG and anti-human CD8-coated Dynal beads (Dynal, Norway)

were added, mixed and incubated with cells for 15 min at room temperature. Cells were then incubated with a Dynal magnet and unbound cells harvested and counted. The $CD4^+$ were expanded by stimulation with anti-CD3 (clone OKT3, A.T.C.C.) and anti-CD28 (clone 9.3, provided by Neil Burden, GlaxoSmithKline, UK) coated on plates at (1 ug/ml and 10 ug/ml respectively) and cultured in medium RPMI 1640 (Life Technologies, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technologies, UK), containing 10 ng/ml purified recombinant IL-2 (GSK, UK).

2.4.2 Preparation of Th1 and Th2 $CD4^+$ subsets

Studies using murine T cells were done from cells isolated from the CAR-1 transgenic mouse strain which expresses the CAR-1 receptor on all cells, kindly provided by Dr James De Gregori (Leon et al., 1998). The CAR-1 transgenic mice have been bred with DO11.10 mice to produce the double transgenic CAR-1-DO11.10 mice. DO11.10 mice are T cell receptor transgenic having a TCR specific to ovalbumin (amino acids 323-339) (Murphy et al., 1990). Naïve $CD4^+$ T cells were purified using mouse $CD4^+$ subset small enrichment columns (R & D Systems, UK) following the manufacturer's instructions. Cells were subsequently differentiated into Th1 or Th2 type cells by culturing the cells in the presence of IA^d and ICAM transfected human fibroblasts, which express endogenous B7.1 and B7.2. Ovalbumin peptide corresponding to amino acid residues 323-339 was added at 1 μM final concentration. For Th1 cells, the medium was supplemented with anti-IL4 antibody at 5 μg/ml and IL-12 at 10 ng/ml final concentration. For Th2 cells, the medium was supplemented with anti-IL-12 antibody at 5 μg/ml, anti-IFNγ at 1 μg/ml and IL-4 at 10 ng/ml final concentration (R & D Systems, UK). The medium for both subsets was supplemented with IL-2 at a final concentration of 20 ng/ml (human recombinant IL-2, GlaxoSmithKline, UK). Cells were harvested at day 6 for infection with adenovirus constructs.

2.4.3 Naïve, Th1 and Th2 murine cell infection with adenovirus

Cells were washed in serum free DMEM and resuspended at 1 x 10^7 cells/ml in serum free DMEM. Cells were aliquoted in 15 ml falcon tubes in 0.1 ml volumes and 0.1 ml virus diluted in DMEM was added to give a m.o.i. of 10. Cells were mixed with virus

by pipetting and incubated at 37°C for 1-2 hours. Cells were washed and centrifuged at 1200 rpm for 5 min at 20°C. Cells were resuspended at a concentration of 1 x 10^6 cells/ml in complete culture medium (RPMI 1640, L-Glu/Pen/Strep/FCS/2-β-mercaptoethanol) containing 10 μg/ml IL-2 and plated in 24 well plates. Cells were incubated for 24 hours, washed and counted before using in experiments.

2.4.4 ELISA – Measurement of human IL-2

Jurkat T cells were activated in flat bottomed 96 well plates coated with 1μg/ml of mouse monoclonal anti-CD3 antibody (clone OKT3 A.T.C.C.) and 3 μg/ml of mouse monoclonal anti-CD28 antibody (clone 9.3, GlaxoSmithKline, UK). Cells were added at 5 x 10^5 cells per well in 200 μl volume, in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin. Supernatants from triplicate wells were taken at 24 hours and pooled. IL-2 production was measured by ELISA. ELISA plates (Nunc, UK) were coated overnight at 4°C, with anti-human IL-2 (Pharmingen, UK) at 2 μg/ml, diluted in PBS. Plates were subsequently blocked with 2% BSA (A-7030, Sigma, UK) diluted in PBS. Dilutions of recombinant human IL-2 standard (GlaxoSmithKline, UK) were added in triplicate and culture supernatants were added to the wells in duplicate. After incubation, plates were washed and anti-human IL-2 biotinylated antibody (Pharmingen, UK) was added at 1 μg/ml per well, diluted in blocking buffer. After incubation, wells were washed and HRP-conjugated streptavidin (Caltag, UK) was added at a dilution of 1:2000, in blocking buffer. Plates were incubated, washed and 3,3',5,5'-tetramethylbenzidine (TMB) substrate Sigma, UK) was added to wells. Reactions were stopped with 0.2 M sulphuric acid (BDH, UK) and read at absorbance 450nm using a Spectramax ELISA plate reader with Softmax Pro 3.2.1 software (Molecular Devices, UK).

2.4.5 ELISA – Measurement of murine IL-2, IL-4, IL-5, IL-13 and IFN-γ

Mouse IL-2, IL-4, IL-5 (Pharmingen, UK), IL-13 (R&D, UK) and IFN-γ (Pharmingen, UK) were measured from culture supernatants using paired capture and detection antibodies and recombinant protein standard following the manufacturer's instructions.

2.4.6 Human T cell proliferation assay

Flat bottomed 96 well plates were coated with 0.3 μg/ml of mouse monoclonal anti-CD3 antibody (clone OKT3) and 1 μg/ml of mouse monoclonal anti-CD28 antibody (clone 9.3). Cells were added at 2 x 10^4 cells per well in 200 μl volume, in RPMI 1640 supplemented with 10% heat inactivated foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin. Plates were incubated for 24, 48, 72 and 96 hours at 37°C, 5% CO_2. 1 μCi/well ^3H thymidine (Amersham, UK) was added for the final 4 hours of culture. Plates were harvested on printed filtermats A (Wallac, Finland) using a semi-automatic Skatron cell harvester (Wallac, Finland). Filters were read using a Betaplate liquid scintillation counter (Wallac, Finland) and data generated in counts per minute (cpm). The mean value for triplicate wells was calculated. Results are expressed as a percentage of the non activated control sample (100%). For one set of experiments, IL-2 was added to the medium at a final concentration of 100 ng/ml.

2.4.7 Mouse proliferation assay

Proliferation was performed as described in section 2.4.6 with the following modifications: activating antibody: 1 μg/ml hamster anti-mouse CD3 (clone 2C11, Pharmingen, UK), 1 μg/ml hamster anti-mouse CD28 (Pharmingen, UK), 1 x 10^5 cells per well.

2.4.8 Extracellular marker staining

Jurkat T cells were activated in 24 well plates coated with 1 μg/ml of mouse monoclonal anti-CD3 antibody (clone OKT3) and 3 μg/ml of mouse monoclonal anti-CD28 antibody (clone 9.3). Cells were added at 5 x 10^5 cells per well in 2 ml volume, in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin. Cells were recovered at 72 hrs and washed in FACS buffer (2.5% fetal calf serum, 0.05% sodium azide in PBS). Anti-CD3, anti-CD28, anti-CD69 and anti-CD25 antibodies conjugated to FITC or PE (Becton Dickinson, UK) were added to the cells and incubated at 4°C for 30 min. Cells were washed and resuspended in FACS buffer. Data was acquired using a Becton-Dickinson FacScan with WinScan software and analysed using Expo32 software.

2.4.9 Intracellular cytokine staining

2 x 10^6 CAR-1 Th1 or Th2 cells were gently pelleted with 0.5 x 10^6 DAP3.ICAM APCs expressing IAd'B7.1 and B7.2 and 10 μM ovalbumin peptide and incubated for 2 hours at 37°C. 10 μg/ml 2 μM Monensin was added and cells incubated for a further 2 hours at 37°C. Cells were centrifuged at 1200 rpm for 5 minutes and washed in PBS/2.5% FCS/0.5% sodium azide. Cells were washed and divided into 5 x 10^5 aliquots and then fixed with 100 μl Fixation reagent (Caltag, UK) for 15 minutes at room temperature. Cells were washed in FACS buffer and permeabilised with 100 μl Permeabilisation reagent (Caltag, UK) added together with the appropriate PE or FITC labelled anti-cytokine antibody (Pharmingen, UK). Staining for IL-2, IL-4 and IFN-γ was used to assess the polarisation of cells to a Th1 or a Th2 phenotype. Cells were incubated with antibody for 15 minutes at room temperature, washed and resuspended in FACS buffer. Data was acquired using a Becton-Dickinson FacScan with WinScan software and analysed using Expo32 software.

2.4.10 Photomicrography of cells

Jurkat T cells were cultured in 24 well plates and photographs were taken at days 1 and 3 following the addition of different stimuli. Cells were visualised using a Nikon Eclipse TS100 microscope (Nikon, UK) with 4x or 10x magnification. Photographs were taken using a Digital Camera Coolpix995 (Nikon, UK). Stable cell lines were stimulated with anti-CD28 (clone 9.3) added either in solution or coated onto plates at a final concentration of 10 μg/ml; anti-CD18 (GlaxoSmithKline, UK) was added in solution at a final concentration of 10 μg/ml; EDTA (Sigma, UK) was added in solution at a final concentration of 4mM.

CHAPTER THREE: RESULTS
PROTEIN INTERACTIONS OF LCPTP IN JURKAT T CELLS AND HUMAN PERIPHERAL CD4$^+$ T CELLS

3.1 Introduction

Previous studies looking at the role of LCPTP in cell signalling have all used systems in which recombinant LCPTP was overexpressed at levels much higher than those found normally in cells (Oh-hora et al., 1999); (Saxena et al., 1999a). This approach does have limitations as seen in the study of Bailyes, in which recombinant prohormone convertase PC2 and the endogenous rat hormone have the same substrate specificity but their pH and calcium sensitivities are different (Bailyes et al., 1995). Also, if a protein is overexpressed in a cell, it is possible that it could form associations which are not physiologically relevant. For example, the cytoplasmic protein, hsp70 which when overexpressed in cells, goes into the nucleus and interacts with a nuclear protein (Personal communication, Dr. Jean-Yves Masson).

LCPTP has been shown in overexpression systems to constitutively form associations with members of the MAP kinase family (Oh-hora et al., 1999); (Saxena et al., 1999a). In order to address the physiological significance of the interaction reported with overexpressed LCPTP protein, experiments presented in this chapter examined protein associations of endogenous LCPTP, rather than transfected recombinant protein.

MAP kinase family members are found widely distributed in different cell types (Keyse, 1998); (Roovers and Assoian, 2000); (Chang and Karin, 2001) including T cells (Keyse, 1998); (Saxena and Mustelin, 2000); (Rincon et al., 2000). However, the distribution of LCPTP appears to be restricted to cells of hematopoietic origin (Zanke et al., 1992); (Adachi et al., 1992b). The Jurkat T cell line was thus chosen as a suitable model system in which to study the interactions of endogenous LCPTP in cells stimulated via the T cell receptor. Jurkats were used in the 1980s to produce and purify IL-2 (Smith, 1988). Since then, Jurkat cell lines, have been used extensively as a model system for studying TCR signal transduction (Landegren et al., 1985); (Astoul et al., 2001). Consequently, the function of many molecules has been characterised, including protein tyrosine kinases, protein tyrosine phosphatases and

adapter proteins using Jurkats (Peterson et al., 1998); (van Leeuwe and Samelson, 1999)(Lin and Weiss, 2001). The use of Jurkat T cell lines as a good model system for examining T cell signalling pathways has been justified by studies in primary T cells where similar functions were attributed for most of the molecules looked at (Astoul et al., 2001).

The Jurkat T cell line available in the laboratory, namely JE6 (American Type Culture Collection), expresses high levels of endogenous LCPTP. In addition, the JE6 Jurkat T cell line expresses a functional T cell receptor and the costimulatory molecule, CD28. Previous studies looking at the role of LCPTP in signalling pathways have used pervanadate to induce phosphorylation of multiple cell proteins, via inactivation of endogenous phosphatases (Pettiford and Herbst, 2000). In order to use a more physiologically relevant stimulus and also in order to allow study of interactions with endogenous LCPTP without interference by pervanadate, which modifies irreversibly the catalytic sulfhydryl group at the active site on the phosphatase (Huyer et al., 1997), cells were activated via antibody to the TCR CD3 molecule.

To date, reported substrates of LCPTP include ERK2 and p38 (Oh-hora et al., 1999); (Saxena et al., 1999a); (Pettiford and Herbst, 2000). Therefore, in attempts to further elucidate the role of LCPTP in T cell signalling, experiments were designed to identify other potential substrates of LCPTP. These experiments made use of a recombinant LCPTP mutant protein which can bind irreversibly to its substrate (Flint et al., 1997) and thus enable isolation of LCPTP with potential substrates and their subsequent identification using Western blotting or protein sequencing techniques.

Jurkat T cells, as mentioned, provide a good system for examining signalling pathways, but nonetheless, they represent a transformed cell line. This means that any conclusions drawn must be interpreted with a cautionary note regarding the relevance of what happens in Jurkat cells compared to primary T cells. Experiments performed in Jurkat T cells were repeated in $CD4^+$ T cells derived from peripheral blood in order to further validate the results obtained in a more physiologically relevant cell type.

3.2. Results

3.2.1 Time course to determine the point of maximal tyrosine phosphorylation in Jurkat T cells

In order to start looking at interactions of LCPTP with other proteins, studies were carried out to select the optimal conditions for Jurkat T cell activation, as measured by an increase in tyrosine phosphorylation in response to TCR ligation. Maximal substrate phosphorylation is desirable with the view to maximise the amount of substrate available for the phosphatase to bind to its substrate. Different concentrations of crosslinking anti-mouse IgG and mouse anti-CD3 (OKT3) antibodies were tested. Anti-CD3 binds to the TCR associated molecule CD3 and is in turn bound by the anti-mouse IgG causing TCR clustering which has been shown to be necessary to drive T cell signalling pathways leading to transcription of IL-2 (Lanzavecchia, 1997). This is illustrated in Figure 3.1.

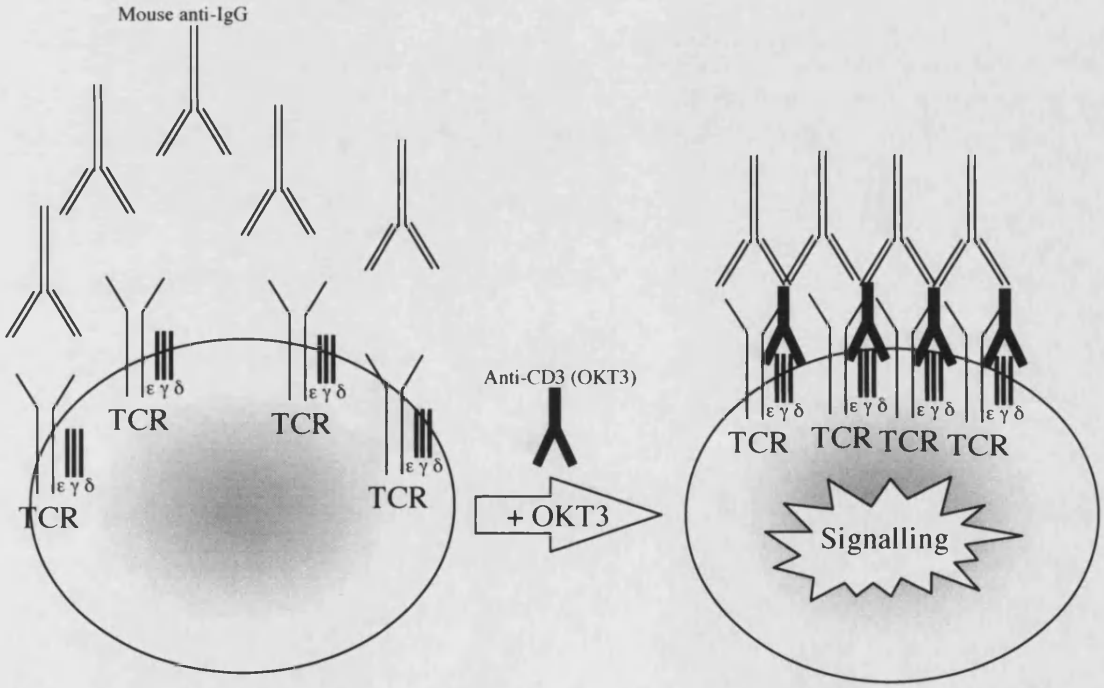


Figure 3.1: TCR clustering initiated by anti-CD3 antibody binding and secondary crosslinking with anti-mouse IgG.

The anti-CD3 antibody binds to the extracellular domain of CD3 molecules. Anti-mouse IgG binds to the mouse anti-CD3 antibody and effectively crosslinks the primary antibody, leading to TCR aggregation. This mimics the binding of peptide presented by an MHC class II molecule on an APC binding to the TCR, the clustering

of TCRs at the point of contact with an APC and the activation of TCR associated kinases, initiating TCR signalling pathways.

Different concentrations of anti-mouse IgG ranging from 1, 3 10, 30 μg/ml and the same concentrations for anti-CD3 (1, 3 10, 30 μg/ml) were tested for their ability to give maximal protein tyrosine phosphorylation as determined by SDS-PAGE and Western blotting using an anti-phosphotyrosine monoclonal antibody (Clone 4G10). An optimal concentration of 10 μg/ml for both antibodies was used in all following signalling experiments (Data not shown). In order to determine the optimal time of activation to achieve maximal tyrosine phosphorylation, a time course was done. The time course shows a rapid rise in protein tyrosine phosphorylation peaking at 1 min (Figure 3.2A, lanes 1 to 5) and a gradual reduction of tyrosine phosphorylation after this time point (Figure 3.2A, lanes 5 to 10). To demonstrate equal protein loading, the gel was stained with Coomassie blue (Figure 3.2B). Resting and cells activated maximally for 1 minute (corresponding to the peak of protein phosphorylation) were used for subsequent immunoprecipitation experiments.

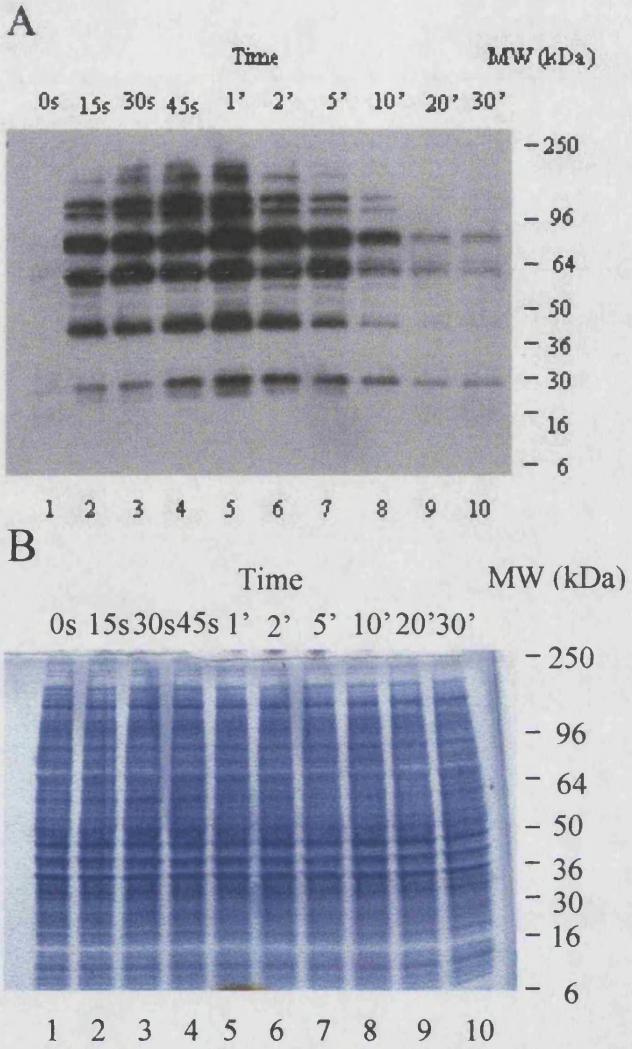


Figure 3.2. Phosphotyrosine blot of time course of anti-CD3 antibody stimulated Jurkat T cells.

A. Phosphotyrosine blot of cell lysates at different times. B. Coomassie blue staining showing equivalent protein loading. Lanes 1 to 10 represent activation times of 0, 0.25, 0.5, 0.75, 1, 2, 5, 10, 20 and 30 minutes at 37°C, respectively. Each lane is the equivalent of 1 x 10^5 cells. The data shown are representative of 4 separate experiments.

3.2.3 Endogenous LCPTP immunoprecipitations in Jurkat T cells

It has been reported in the literature, that overexpressed LCPTP binds constitutively to MAP kinases (Oh-hora et al., 1999); (Saxena et al., 1999a). In order to determine whether the MAP kinases could be shown to constitutively associate with endogenous LCPTP, Jurkat T cells were activated for 1 minute with anti-CD3

antibody, cells were lysed and LCPTP complexes were isolated using a rabbit polyclonal anti-LCPTP antibody. Following protein separation by SDS-PAGE, Western blots were performed and probed with antibodies raised against ERK1, ERK2 and p38. As shown in Figure 3.3A, ERK1 was not detected in LCPTP immunoprecipitates (lanes 1 and 2), but was seen in the total cell lysate control (lane 6), containing 100 times less total protein than the IPs. A similar pattern was seen when LCPTP IPs were probed for ERK2 protein and p38 (Figure 3.3B and 3.3C, respectively, lane 6). Some p38 was detected binding non-specifically to coupling gel (Figure 3.3C, lane 4). In order to confirm the presence of LCPTP in the immunoprecipitates, membranes were stripped and reprobed for the presence of LCPTP. Figure 3.3D shows one representative example. LCPTP was brought down by the coupled antibody and the amount was enriched in the immunoprecipitation compared to the total lysate (Figure 3.3D, lanes 1 and 2 and 6); LCPTP did not interact non-specifically with the coupling gel (lanes 3 and 4) and could be detected in the cell lysate (lane 6).

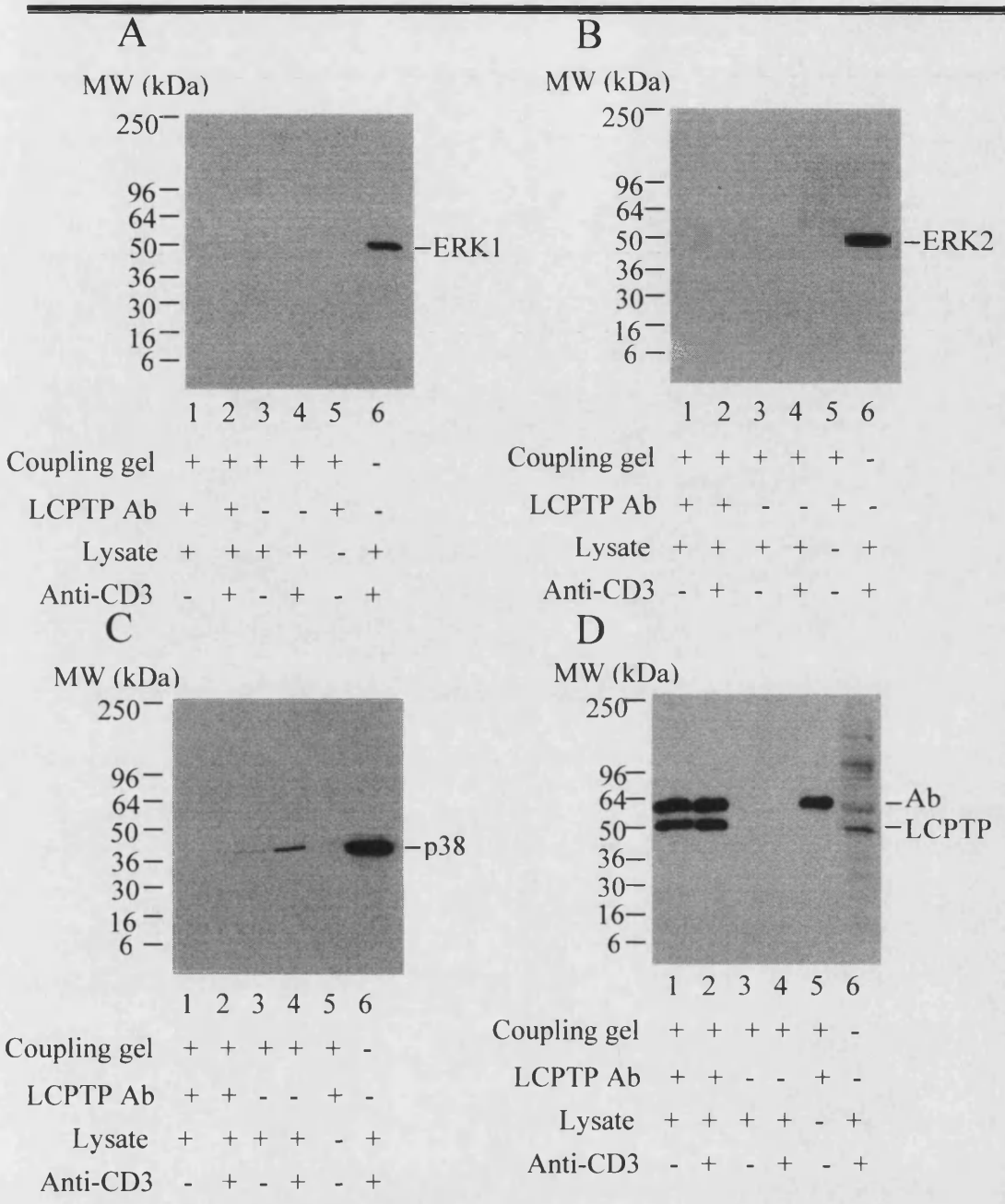


Figure 3.3. LCPTP immunoprecipitates from Jurkat T cells do not contain ERK1, ERK2 or p38.

LCPTP antibody immunoprecipitated complexes from Jurkat T cells were blotted for A. ERK1; B. ERK2; C. p38; D. LCPTP. Cell equivalents corresponding to 1 x 10^8 cells for Jurkat T cells were immunoprecipitated with rabbit antisera raised against LCPTP protein from either unstimulated or anti-CD3 antibody-stimulated Jurkat T cells. Figures A, B, C, D: lane 1- LCPTP immunoprecipitations non-activated. Lane 2- LCPTP immunoprecipitations activated 1 min with anti-CD3 antibody. Lanes 3, 4

and 5 represent controls for non-specific binding and lane 6, soluble cell lysate equivalent to 1 x 10^6 cells.

3.2.4 Microsequencing of LCPTP IPs in Jurkat T cells

The presence of LCPTP in immunoprecipitations was confirmed by microsequencing using tandem on line mass spectrometry. LCPTP immunoprecipitations were done using 1 x 10^9 cells per IP and proteins separated by 4-20% gradient SDS-PAGE. Only one band was visualised which was specific for LCPTP IPs, with or without anti-CD3 activation (Figure 3.4A, lanes 1 and 2) and this band was excised and processed for mass spectrometry protein sequencing. Results obtained confirmed the presence of LCPTP (Table 3.1A and B) and demonstrated the specificity of the rabbit polyclonal antisera used for immunoprecipitations. A number of peptides from B-amyloid precursor protein (Table 3.1B) were also present in the band excised. This was due to contamination during the preparation of the sample by the Cell Map Incubator Project Group (Personal communication, Malcolm Ward). No peptides corresponding to MAP kinase family members were detected even though ERK1 migrates on SDS-PAGE at the same molecular weight as LCPTP. A number of other proteins identified in the excised band were deemed to be nonspecific contaminants (see Table 3.1 A and B), as these proteins are commonly seen in other non related samples analysed by the Cell Map Incubator (Cell Map Incubator, personal communication).

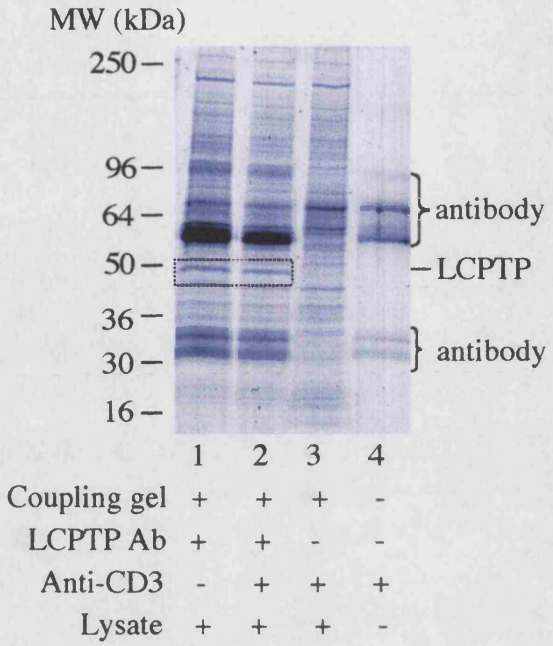


Figure 3.4: Microsequencing of band in LCPTP IPs.

Colloidal blue staining of LCPTP immunoprecipitations using 1 x 10^9 cells/IP. Cell equivalents corresponding to 1 x 10^9 cells were immunoprecipitated with rabbit antisera raised against GST-LCPTP fusion protein. Lane 1- LCPTP immunoprecipitations non-activated. Lane 2- LCPTP immunoprecipitations activated 1 min with anti-CD3 antibody. Lane 3 represents control for non specific binding and lane 4 is soluble cell lysate equivalent to 1 x 10^6 cells. The data shown are representative of two separate experiments.

Sample 1: LCPTP IP non activated	
Protein identification	Peptides matches
Human keratin, type II cytoskeletal 1	34
Human keratin, type I cytoskeletal 9	19
Protein-tyrosine phosphatase LCPTP	**19**
Bovin trypsinogen, cationic precursor	44
Human keratin, type I cytoskeletal 10	8
AS1-casein	2

Sample 2: LCPTP IP activated 1 min with anti-CD3	
Protein identification	Peptides matches
Amyloid B-protein precursor	43
Protein-tyrosine phosphatase LCPTP	**18**
Bovin trypsinogen cationic precursor	47
Human PTB-associated splicing factor	6
Human keratin, type II cytoskeletal I	4
IG kappa chain I (fragment)	1
KIAA1482 protein (fragment)	1
Signal peptidase I	1

Table 3.1: Identification of proteins by mass spectrometry sequence analysis.

Bands excised from the gel, were trypsinised and then applied to tandem on line mass spectrometry analysis. Proteins were identified by sequence homology using GenBank database. Microsequencing of non-activated sample (sample 1) and Microsequencing of activated sample with anti-CD3 (sample 2).

3.2.5 Endogenous MAPK immunoprecipitations in Jurkat T cells

One possible explanation for the inability to demonstrate any constitutive interaction between endogenous LCPTP and members of the MAP kinase family was that the rabbit polyclonal anti-LCPTP antibody used for immunoprecipitations was interfering with LCPTP-MAPK interactions. Therefore, reverse

immunoprecipitations were carried out using antibodies to the different members of the MAP kinase family to immunoprecipitate putative MAPK-LCPTP complexes. LCPTP was not detected in MAPK immunoprecipitations (Figure 3.5A, lane 1-6), or control precipitations (Figure 3.5A, lane 7 and 8), but was seen in the total cell lysate (Figure 3.5A, lane 10), containing 100 times less protein than the IPs. To confirm the presence of appropriate kinases in the immunoprecipitations, the membranes were stripped and reprobed for the presence of ERK1, ERK2 and p38 protein. Figure 3.5B shows the presence of each MAPK family member in the appropriate immunoprecipitation and in the total cell lysate (Figure 3.5B, lane 1-6 and 10) but not in control precipitations (Figure 3.5B, lane 7 and 8).

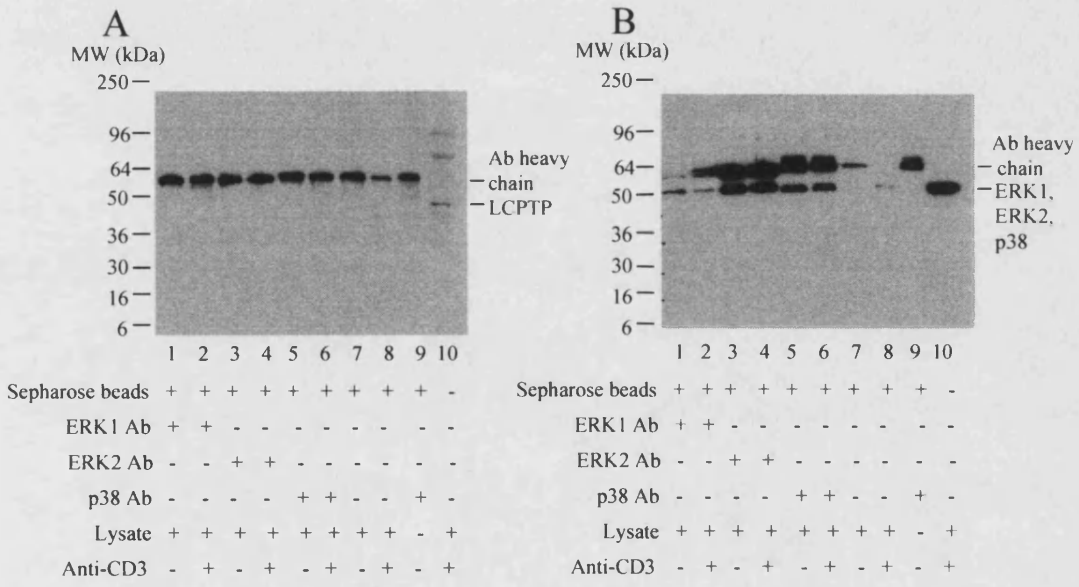


Figure 3.5: Endogenous MAPK immunoprecipitations

A. LCPTP blot of MAPK immunoprecipitates. B. MAPK blot of MAPK IPs. Cell equivalents corresponding to 1 x 10^8 cells were immunoprecipitated. Immunocomplexes were isolated using monoclonal mouse antisera raised against ERK1, ERK2 or p38 protein/Protein A Sepharose in unstimulated, or anti-CD3 antibody-stimulated Jurkat T cells. Lane 1- ERK1 immunoprecipitations non-activated. Lane 2- ERK1 immunoprecipitations activated 1 min with anti-CD3 antibody. Lane 3– ERK2 immunoprecipitations non-activated. Lane 4- ERK2 immunoprecipitations activated 1 min with anti-CD3 antibody. Lane 5- p38 immunoprecipitations non-activated. Lane 6- p38 immunoprecipitations activated 1

min with anti-CD3 antibody. Lanes 7, 8 and 9 represent controls for non specific staining/binding. Lane 10 - Cell lysate equivalent to 1 x 10^6 cells.

3.2.6 Intracellular cAMP measurement in Jurkat T cells

Previous studies have suggested that cAMP may regulate LCPTP interactions and that increased levels resulted in the dissociation of MAP kinase and LCPTP complexes (Saxena et al., 1999b). High levels of cAMP in Jurkat T cells could therefore explain why we cannot demonstrate the interaction between endogenous LCPTP and MAP kinases. To address whether Jurkat T cells have elevated cAMP levels thus favouring release of LCPTP from MAP kinase complexes, the levels of cAMP in non activated and anti-CD3 stimulated cells were measured. As a positive control, cells were activated in the presence or absence of forskolin which induces raised cAMP levels (Kvanta et al., 1990). There was a five fold increase in the concentration of cAMP in cells activated with anti-CD3 antibody for 1 minute compared to resting cells. In contrast, cAMP levels were increased 10 000 fold after forskolin stimulation. PKA activity was not measured and so it was not possible to confirm whether the levels of cAMP detected, albeit very small would still be sufficient to activate PKA and phosphorylate LCPTP

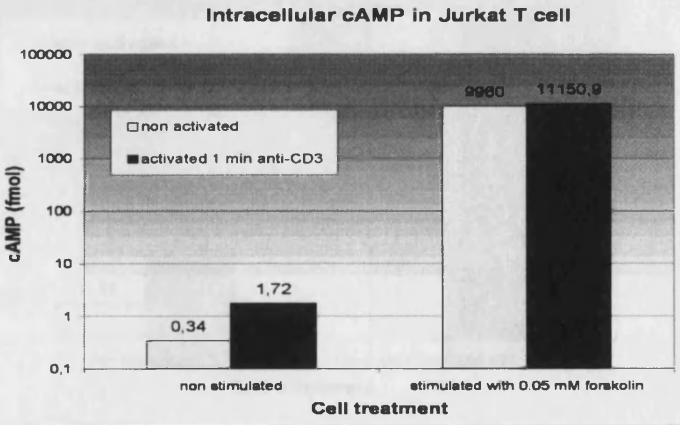


Figure 3.6: Intracellular cAMP measurement in Jurkat T cell

A. Intracellular cAMP from cells was measured: Unstimulated cells (blue bars) and cells activated 1 min with anti-CD3 antibody (pink bars). cAMP levels are compared between unstimulated (left) or cells stimulated (right) with 0.05 mM forskolin. The amount of cAMP is measured in fmol and the results are the mean of two experiments.

3.2.7 Bulk-up of endogenous LCPTP immunoprecipitations in Jurkat T cells

In a final attempt to show an interaction between endogenous LCPTP with ERK by Western blotting, lysates equivalent to 5 x 10^8 cells from both resting and 1 min anti-CD3 antibody activated samples were used. LCPTP immunoprecipitations were probed by Western blotting for the presence of ERK1 protein. Figure 3.7A shows the faint presence of ERK1 in LCPTP immunoprecipitations (lanes 1 and 2), but the level detected is much lower than compared to the cell lysate control (lane 4) which contains 500 times less protein than the immunoprecipitates. The presence of LCPTP in the same immunoprecipitations is clearly detectable (Figure 3.7B lanes 1 and 2) and is much higher than what is found in the lysate control. In order to look for other potential substrates or associating proteins for LCPTP, the bulk-up immunoprecipitations were probed with a phosphotyrosine antibody (Figure 3.7C). No phosphotyrosine proteins were detected by probing blots with the 4G10 anti-phospho tyrosine antibody coprecipitating with LCPTP.

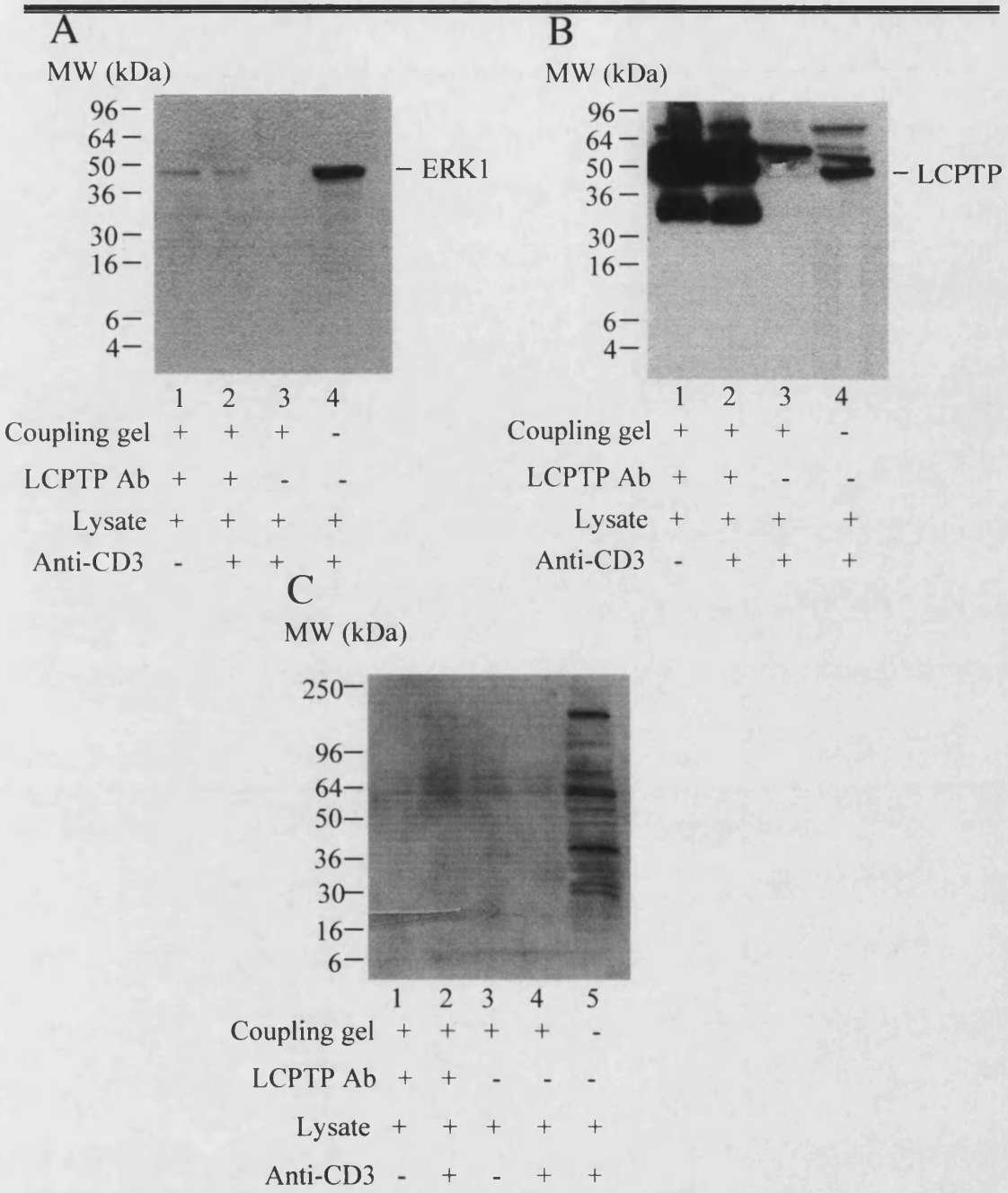


Figure 3.7: Detection of ERK1 and phosphotyrosine proteins in LCPTP immunoprecipitations using 5 x 10^8 cells.

Cell equivalents corresponding to 5 x 10^8 cells were immunoprecipitated with rabbit antisera raised against LCPTP protein in either unstimulated or anti-CD3 antibody-stimulated Jurkat T cells. Lane 1- LCPTP immunoprecipitations non-activated. Lane 2- LCPTP immunoprecipitations activated 1 min with anti-CD3 antibody. Lane 3 represents a control for non specific binding and lane 4 is soluble cell lysate equivalent to 5 x 10^6 cells. Membrane probed with A. ERK1 antibody; B. LCPTP

antibody; C. anti-phosphotyrosine 4G10 antibody. The data shown are representative of two separate experiments.

3.2.8 Immunoprecipitations of overexpressed LCPTP in Jurkat T cells

In order to ascertain that the conditions we used could maintain protein:protein interactions, we repeated the experiments published in the literature in which LCPTP was overexpressed in cells. Jurkat T cells were transiently transfected with cmyc, cmyc-LCPTP WT or cmyc-LCPTP substrate-trap mutant proteins. Unstimulated cells and cells activated for 1 minute by CD3 ligation were used for LCPTP immunoprecipitations. Immunoprecipitations were probed for the presence of the MAP kinase ERK1. ERK1 was not brought down when only the myc tag protein was overexpressed (Figure 3.8 lanes 1 and 2) but interacted constitutively with both LCPTP wild-type and substrate-trap proteins (Figure 3.8 lanes 3 to 6).

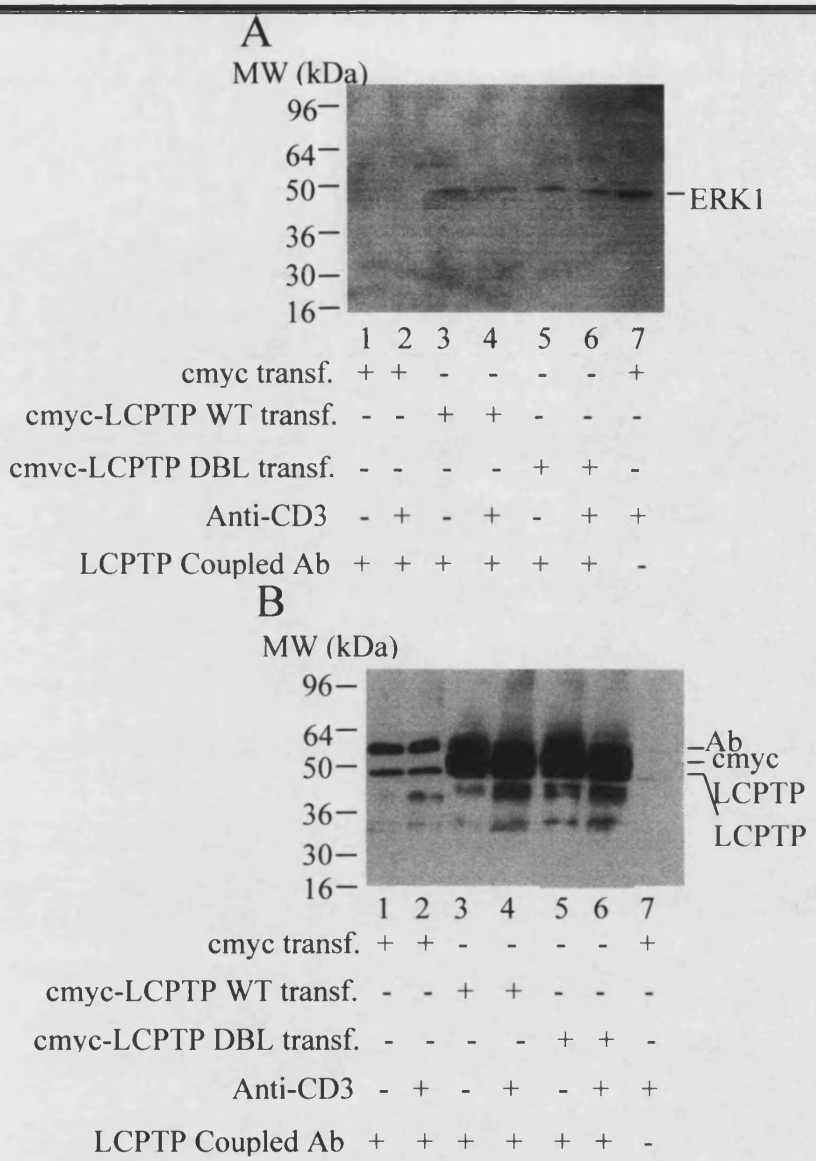


Figure 3.8. ERK1 is found in immunoprecipitates of overexpressed LCPTP.

LCPTP immunoprecipitates were blotted for A. ERK1; B. LCPTP. Cell equivalents corresponding to 1 x 10^7 cells were immunoprecipitated with rabbit antisera raised against LCPTP protein in either unstimulated or anti-CD3 antibody stimulated Jurkat T cells. Lanes 1 and 2- cells overexpressing cmyc tag. Lanes 3 and 4- cells overexpressing cmyc-LCPTP wild-type. Lanes 5 and 6- cells overexpressing cmyc-LCPTP substrate-trap protein (DBL). Lanes 1, 3 and 5- LCPTP immunoprecipitations non-activated. Lanes 2, 4 and 6- LCPTP immunoprecipitations activated 1 min with anti-CD3 antibody. Lane 7 represents soluble cell lysate equivalent to 1 x 10^5 cells. The data shown are representative of two separate experiments.

3.2.9 GST-LCPTP substrate-trap (C291S, D257A) pull-downs in Jurkat T cells

To further confirm that the lysis and immunoprecipitation conditions used, preserved protein complexes, we looked for the presence of MAPK proteins in GST pull-down assays from Jurkat T cells, using a GST-LCPTP fusion substrate-trap protein (C291S, D257A). Pull-down complexes were separated by SDS-PAGE and Western blotted using monoclonal ERK1, ERK2 and p38 antibodies. As shown in Figure 3.9A, lanes 1 and 2, ERK1 did not interact constitutively with GST-LCPTP wild-type protein. When the GST-LCPTP substrate-trap protein was used to immunoprecipitate from cell lysates (Figure 3.9A, lanes 4 and 5), ERK1 was brought down from both the non-activated and anti-CD3 activated cell lysates. More ERK1 was seen associating with the GST-LCPTP substrate-trap from lysate from activated cells compared to non-activated. ERK1 protein was not brought down by GST protein alone (lanes 7 and 8). ERK2 interacted constitutively with GST-LCPTP substrate-trap protein, but in contrast to ERK1, more ERK2 was captured from the non-activated lysate compared to the activated lysate (Figure 3.9B, lanes 4 and 5). The presence of p38 protein was also detected in pull-downs using GST-LCPTP substrate-trap protein but the amount appeared to be much less compared to that seen with ERK1 and ERK2 (Figure 3.9C, lanes 4 and 5). Similarly to ERK2, more p38 bound to the phosphatase-trap when the cells were not activated. A phosphotyrosine protein blot revealed that there was an increase in the phosphorylation state of the MAPK family members following CD3 activation of cells from a basal phosphorylation state (Figure 3.9D, lanes 4 and 5).

In order to semi-quantitate the data obtained looking at LCPTP-MAPK interactions, protein bands were scanned using a densitometer. Changes in the association of LCPTP with the different MAPK family members, dependent on the state of cell activation and cell phenotype are represented graphically in Figure 3.9E. The lysate for each blot was used as a reference and given an arbitrary number of one. This enabled a comparison of the densitometric figures obtained from bands from immunoprecipitated samples relative to the reference.

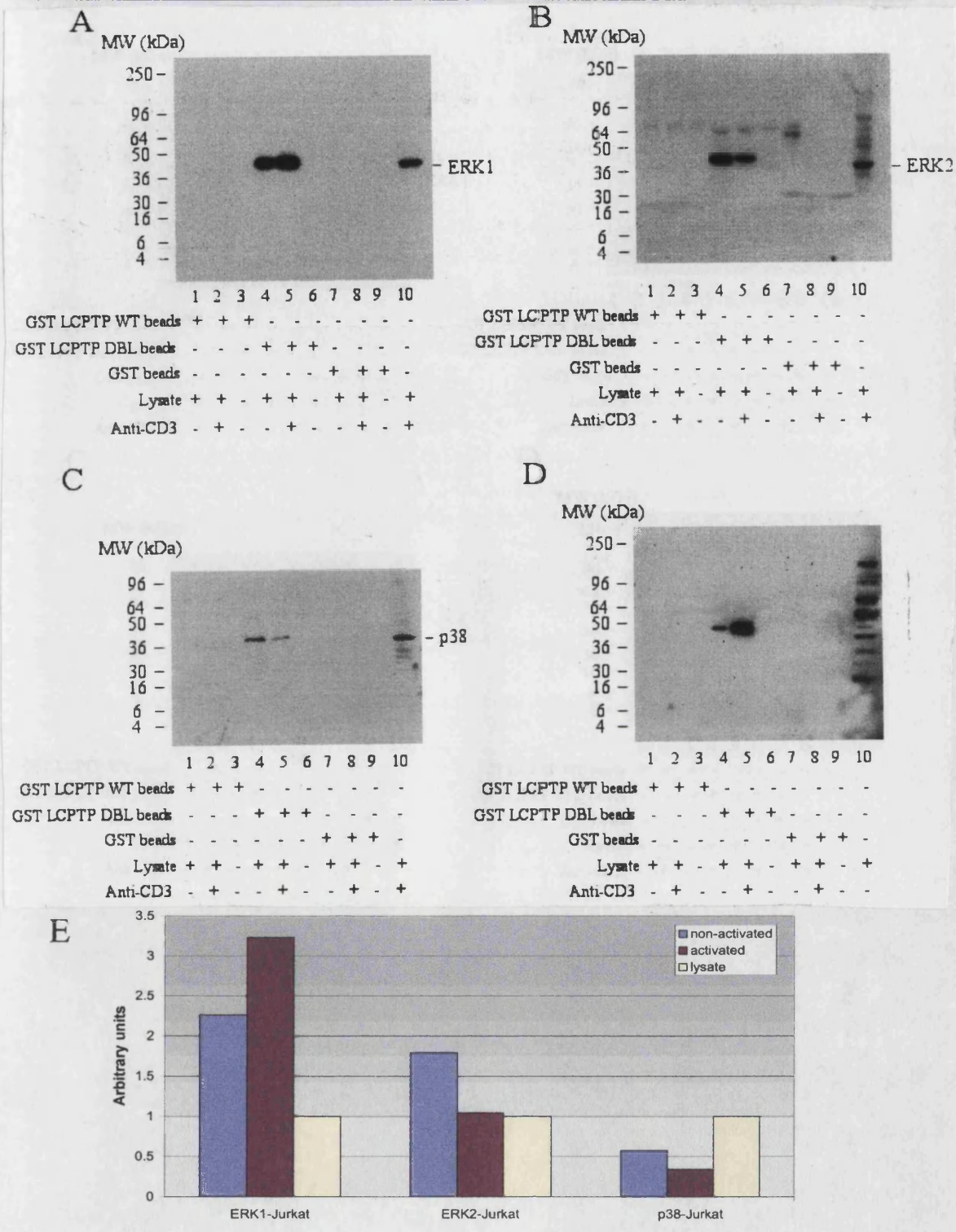


Figure 3.9: ERK1, ERK2 and p38 associate with a recombinant GST-LCPTP substrate-trap protein in Jurkat T cells

GST fusion protein pull-downs in Jurkat T cells blotted for A. ERK1; B. ERK2; C. p38 and D. Phosphotyrosine (4G10). Graph representing the intensity of each band

probed for a particular member of the MAPK family measured by densitometry compared to the lysate control in E. Unstimulated or anti-CD3 antibody-stimulated Jurkat T cells corresponding to 1 x 10^8 cells were used in pull-down experiments using recombinant GST-LCPTP wild-type, substrate-trap (C291S, D257A) and GST proteins. GST fusion proteins in the absence of lysates were also run to control for non-specific protein binding. Figures A, B, C and D: lane: 1- GST-LCPTP wild-type protein pull-downs non-activated. 2- GST-LCPTP wild-type protein pull-downs activated 1 min with anti-CD3 antibody. 3- Control: recombinant GST-LCPTP wild-type protein. 4- GST-LCPTP substrate-trap protein (C291S, D257A) pull-downs from non-activated cells. 5- GST-LCPTP substrate-trap protein (C291S, D257A) pull-downs from cells activated 1 min with anti-CD3 antibody. 6- Control: recombinant GST-LCPTP substrate-trap protein (C291S D257A). 7- GST protein pull-downs from non-activated cells. 8- GST protein pull-downs from cells activated 1 min with anti-CD3 antibody. 9- Control: recombinant GST protein. 10- Cell lysate equivalent to 1 x 10^6 cells. The data shown are representative of two separate experiments. Figure E: Non-activated samples (blue bars), activated samples (pink bars) and lysate control (cream bars).

3.2.10 ERK1 does not associate constitutively with endogenous LCPTP in human peripheral CD4$^+$ T cells

Having shown that MAP kinase family members can associate with LCPTP in Jurkat T cells, it was of interest to investigate whether interactions occur between LCPTP and MAP kinases in primary T cells. Experiments were performed to determine whether any constitutive interactions occur between ERK1 and endogenous LCPTP. ERK1 did not interact constitutively with endogenous LCPTP (Figure 3.10A, lanes 1 and 2) although ERK1 could be detected in the lysate containing 100 times less protein (lane 4). This result supported our findings in Jurkat T cells, confirming that with the experimental conditions used, LCPTP does not form constitutive interactions with ERK1. As in Jurkat T cells, we were able to show that LCPTP was brought down by the coupled LCPTP antibody from a primary human CD4$^+$ T cell lysate (Figure 3.10B, lanes 1 and 2).

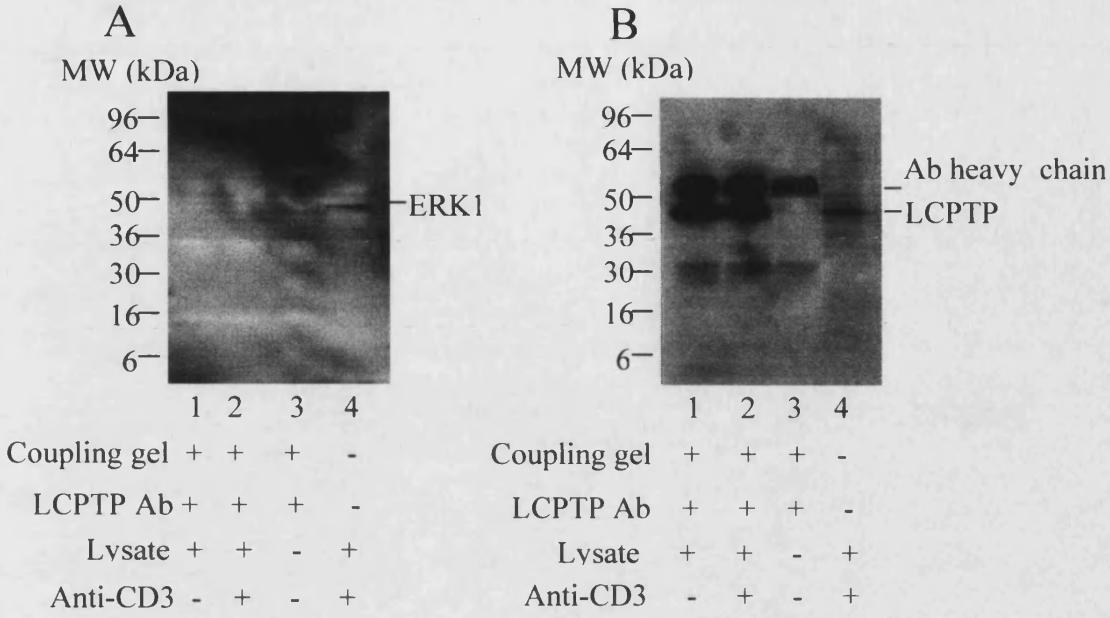


Figure 3.10. LCPTP immunoprecipitates from human peripheral CD4⁺ T cells do not contain ERK1.

LCPTP complexes immunoprecipitated from peripheral CD4⁺cells were blotted for A. ERK1 and B. LCPTP. LCPTP protein was immunoprecipitated using rabbit antisera raised against LCPTP from 5 x 10^7 CD4⁺ T cells either unstimulated or stimulated with anti-CD3 antibody. Figures A and B: lane 1- LCPTP immunoprecipitations, non-activated. Lane 2- LCPTP immunoprecipitations, activated 1 min with anti-CD3 antibody. Lane 3- antibody control and lane 4- lysate control. The data shown are representative of four separate experiments.

3.2.11 ERK1 and ERK2 from peripheral CD4⁺ T cells form transient associations with LCPTP

Having determined that LCPTP and ERK1 do not associate constitutively in peripheral CD4⁺ T cells, experiments were performed to see if transient associations could be detected dependent on phosphatase activity, as shown to occur in Jurkat T cells. In contrast to what was seen in the Jurkat T cell line, both ERK1 and ERK2 interacted more strongly with the phosphatase-trap in pull-downs from unactivated cell lysates compared to activated cell lysates (Figure 3.11A and 3.11B, lanes 1 and 2). In comparison to what was seen in Jurkat T cells, both in activated and unactivated cells, p38 interacted very weakly with the substrate-trap (Figure 3.11C, lanes 1 and 2). To semi-quantitate the data obtained, densitometry was performed on

each band. The graph in Figure 3.11D shows the changes in the association of LCPTP with the different MAPK family members. The lysate for each blot was used as a reference and given an arbitrary number of one.

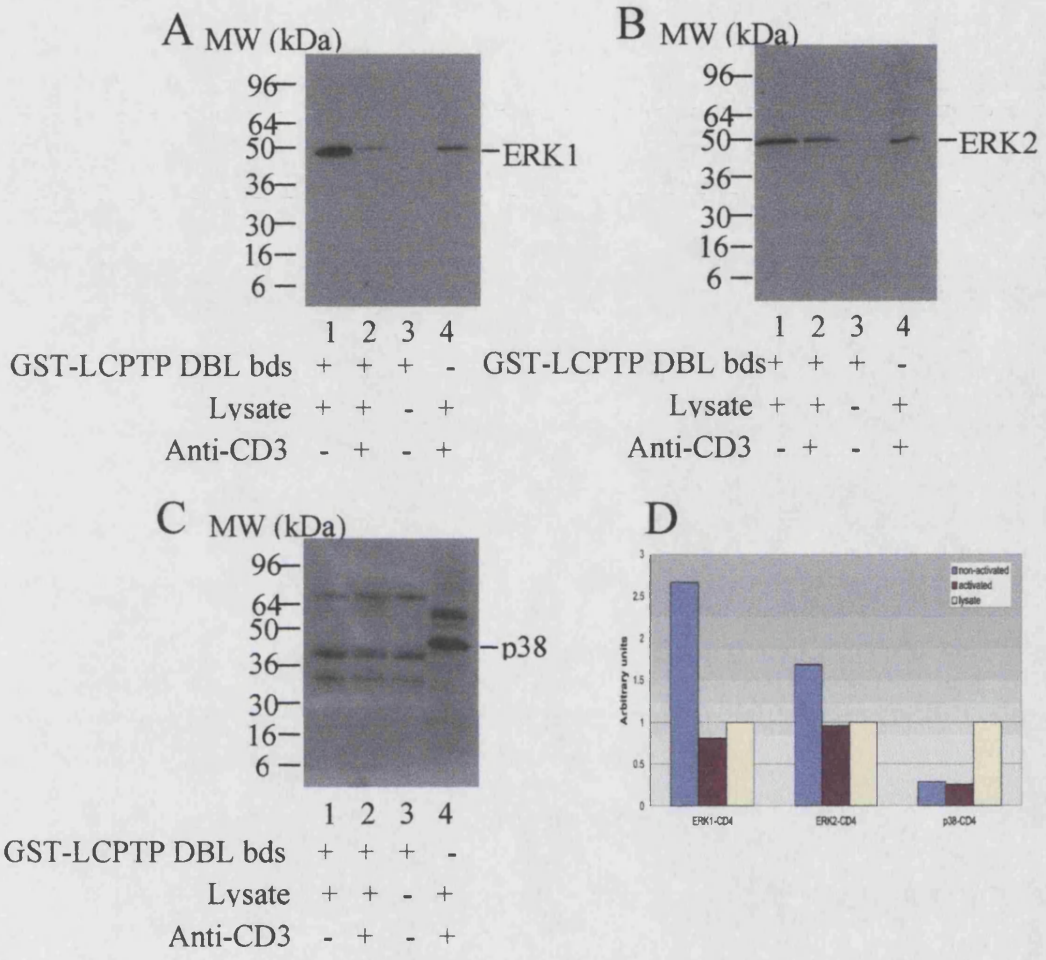


Figure 3.11: ERK1, ERK2 and p38 associate differentially with a recombinant GST-LCPTP substrate-trap protein in peripheral CD4⁺ T cells dependent on the state of cell activation.

GST fusion protein pull-downs in peripheral $CD4^+$ T cells blotted for A. ERK1; B. ERK2 and C. p38. D. Graph representing the intensity of each band probed for a particular member of the MAPK family measured by densitometry compared to the lysate control. Unstimulated or anti-CD3 antibody-stimulated peripheral $CD4^+$ T cells corresponding to 5×10^7 cells were used in pull-down experiments with GST-LCPTP substrate-trap protein. GST fusion proteins in the absence of lysates were also run to control for non-specific protein binding. Figure A, B and C: lane 1- GST-LCPTP substrate-trap protein (C291S, D257A) pull-downs, non-activated. 2- GST-LCPTP

substrate-trap protein (C291S, D257A) pull-downs, activated 1 min with anti-CD3 antibody. 3- Control: recombinant GST-LCPTP substrate-trap protein (C291S D257A). 4- Cell lysate equivalent to 5 x 10^5 cells. The data shown are representative of three separate experiments. Figure D: non-activated samples (blue bars), activated samples (pink bars) and lysate control (cream bars).

CHAPTER THREE

3.3. Discussion

Following recognition by the TCR of an antigenic peptide presented by MHC molecules, a cascade of signal transduction events occurs. The early stages of this cascade are represented by a dramatic but transient increase in the total protein tyrosine phosphorylation (Isakov et al., 1994); (Mustelin et al., 1998). The Jurkat T lymphocyte cell line, activated with an anti-CD3 antibody, has been used extensively as a model system for studying TCR signal transduction (Landegren et al., 1985). This provided us with a good model system for looking at LCPTP function, since Jurkat T cells express high levels of endogenous LCPTP. Studies were performed looking at time courses following cell stimulation in order to determine the point at which maximal tyrosine phosphorylation occurred in Jurkat T cells. Maximal total protein tyrosine phosphorylation occurred at one minute following anti-CD3 stimulation and this time point was selected as the optimal time for studies looking for interactions of LCPTP with other proteins and potential substrates (Figure 3.2).

Previous studies have suggested a model in which members of the MAP kinase families (ERK1, ERK2 and p38) interact constitutively with recombinant LCPTP (Oh-hora et al., 1999); (Saxena et al., 1999a). Both wild-type LCPTP protein and an LCPTP substrate-trap (C200S) protein formed complexes with ERK1, ERK2 and p38 (Model 1) (Oh-hora et al., 1999); (Saxena et al., 1999a). In our experiments, we endeavoured to demonstrate an association between endogenous LCPTP and members of the ERK and p38 MAP kinase families in order to support this model.

We were not able to demonstrate any constitutive interaction with endogenous LCPTP and members of the ERK and p38 MAP kinase families in either Jurkat T cells or in peripheral human $CD4^+$ T cells (Figures 3.3 and 3.10). However, we were able to show that LCPTP/MAP kinase interactions occurred when LCPTP was overexpressed in cells (Figure 3.8) (Oh-hora et al., 1999); (Saxena et al., 1999a). We concluded from this, that if a constitutive interaction occurs between endogenous LCPTP and MAP kinase family members, only a very small percentage of total LCPTP protein associates with MAP kinases. This could be demonstrated by increasing the number of Jurkat T cells and performing immunoprecipitations (Figure 3.7). One explanation for the failure to detect the constitutive interaction of LCPTP

and MAP kinases reported previously, is that the LCPTP antibodies disrupt the association of LCPTP with other protein. This remains a possibility, but is thought to be unlikely as performing the reciprocal immunoprecipitations with antibodies to MAP kinase family members and probing for LCPTP also did not reveal any constitutive interaction between LCPTP and MAP kinases (Figure 3.5). Our data supports a second model proposed recently in which LCPTP interacts transiently with ERK2 (Model 2). This interaction could be seen only with a substrate-trap mutant form of LCPTP (C200S) (Pettiford and Herbst, 2000). In addition to ERK2, we propose that ERK1 is also an LCPTP substrate and forms a transient association with LCPTP. The intensity of interaction between the MAP kinases and the phosphatase-trap is dependent on the state of activation of the cells and the cell phenotype. Thus, there is an increased amount of ERK1 associating with LCPTP following anti-CD3 cell activation in Jurkats. However, in peripheral $CD4^+$ T cells, the situation is reversed, with less ERK1 associating with LCPTP following cell activation relative to the unstimulated cells. This discrepancy is likely to reflect the fact that Jurkat T cells are a transformed cell line compared to isolated resting peripheral $CD4^+$ T cells (Figures 3.9 and 3.11). These results suggest that LCPTP has a role in dephosphorylating the MAP kinases ERK1 and ERK2 and maintaining the kinases in an inactive state in unstimulated $CD4^+$ T cells.

The presence of LCPTP in anti-LCPTP immunoprecipitations has been demonstrated by Western blotting but to further confirm the presence of LCPTP in immunoprecipitations, studies were done using mass spectrometry to obtain protein sequence data. The only clear band to be found uniquely in LCPTP IPs was shown to be LCPTP protein (Figure 3.4). There was a notable absence of the MAP kinase, ERK1, in the sequence analysis, despite the fact that ERK1 has a molecular weight of 44 kDa ie. equivalent to LCPTP and would therefore, be expected to comigrate on a SDS-PAGE (Table 3.1).

There are two models in the literature which suggest two alternative interactions which could occur between LCPTP and protein kinases. If the kinases act as substrates for LCPTP, the interaction will occur transiently, and LCPTP will inactivate the target by dephosphorylating phosphotyrosine residues of the MAP

kinase (Haneda et al., 1999). Alternatively, more stable constitutive interactions may occur if the protein which interacts with LCPTP is not solely a substrate for the phosphatase but contains other specific protein/protein interaction sites as suggested by Oh-hora et al. (1999). Examples include recombinant PTPBR7 which forms stable complexes with ERK1 and ERK2 both in its wild-type or substrate-trap conformation (Ogata et al., 1999); (Blanco-Aparicio et al., 1999). This is also the case for PTP-SL, STEP protein phosphatase and the *Drosophila* PTP-ER protein phosphatase, which have all been shown in overexpression systems to interact with MAP kinases via their respective KIM motifs (Blanco-Aparicio et al., 1999); (Karim and Rubin, 1999).

One possible explanation for our inability to detect a specific interaction between LCPTP and the MAP kinase family members ERK1, ERK2 and p38, is insufficient sensitivity of detection. However, the amount of total protein present in each immunoprecipitation was 100 times higher compared to protein visualised by blotting (LCPTP or MAP kinase) in the total lysate. Since the MAP kinase members in the total cell lysate are still clearly detectable, the experimental system should therefore detect even weak interactions between LCPTP and MAP kinases.

There is a report indicating that elevated intracellular cAMP levels inhibit the interaction of LCPTP with ERK, by increasing the phosphorylation of LCPTP on Ser-34 and thereby releasing ERK from its association with the phosphatase (Saxena et al., 1999b). We therefore, measured the amount of intracellular cAMP in the Jurkat T cells used for immunoprecipitating LCPTP to see if the absence of interaction could be explained by the fact that the cells contained high levels of cAMP preventing this association. The amount of 8-CPT-cAMP added in the aforementioned study, to measure the release of ERK from recombinant LCPTP, was 1 mM (Saxena et al., 1999b). The amount of cAMP detected in our system was in the range of 1 fmol of cAMP per 10^5 cells (Figure 3.6). Adjusting the amount of cAMP for 10^8 cells, immunoprecipitations were performed in the presence of 1 pmol of cAMP per IP. This is 10^9 times less cAMP per IP than in the experiments described in the literature (Saxena et al., 1999b). The amount of cAMP is therefore, unlikely to be a significant factor inhibiting endogenous LCPTP from interacting with ERK in the cell.

An alternative explanation for the negative results obtained could be differences in lysis conditions. However, endogenous LCPTP immunoprecipitations using the same experimental conditions as in previous studies (Oh-hora et al., 1999) did not reveal any MAP kinase association. Thus our results cannot be explained by differences in lysis conditions. Furthermore, the lysis conditions used in this study did allow the interaction of ERK1, ERK2 and p38 proteins with a recombinant GST-LCPTP substrate-trap protein in Jurkat T cells and peripheral $CD4^+$ T cells.

We conclude that in our conditions, endogenous LCPTP does not interact constitutively with ERK1, ERK2 and p38. However, MAP kinase family members, ERK1 and ERK2 can interact constitutively with LCPTP as phosphatase substrates in Jurkat T cells. We have extended these studies to include peripheral $CD4^+$ T cells in which both ERK1 and ERK2 can act as substrates. In both Jurkat T cells and $CD4^+$ T cells there is a weak interaction detected with p38, but this appears insignificant when compared to levels of p38 in the total cell lysate. The association of ERK1 and ERK2 with LCPTP in $CD4^+$ T cells is reduced following cell activation with anti-CD3 antibody suggesting that LCPTP activity is regulated by signalling via the T cell receptor. Finally, our study provides a cautionary note to the interpretation of protein/protein interactions seen under conditions where one partner is expressed at levels many times higher than is normally present. Such interactions have been documented for many other proteins, including other phosphatases such as the *Drosophila* PTP-ER protein phosphatase (Karim and Rubin, 1999). More sophisticated approaches which allow measurement of such interactions under more physiological conditions will be important in determining the contribution of such interactions to the regulation of cellular activity *in vivo*.[1]

[1] Part of the work described in this chapter has been published in Brodeur, I., Boyhan, A., Heinrichs, N., Plumpton, C., Chain, B., and Rowan, W. C. (2002). LCPTP - MAP kinase interaction: permanent partners or transient association? Mol Immunol *1188*, 1-9.

CHAPTER FOUR: RESULTS
Effect of overexpressing LCPTP on MAP kinase activation and T cell function in the human leukaemic Jurkat T cell line

4.1 Introduction

Several approaches have been used in studies to evaluate the function of phosphatases in cells (Fauman and Saper, 1996); (Conroy et al., 1997) (Frearson and Alexander, 1997); (Mustelin et al., 1998); (Mustelin et al., 1999). One approach is to stably overexpress the protein of interest in cells and look for phenotypic changes (Cohen et al., 1999); (Baker et al., 2001). As well as overexpressing wild-type phosphatase protein, phosphatases have been mutated to remove phosphatase activity. Two forms of phosphatase inactive protein can be generated: firstly one that binds irreversibly to its substrate (catalytically inactive phosphatase able to trap the substrate in the phosphatase pocket) and secondly, a mutant form which forms transient associations with its substrate but can not dephosphorylate it (inactive phosphatase) (called here null) (Flint et al., 1997); (Hoff et al., 2000). In addition to their phosphatase activity, phosphatases include domains via which they can interact with other proteins independently of their enzyme activity. For example, SHP-1 contains two tandem SH homology (SH2) domains by which it can interact with proteins, including ZAP-70 (Jin et al., 1999). Overexpressing the different mutant forms of phosphatases can give an indication of when a phosphatase is involved in signalling pathways, possibly acting as an adaptor protein (Peterson et al., 1998); (Bosselut et al., 1999); (Bruhns et al., 2000). Thus, it is possible for a phosphatase to influence signalling pathways independent of intrinsic enzyme activity.

This approach has been performed with two hematopoietic phosphatases: CD148 and Lyp. Baker et al. showed that CD148 inhibited IP_3 production, calcium mobilisation and phosphorylation of the MAPK family member, ERK1. They observed no upregulation of the CD69 marker and the pattern of protein tyrosine phosphorylation in response to anti-TCR MAb C305 (Baker et al., 2001) was not grossly affected. These phenotypes were restricted to the CD148 wild-type form. Overexpressing the catalytically inactive form had no effect on cell phenotype (Baker et al., 2001). In contrast, overexpression of both the wild-type and substrate-trap D/A Lyp phosphatase inhibited IL-2 promoter activity using a luciferase readout in a

transcriptional reporter assay. However, the substrate-trap C/S protein or the null phosphatase did not inhibit IL-2 promotor activity (Hill et al., 2002).

In order to investigate the role of LCPTP in T cells, stable cell lines expressing either wild-type, a substrate-trap (D257A-C291S) or a null mutant (R297M) form of LCPTP were established. For the purpose of these experiments, the human Jurkat T cell line was used as it is readily amenable to transfection (Tangye et al., 1998); (Plyte et al., 2000); (Denny et al., 2000) and because Jurkat cells were used successfully to detect interactions of LCPTP with members of the MAP kinase family (Brodeur et al., 2002). MAP kinase activation occurs within minutes of TCR ligation (Isakov et al., 1994); (Mustelin et al., 1998) and its association with LCPTP suggested that the phosphatase could have a key role in regulating kinase activity controlling downstream events such as IL-2 transcription (Li et al., 1999b). The effect of overexpressing LCPTP was monitored by following the responses of cells to TCR ligation and CD28 costimulation. Stable cell lines overexpressing the different forms of LCPTP were used to determine the effect of the phosphatase on MAP kinase activation, IL-2 production, expression of T cell surface activation markers, including CD25 and CD69 and cell proliferation.

4.2 Results

4.2.1 Establishment of stable cell lines overexpressing LCPTP

In order to investigate the role of LCPTP in T cell signalling, LCPTP constructs were made incorporating GFP with the view to be able to readily isolate transfected cells using a FACS Cell Sorter. Following transfection and antibiotic selection, stable cell lines were established. However, flow cytometric analysis revealed the expression of the GFP to be very weak. After two to three weeks in culture, clones began to die suggesting that the transfected protein was toxic to cell growth. As LCPTP is endogenously expressed in Jurkat T cells, expression of foreign GFP could be a possible cause of cell death.

In a second attempt to make stable cell lines overexpressing LCPTP protein, constructs were redesigned to replace the GFP tag with cmyc. cmyc-LCPTP vectors were transfected into Jurkat cells and clones were obtained for each of the different forms of LCPTP protein. These included cmyc-LCPTP wild-type, cmyc-LCPTP substrate-trap (D257A-C291S) (Trap) and cmyc-LCPTP null mutant phosphatase (R297M) (Null). The cell lines were probed for the presence of recombinant cmyc-LCPTP using a cmyc antibody (Figure 4.1A) and also with an LCPTP antibody, which detects endogenous LCPTP at a slightly lower molecular weight compared to the recombinant protein (Figure 4.1B). No protein was detected using the anti-cmyc antibody in untransfected Jurkat cells (Figure 4.1A lane 1) but recombinant cmyc-LCPTP wild-type protein was shown to be strongly expressed in four independent stable clones (Figure 4.1A lanes 2-5). Similarly, cmyc-LCPTP substrate-trap and null mutant proteins were shown to be expressed in Jurkat T cell clones (Figure 4.1A lanes 6 and 7 respectively). The densitometry of the cmyc-LCPTP protein taken from the cmyc antibody western blot is shown in Fig. 4.1C and is expressed as arbitrary units. cmyc-LCPTP substrate-trap protein was expressed at approximately the same level as the endogenous LCPTP (Figure 4.1B lane 6) and was at a much lower level compared to the levels of both cmyc-LCPTP wild-type and c-myc-LCPTP null mutant recombinant proteins.

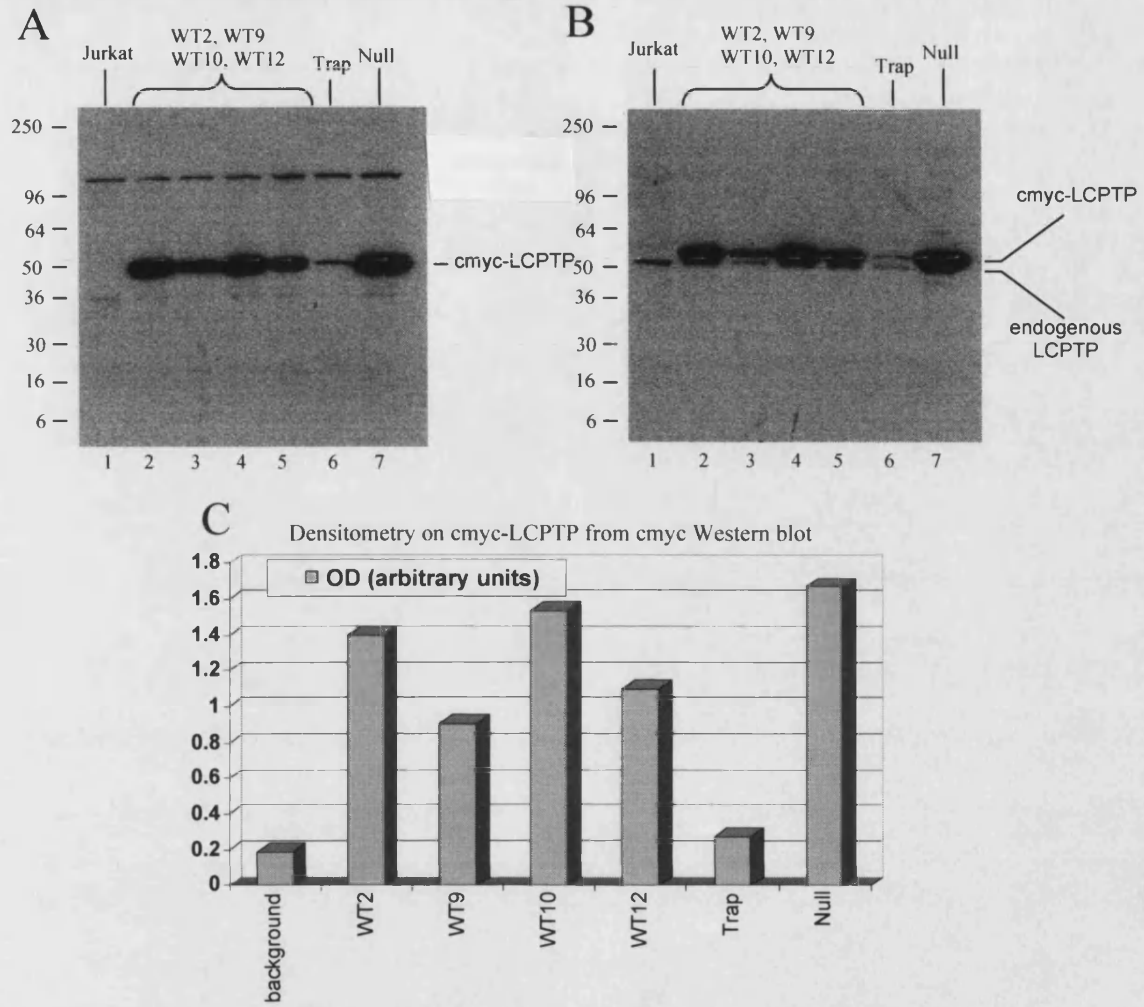


Figure 4.1. Establishment of stable cell lines overexpressing LCPTP.

Western blot of cell lysate of each stable cell line, probed with A. anti-cmyc antibody. B. anti-LCPTP antibody. Lane 1 represents the Jurkat control cell line transfected with cmyc vector alone. Lanes 2 to 5 show protein expression in stable cell lines overexpressing cmyc-LCPTP wild-type. Lane 6 is the stable cell line containing cmyc-LCPTP substrate-trap and lane 7 is a stable cell line containing cmyc-LCPTP null mutant phosphatase. Each lane is the equivalent of 1 x 10^5 cells C. Graph representing the intensity of each band probed with cmyc antibody, measured by densitometry and expressed as arbitrary units. The data shown are representative of 3 separate experiments.

4.2.2 The effect of overexpressing LCPTP on cell division

Jurkat T cells are a transformed cell line and are not dependent on exogenous factors for cell division and growth. In order to determine whether overexpression of

LCPTP phosphatase affects cell division, the different stable Jurkat cell lines were monitored for a period of five days and counted daily. Cells were plated at a starting concentration of 1.5 x 10^5 cells per ml. Figure 4.2 shows the progression in cell growth over five days. The doubling time was the same for the control cmyc cell lines, the cell lines overexpressing LCPTP wild-type, substrate-trap or null mutant.

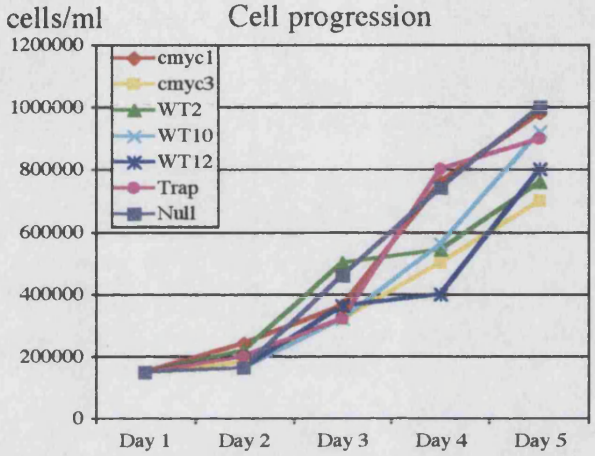


Figure 4.2. Cell division of stable cell lines overexpressing LCPTP.

Cells were seeded at a starting concentration of 1.5 x 10^5 cells per ml in 6 well plates and cell growth was monitored for 5 days by counting the number of viable cells. The graph shows the total cell numbers in cultures at each time point for control cell lines, cmyc1 and cmyc3 (red diamonds and yellow squares), LCPTP wild-type (green triangles, light blue crosses, dark blue crosses), substrate-trap (pink circles) and null mutant (grey squares) cells.

4.2.3 Phosphorylation of active MAPK family members is dependent on LCPTP

The only identified LCPTP substrates to date are the MAP kinase family members, ERK1, ERK2 and p38 (Brodeur et al., 2002). In order to determine whether the recombinant cmyc-LCPTP was active in Jurkat T cells, we investigated the phosphorylation levels of MAPK following TCR activation with anti-CD3/CD28 antibodies. Cells were activated for 5 minutes. Cell lysates were probed with an antibody recognising the phosphorylated forms of ERK1 and ERK2. Control cell lines expressing only the cmyc tag showed the normal levels of MAPK phosphorylation following anti-CD3/CD28 stimulation (Figure 4.3A, lanes 1 and 2). Following expression of cmyc-LCPTP wild-type, a sharp decrease in ERK1/2 phosphorylation

was observed (Figure 4.3A lanes 3-5). The reduction of phosphorylation seen was not due to differences in total protein loading as protein loaded in each lane was equivalent as determined by Coomassie blue staining (Figure 4.3B). Consistent with the inability of cmyc-LCPTP substrate-trap and null mutants to dephosphorylate their target substrates, an increase of MAPK phosphorylation was observed in cell lysates expressing these proteins (Figure 4.3A, lanes 6 and 7 respectively). The MAPK phosphorylation is assumed to increase because of a competition occurring between the overexpressed cmyc-LCPTP substrate-trap or null mutant proteins, which are unable to dephosphorylate the MAPK and the endogenous LCPTP. The variation of total MAPK phosphorylation (ERK1 and ERK2) assessed by densitometry is shown in Figure 4.3C.

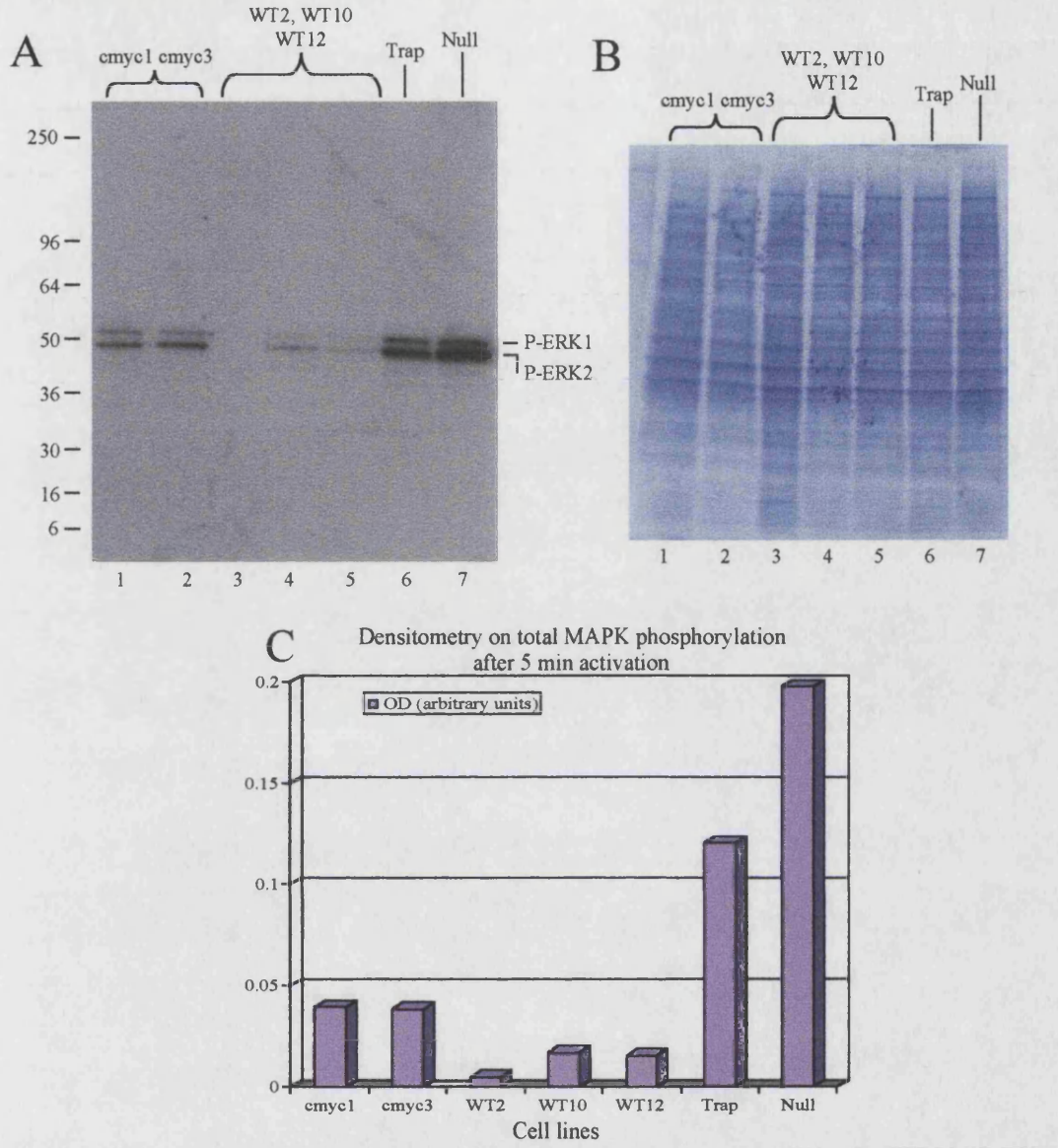


Figure 4.3. Stable expression of wild-type but not catalytically inactive mutants of LCPTP reduces levels of phosphorylated MAP kinase in Jurkat T cells.

A. Western blot of cell lysate of each stable cell line, activated with anti-CD3 for 5 minutes and probed with anti-active MAPK antibody. B. Coomassie stain of SDS-PAGE gel controlling for equal protein loading. Lanes 1 and 2 represent the cmyc control cell lines. Lanes 3 to 5 represent stable cell lines overexpressing cmyc-LCPTP wild-type. Lane 5 is cmyc-LCPTP substrate-trap and lane 6 is cmyc-LCPTP null mutant phosphatase. Each lane represents the equivalent of 1 x 10^5 cells. C. Graph represents the intensity of total MAPK phosphorylation for each cell line measured by densitometry and is expressed as arbitrary units. The data shown is representative of 5 separate experiments.

4.2.4. Overexpressing LCPTP does not have gross effects on total protein tyrosine phosphorylation or LCK phosphorylation in Jurkat T cells

As mentioned in the section above, to date, the only substrates identified for LCPTP include members of the MAP kinase family. This does not exclude the possibility that LCPTP can dephosphorylate other proteins. To investigate whether LCPTP has a general effect on tyrosine phosphorylation, lysates from cells overexpressing the different forms of LCPTP and activated with anti-CD3 were probed with an anti-phosphotyrosine antibody (4G10). Similar levels of tyrosine phosphorylation were observed in cmyc control (Figure 4.4A lanes 1 and 2), cmyc-LCPTP wild-type (lanes 3-5), cmyc-LCPTP substrate-trap (lane 6) and cmyc-LCPTP null mutant cell lines (lane 7). The effect was not time dependent as the same pattern of phosphorylation was seen at other other time points and supports the findings that LCPTP has a limited number of substrates and that LCPTP is not affecting the activity of kinases, such as Lck, involved in TCR proximal signalling.

It has been reported in the literature that ERK can phosphorylate Lck on serine residues leading to changes in Lck activity (Kesavan et al., 2002). Lck activation is believed to be one of the earliest events to occur following TCR ligation and the increase in Lck kinase activity is largely responsible for activating downstream kinases, including ZAP-70, which in turn phosphorylate a number of proteins and give rise to the characteristic patterns of tyrosine phosphorylation which can be seen in Figure 4.4A (Hall et al., 1999); (Brodeur et al., 2002). It was of interest to determine whether overexpressing LCPTP influenced MAP kinase activity and the subsequent serine phosphorylation of Lck. The results are shown in Figure 4.4B. No changes in serine phosphorylation were observed when the same blot used for looking at patterns of protein tyrosine phosphorylation was stripped and reprobed with a phospho-Lck antibody (Figure 4.4B). Equal protein loading in each track is shown in Figure 4.4C by Coomassie staining.

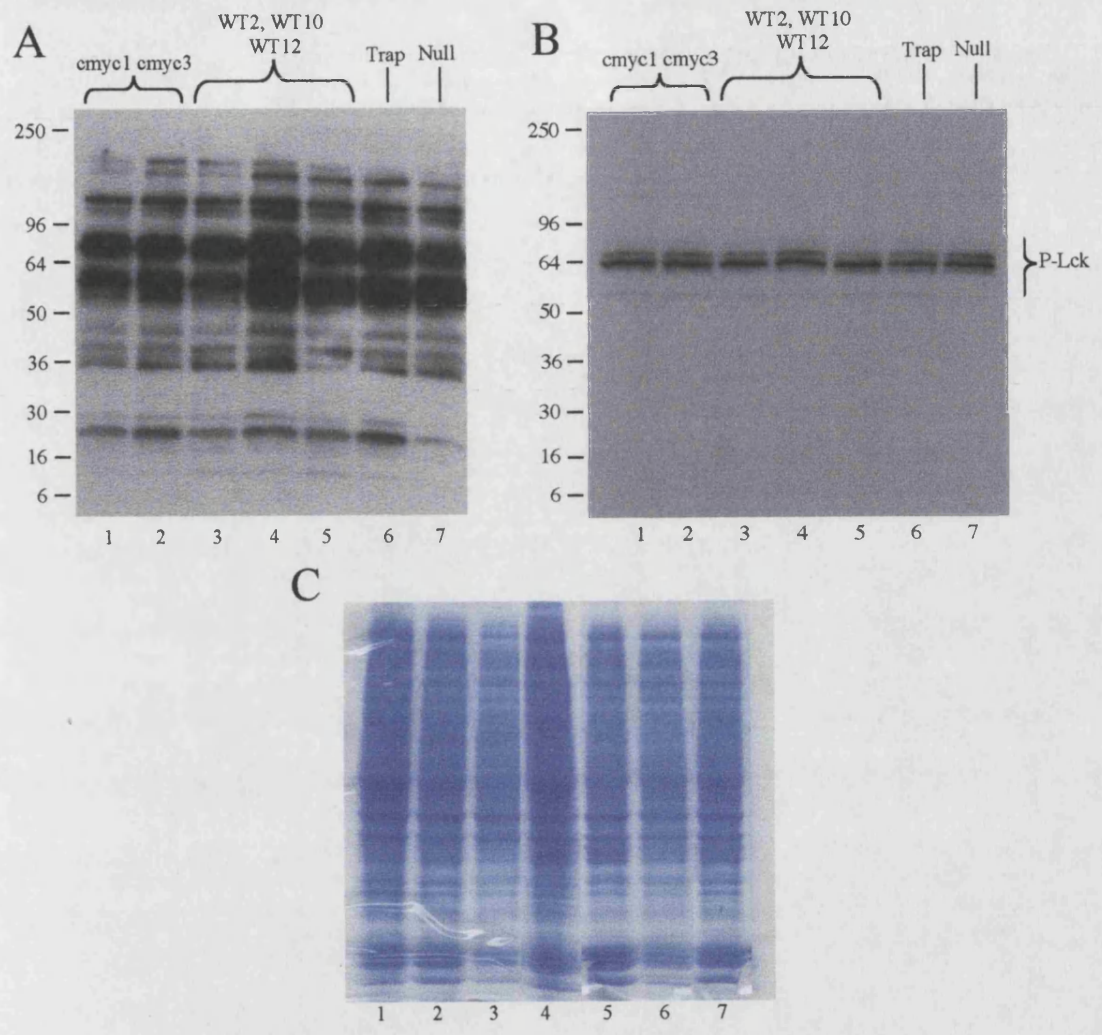


Figure 4.4. The effect of overexpressing LCPTP does not affect total protein tyrosine phosphorylation or LCK phosphorylation in Jurkat T cells.

A. Western blot of cell lysate of the different LCPTP cell lines, activated with anti-CD3 for 1 minute and probed with anti-phosphotyrosine antibody 4G10. B. Western blot probed with anti-phospho-serine Lck antibody. C. Coomassie staining of SDS-PAGE gel showing equal protein loading. Lanes 1 and 2 represent the cmyc control, lanes 3 to 5 represent cmyc-LCPTP wild-type cell lines, lane 6 is the cmyc-LCPTP substrate-trap and lane 7 is the cmyc-LCPTP null mutant phosphatase cell line. Each lane represents the equivalent of 1 x 10^5 cells. The data shown are representative of 3 separate experiments.

4.2.5. LCPTP regulates IL-2 production

The production of interleukin-2 is used as a measure to characterise the

proliferation state of the cell and its ability to respond to stimulation. Following stimulation via CD3 and CD28, the Jurkat cell line is stimulated to release IL-2. Anti-CD3 and CD28 antibodies were titrated to determine the optimal concentration for stimulating IL-2 release and accordingly 1 μg/ml anti-CD3 and 3 μg/ml anti-CD28 were selected for experiments. The optimal time point for detecting IL-2 release was determined to be 24 hours post-stimulation. Unlike in primary T cells, IL-2 appeared to accumulate in the medium and was not consumed by Jurkat T cells (Figure 4.5A). A significant reduction in IL-2 release was observed in all cmyc-LCPTP expressing cell lines, including wild-type, substrate-trap and null mutant compared to the c-myc controls (Figure 4.5B).

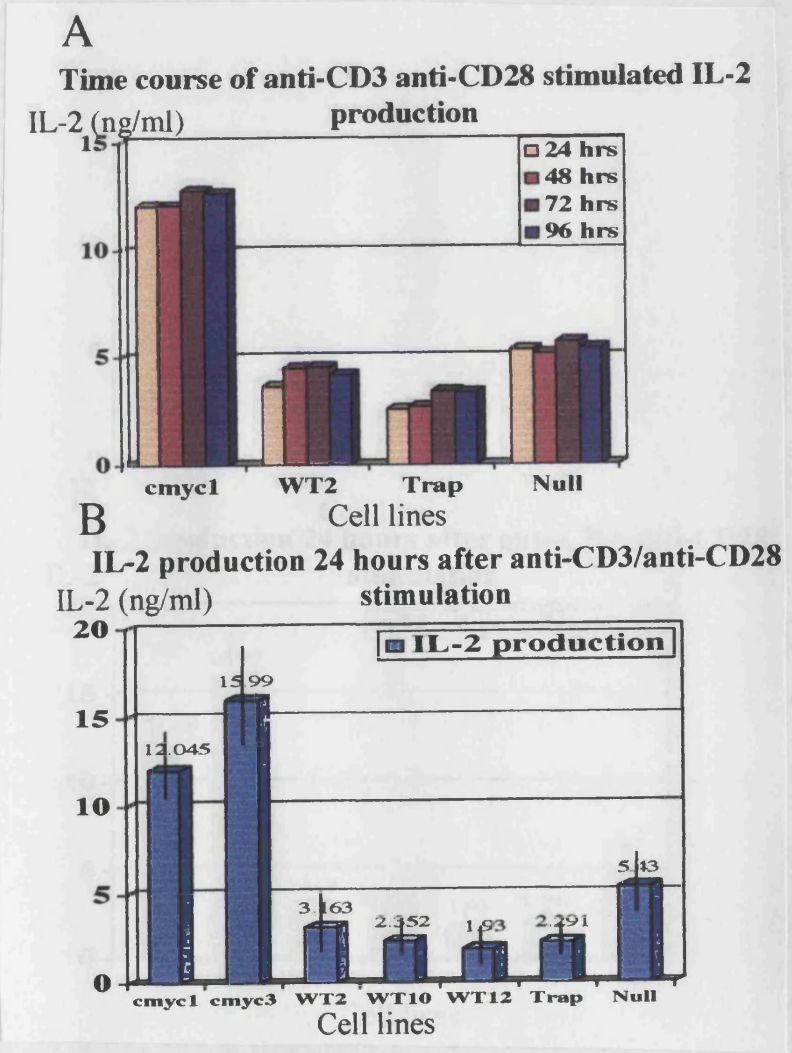


Figure 4.5. LCPTP regulates IL-2 expression.

Production of IL-2 following anti-CD3 and anti-CD28 stimulation was measured and compared to the control cell lines. A. IL-2 production was measured at: 24 (cream

bars), 48 (pink bars), 72 (purple bars) and 96 (blue bars) hrs post-stimulation. B. IL-2 production of all cell lines 24 hrs post-stimulation with error bars. Graphs show the mean values of 5 independent experiments.

4.2.6. LCPTP regulates cell growth following anti-CD3/anti-CD28 stimulation

As a consequence of T cell receptor ligation, both in the presence, or absence of CD28 costimulation, Jurkat T cells undergo growth inhibition. It was of interest to determine the consequences of LCPTP overexpression on this effect. Cell proliferation was monitored by measuring ^{3}H-thymidine incorporation into cells over a time course up to 96 hours following stimulation with anti-CD3 and anti-CD28. The optimal concentrations of antibodies for inducing growth inhibition were 0.3 μg/ml anti-CD3 and 1 μg/ml anti-CD28. Results are plotted as a percentage of the growth of each stimulated cell line at a given time relative to the unstimulated growth at the same time point (Figure 4.6A). In contrast to the control cell lines, when cmyc-LCPTP wild-type was overexpressed in the Jurkat cells, the CD3/CD28 mediated growth inhibition effect was reduced. This reduction in growth inhibition was also seen in the cell line overexpressing the cmyc-LCPTP substrate-trap. However, the cmyc-LCPTP null mutant cell line behaved similarly to the cmyc control cell lines.

One explanation for the loss of sensitivity of cell lines overexpressing LCPTP to anti-CD3/CD28 mediated growth inhibition is that dependent on the state of cell activation, IL-2 can render cells more sensitive to activation-induced death (Wang et al., 1996). Thus, a reduction in IL-2 production as a consequence of overexpressing LCPTP might favour Jurkat cell survival. To investigate whether the Jurkat cells were responsive to the effects of IL-2, the experiment was repeated with the addition of exogenous IL-2 into cultures. Addition of IL-2 had no effect on cell growth of either the cmyc control cell lines, or cell lines overexpressing LCPTP indicating that although the cells are capable of producing IL-2, they are not responsive to it (Figure 4.6B).

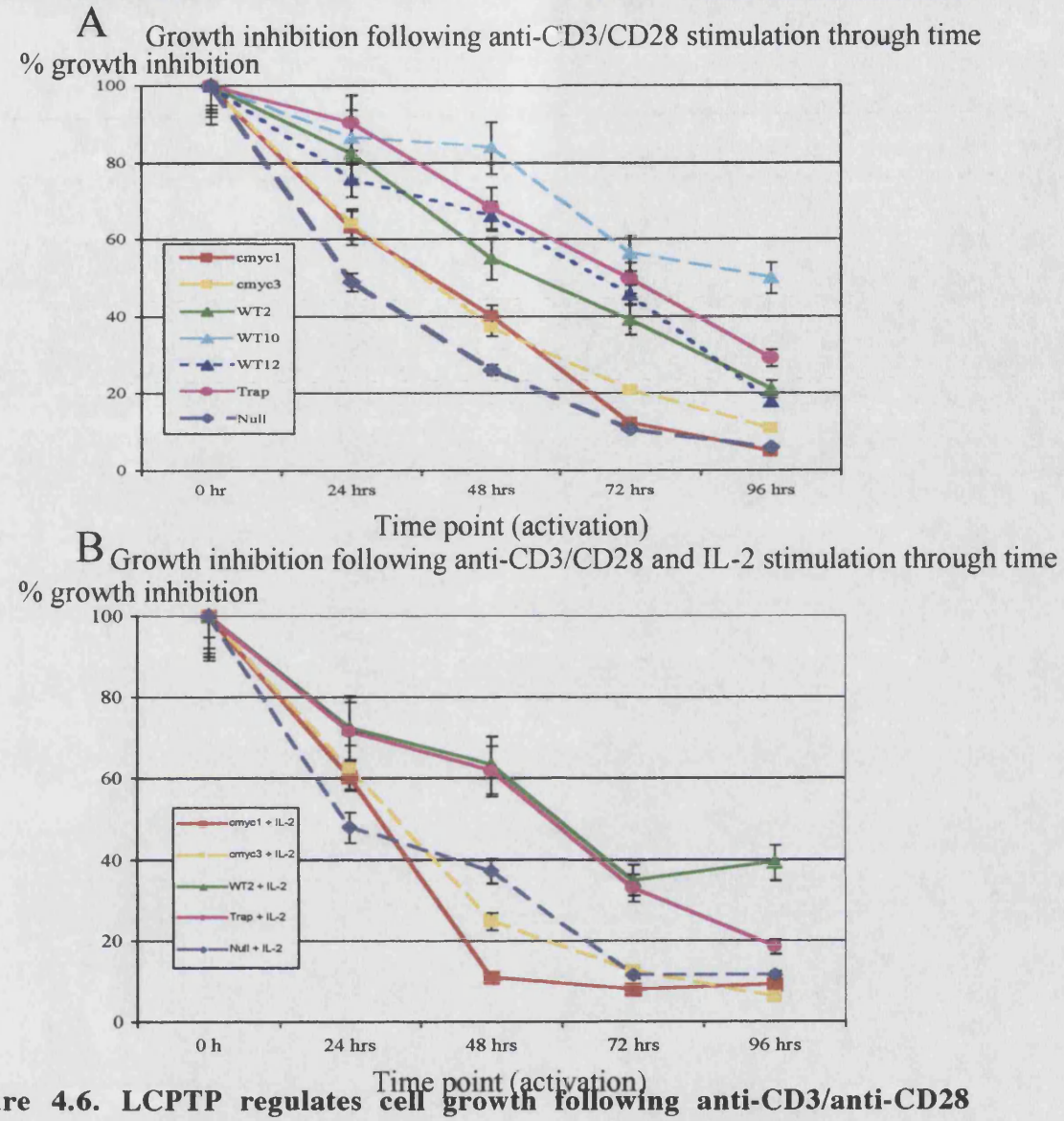
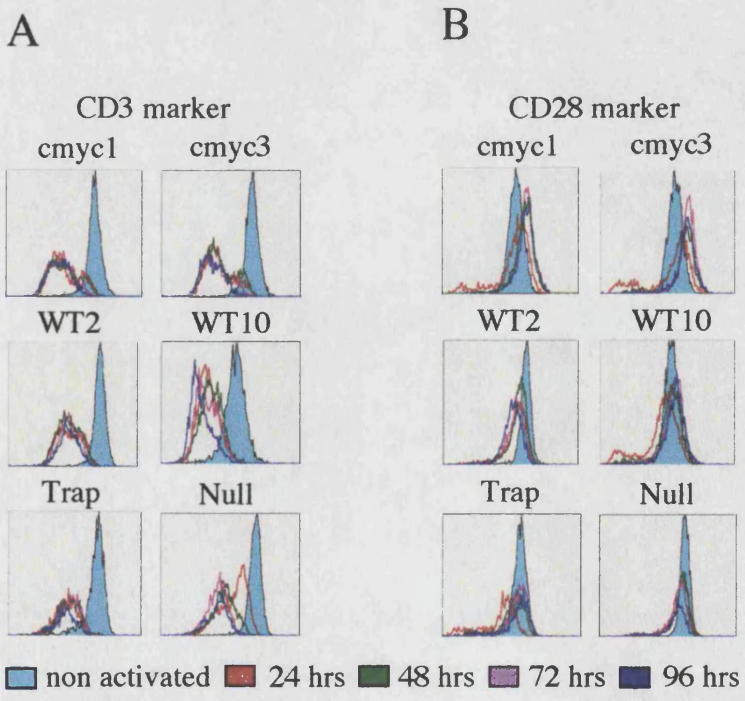


Figure 4.6. LCPTP regulates cell growth following anti-CD3/anti-CD28 stimulation.

The growth of the different cell lines in response to anti-CD3/CD28 stimulation was monitored over a period of 96 hours by measuring (Koch et al.1991) thymidine incorporation (cpm),added during the last 4 hours of culture. Cpm are expressed as a percentage of the non activated control sample (100%). A. Growth curves for each cell line following anti-CD3/CD28 stimulation: cmyc1 (red squares), cmyc3 (yellow squares), WT2 (green triangles), WT10 (light blue triangles), WT12 (dark blue triangles), Trap (pink circles), Null (grey diamonds). B. Growth curves for each cell line following anti-CD3/CD28 stimulation in the presence of 100 ng/ml IL-2: cmyc1 (red squares), cmyc3 (yellow squares), WT2 (green triangles), Trap (pink circles), Null (grey diamonds). Results are the mean of 6 independent experiments.

4.2.7. Effect of LCPTP on surface marker expression in unstimulated and anti-CD3/CD28 stimulated cells

Following T cell activation, the TCR is downregulated and there is an increase in expression of so-called activation markers, including the IL-2 receptor (CD25) and CD69 (Reem et al., 1985); (Ashwell et al., 1986); (Kabouridis and Tsoukas, 1990); (Cerdan et al., 1995); (Villalba et al., 2000). Experiments were performed to determine whether overexpressing LCPTP had any effect on expression of selected surface markers, both in unstimulated and anti-CD3/anti-CD28 stimulated cells. CD3 was expressed in all cell lines and was downregulated following stimulation (Figure 4.7A). CD28 was expressed on all cell lines at an equivalent level and was not downregulated following stimulation (Figure 4.7B). Expression of the CD69 activation marker whose expression rapidly increases following stimulation was likewise not affected by overexpression of LCPTP (Figure 4.7C). In contrast, CD25 was not upregulated in cells overexpressing either wild-type, the substrate-trap or null mutant form of LCPTP. In the cmyc control cell lines, the expression of CD25 was upregulated (Figure 4.7D). The expression pattern of CD25 in the different cell lines following CD3/CD28 ligation correlated with the production of IL-2.



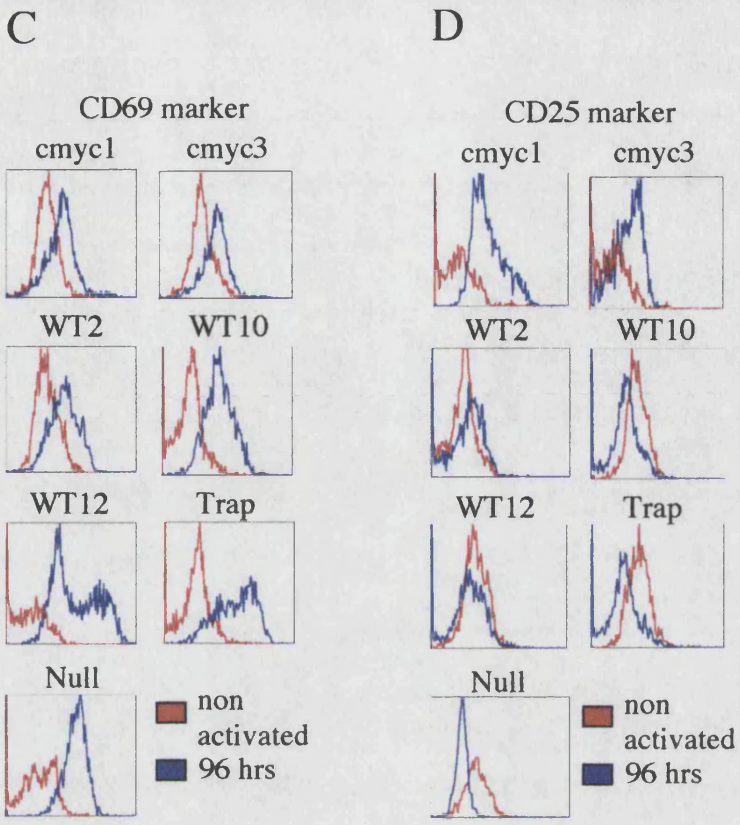


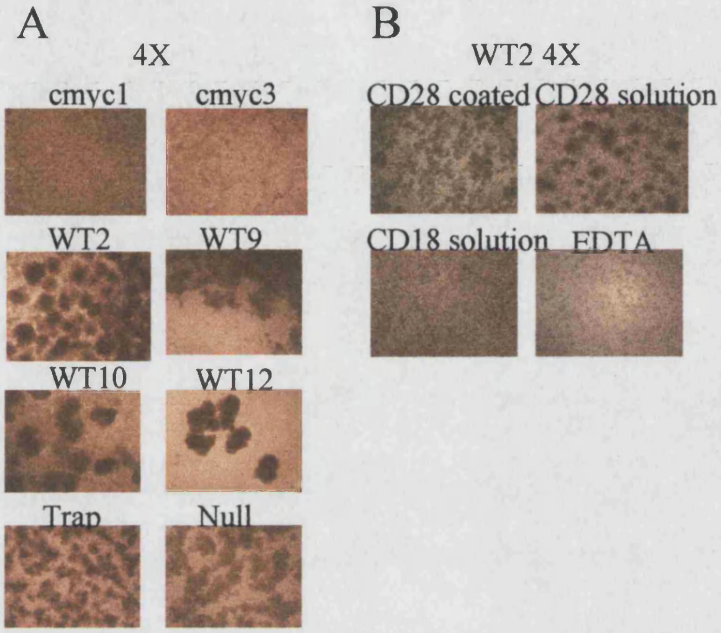
Figure 4.7. Effect of LCPTP on surface marker expression in unstimulated and anti-CD3/CD28 stimulated cells.

Flow cytometric analysis of surface marker expression of stable cell lines A. CD3. B. CD28. C. CD69. D. CD25. A and B: non activated sample (light blue), 24 hrs post-stimulation (red), 48 hrs post-stimulation (green), 72 hrs post-stimulation (purple), 96 hrs post-stimulation (dark blue). C and D: non activated sample (red), 96 hrs post-stimulation (dark blue). Data are representative of 3 separate experiments.

4.2.8. Overexpression of LCPTP induces integrin dependent cell aggregation.

We observed during cell culture of cmyc-LCPTP stable cell lines, an increase in cell aggregation. Control cmyc stable cell lines grow in single cell suspension (Figure 4.8A). In contrast, the cmyc-LCPTP wild-type, the cmyc-LCPTP substrate-trap and null mutant stable cell lines grow in clumps. The aggregation was integrin dependent demonstrated by adding an anti-CD18 antibody to the cultures (Figure 4.8B). It was also noted that anti-CD28 antibody coated onto the plate, partially

disaggregated the clumps while, when added in solution had no effect (Figure 4.8B). Anti-CD3 antibody coated onto wells had no effect on aggregation. The effect was divalent cation dependent, demonstrated by adding the chelating agent EDTA to cultures, which resulted in de-aggregation (Figure 4.8B).



4.8. Overexpression of LCPTP induces integrin dependent cell aggregation.

Photomicroscopy of stable cell lines. A. cmyc1, cmyc3, WT2, WT9, WT10, WT12, Trap and Null mutant cell lines (x4 magnification). B. WT2 stable cell line (x4 magnification) with anti-CD28 coated on plate or added in solution, or anti-CD18 added in solution, or EDTA added in the medium.

4.2.9 Overexpressed LCPTP has no effect on surface expression of adhesion molecules or Fas/Fas ligand

Following the observation that overexpressing LCPTP in Jurkats has an effect on integrin-mediated aggregation, demonstrated by the ability of anti-CD18 antibody to inhibit homotypic cell adhesion, it was of interest to determine whether this was due to upregulation of adhesion molecules relative to control cells. In addition, one possible explanation for the reduction in growth inhibition seen when cells overexpressing LCPTP were stimulated with anti-CD3/CD28 could be changes in Fas (CD95)/Fas ligand (CD178) expression relative to control cells. The expression of CD2 (LFA-2), CD11a (LFA-1 alpha chain), CD62L (L-selectin), CD152 (CTLA-4),

CD154 (ICAM) and Fas and FasL were analysed by flow cytometry (Table 4.1). All cell lines expressed CD2, CD11a, CD62L, CD152 and CD154 at comparable levels, in activated or unactivated samples. The level of expression did not change following 24 hours stimulation with anti-CD3/CD28. Fas and Fas ligand were not expressed on any of the cell lines. The expression of adhesion molecules and Fas and Fas ligand in the different cell lines is summarised in Table 4.1.

Cell lines	Markers							
	CD2	CD11a	CD18	CD62L	CD152	CD154	Fas	FasL
cmyc1	253	364	448	327	55	90	77	85
cmyc3	231	342	475	328	64	83	71	75
WT2	266	349	432	309	87	75	59	64
WT9	283	310	452	325	76	81	63	69
WT10	265	327	437	353	85	76	65	71
WT12	264	328	436	364	81	77	63	74
Trap	258	338	440	329	90	65	84	68
Null	273	359	419	374	63	81	75	72

Table 4.1. Effect of overexpressing LCPTP on adhesion molecules and Fas and Fas ligand.

The table shows the median fluorescence intensity of selected adhesion molecules and Fas and Fas ligand on cell lines.

4.3 Discussion

LCPTP is one of the few tyrosine phosphatases, which is expressed exclusively in hematopoietic cells. It appears to have a restricted number of substrates limited to the MAP kinase family members (Brodeur et al., 2002). Most studies done previously have involved transiently overexpressing the phosphatase, either alone or with the MAP kinase substrate. We have overexpressed LCPTP in Jurkat T cells and generated stable cell lines in order to look at the functional consequences of LCPTP activity in both unstimulated and anti-CD3/anti-CD28 stimulated cells. Initial attemps to make GFP tagged LCPTP constructs (WT, substrate-trap and Null mutant) and make stable cell lines overexpressing these proteins were not successful. We obtained several cell lines overexpressing LCPTP but failed to produce any cells expressing GFP alone. After two to three weeks in culture, clones expressing LCPTP tagged to GFP stopped dividing and died. There are two possible explanations: either LCPTP overexpression inhibits cell growth, or, the presence of GFP is toxic to the cells and when tagged to LCPTP, the construct was less toxic than GFP alone, but ultimately, cell survival was affected and longterm survival of clones was not possible (Stratagene, Drs Nigel Sharp and Madeleine Carreau, personal communication, (Bell et al., 2001)).

In order to ascertain whether it was the GFP tag or the LCPTP protein itself which was affecting longterm cell survival, efforts were made to generate alternative c-myc tagged constructs for transfection studies. These included c-myc alone as a control, c-myc-LCPTP wild-type and two mutant forms of LCPTP, both lacking phosphatase activity: the c-myc-LCPTP substrate trap protein, as the name implies, binds irreversibly to its substrate; in contrast, the c-myc-LCPTP null mutant binds reversibly to its substrate. A number of independent clones were obtained expressing the cmyc-LCPTP wild-type recombinant protein but despite repeated attempts, only one surviving clone was obtained for the substrate-trap recombinant protein. The clone obtained expressed the substrate-trap form of LCPTP at equivalent levels to the endogenous protein and so it is possible that the reason this clone survived was that the levels were sufficiently low enabling signalling via the endogenous LCPTP, or sufficiently low that some ERK was still free. Overexpression of the LCPTP substrate-trap could block all downstream signalling from its substrate MAP kinases

and effectively stop MAP kinase dependent cell proliferation (Dumont et al., 1998); (DeSilva et al., 1998). Although LCPTP phosphatase is overexpressed in cells, its activity does not appear to affect cell cycle progression, as judged by the observation that the growth of cmyc-LCPTP WT, Trap and Null mutant cells is similar to the growth of cmyc control cell lines. Thus, LCPTP overexpression in Jurkat T cells is not detrimental to cell survival. This points to GFP being toxic to cell growth as the explanation for why it was not possible to establish cell lines overexpressing GFP tagged LCPTP proteins.

Cell lines overexpressing cmyc-LCPTP wild-type protein have been shown to reduce the level of MAPK phosphorylation after stimulation with anti-CD3/anti-CD28 antibodies compared to control cell lines expressing the cmyc tag alone. This provides evidence that LCPTP is active in cells in which it is overexpressed. As would be predicted, the phosphorylation of the MAPK family members was increased in the presence of the cmyc-LCPTP substrate-trap. LCPTP substrate trap binds the phosphorylated forms of ERK1 and ERK2 but does not remove the phosphate and thus the tyrosine phosphorylated forms of ERK1 and ERK2 accumulate in cells. The interaction of the null mutant is reversible and so it can bind to its substrate but will disassociate without dephosphorylating its target. There are two possible explanations why an increase in ERK phosphorylation is seen when the null mutant is overexpressed in cells. Firstly, LCPTP contains a kinase interaction motif (KIM). KIMs were first identified in the phosphotyrosine phosphatases, PTP-SL and STEP, which are involved in regulating MAP kinase activity (Pulido et al., 1998). Previous studies looking at interactions of overexpressed LCPTP with MAP kinases proposed a model in which LCPTP interacted constitutively with its protein substrates (Oh-hora et al., 1999); (Saxena et al., 1999). If this was the case, then the null mutant could bind to MAP kinases irreversibly via the KIM motif and sequester the MAP kinase away from endogenous LCPTP activity. We do not believe this to be the case, as we were not able to show a constitutive interaction of endogenous LCPTP with MAP kinases and concluded that LCPTP forms transient associations with its substrates via its phosphatase domain (Brodeur et al., 2002). A second explanation why overexpression of the null mutant leads to an increase in MAP kinase phosphorylation is that the protein is overexpressed compared to the endogenous LCPTP and will

therefore compete with the endogenous LCPTP for its substrate. The competition prevents active LCPTP from encountering its substrate and the balance of MAP kinase phosphorylation is shifted.

Jurkat T cells do not require IL-2 for proliferation although they are capable of producing IL-2 following anti-CD3 and anti-CD28 stimulation. Previous studies have shown that both LCPTP mRNA and protein were increased after TCR or IL-2 stimulation (Adachi et al., 1994). Also, transiently overexpressed LCPTP had a strong inhibitory effect on the transcriptional activation of the luciferase gene driven by the NFAT/AP-1 promotor region derived from the IL-2 gene (Saxena et al., 1998). This suggested that LCPTP could be involved in negatively regulating IL-2 production. We have shown in the present study that there is a significant reduction in IL-2 production following stimulation of cells expressing either the wild-type or the substrate-trap form of LCPTP. There is also an inhibitory effect mediated by overexpressing the null mutant form of LCPTP. This data supports previous studies in Jurkat T cells in which a dominant negative form of ERK1 was overexpressed resulting in a reduction in TCR stimulated IL-2 production (Li et al., 1999a). Thus, a reduction in MAPK activity in Jurkat cells has a direct effect on the production of interleukin-2. Although a visible effect is seen on the MAPK phosphorylation, no change is observed in the general tyrosine phosphorylation pattern in activated cell lines.

When we initiated our studies to elucidate the functional effects of LCPTP activity in cells, the plan was to control experiments by comparing the differences seen when overexpressing wild-type protein, compared to mutant forms of LCPTP lacking phosphatase activity. The results were unexpected in that overexpressing the wild-type and both the substrate trap and the null mutant LCPTP proteins resulted in inhibition of IL-2 production. However, this can be explained by considering how signalling via the MAP kinases works. Upon phosphorylation by MAP kinase kinases (MAPKK), the MAP kinases translocate to the nucleus and phosphorylate transcription factors, which initiate gene transcription (DeSilva et al., 1998). The wild-type protein prevents activation of the MAP kinase by keeping it in an inactive state by dephosphorylation of the regulatory tyrosine residues. With the substrate-

trap protein, MAP kinase can be phosphorylated, but is then trapped irreversibly by the mutant LCPTP protein in the cytoplasm, preventing the translocation of the active MAP kinase to the nucleus. This in turn, prevents the phosphorylation and activation of the transcription factors necessary for inducing IL-2 gene transcription. A similar explanation can be proposed for the null mutant having the same effect on reducing IL-2 production compared to the wild-type and the substrate-trap LCPTP proteins. There was an increase in MAP kinase activity in cells overexpressing the mutant protein, but the active kinase could be hindered from translocating to the nucleus because of continuous transient associations with the overexpressed null mutant. There is a suggestion that this could indeed be the case by the relative inhibition seen with the null mutant compared to wild-type protein. There is less inhibition seen with the null mutant, which can be explained by some of the active MAP kinase being able to translocate to the nucleus and fulfil its signalling functions (see Figure 4.8).

Depending on the activation state of T cells and external factors such as the presence of costimulation, cytokines, etc., TCR stimulation can induce cells to proliferate, become anergic or enter into apoptosis. Jurkat T cells when activated with antibodies to the invariant CD3 chain of the TCR complex and an antibody, which ligates the CD28 costimulatory molecule, undergo growth inhibition (Makover et al., 1991). This growth inhibition in Jurkat T cells has been shown to be due to a reduction in expression of cyclin D3 resulting in cells arresting in the G1 phase of the cell cycle (Boonen et al., 1999). ERK activity is required for activation of cyclin D3 and cyclin D3 activity can be blocked using a MAP kinase inhibitor (Dolci et al., 2001). Since ERK activity is reduced by overexpressed LCPTP, one would predict that overexpressing wild-type LCPTP protein in cells would increase the growth inhibition effect mediated by CD3/CD28 ligation. Contrary to what was expected, overexpressing both wild-type and substrate-trap forms of LCPTP appeared to protect against CD3/CD28 stimulated growth inhibition. The reason for this observation remains unclear.

MAP kinases are involved in mediating signals triggered by diverse stimuli, including cytokines, growth factors and environmental stress leading to growth, differentiation and cell death (Rincon et al., 2000). The functional effects seen when

LCPTP is overexpressed in Jurkat T cells, could be due to any number of explanations, all of which require further investigation. One explanation for a more sustained growth in cell lines overexpressing LCPTP following anti-CD3/anti-CD28 stimulation could be that something other than cyclin D3, signalling downstream from LCPTP and MAP kinases is involved in regulating cell cycle progression. The LCPTP gene is often duplicated in cases of myeloid leukemia, which results in changes in LCPTP activity, which might contribute to unregulated growth and establishment of clonal dominance (Zanke et al., 1994). We also considered whether a change in expression of Fas and its ligand on the surface of cells could contribute to prolonged survival, but flow cytometric analysis showed there were no differences in surface expression following activation for Fas, FasL or other extracellular markers looked at.

One of the downstream functional effects of MAPK activation, is the upregulation of the early activation marker CD69 on the cell surface (Villalba et al., 2000). In our studies, we would have expected to see some effect on the expression of CD69 following cell activation in the presence of overexpressed LCPTP. The expression of CD69 on the surface of cells is the same in all cell lines following CD3/CD28 stimulation. This suggests that in Jurkat T cells, expression of CD69 is not influenced by LCPTP dephosphorylating the MAP kinases. Iwashima and collaborators have identified an adapter protein, Shc, which like LCPTP, influences the MAPK pathway but does not affect CD69 expression. They found that Shc is essential for IL-2 production in J.SL1 T cells, but is dispensable for cell surface expression of CD69 and CD25 (Iwashima et al., 2002). In contrast, inducible expression of another tyrosine phosphatase, CD148, in Jurkats, has been shown to inhibit TCR induced ERK phosphorylation and CD69 upregulation (Baker et al., 2001).

In contrast to CD3, CD28 and CD69, we saw changes in the expression of the IL-2 receptor, CD25. CD25 is upregulated on the surface of activated T cells in response to TCR/CD28 stimulation and is maintained on the cell surface in the presence of IL-2 (Reem et al., 1985); (Ashwell et al., 1986); (Kabouridis and Tsoukas, 1990); (Cerdan et al., 1995). Expression of CD25 also controls T cell proliferation (Reem et al., 1985); (Cerdan et al., 1995). CD25 was upregulated on the

surface of cells expressing the cmyc control protein, but failed to be upregulated following cell activation in the presence of the overexpressed wild-type, trap and null mutant LCPTP proteins. This data correlated with the reduction in the production of IL-2 from the cells and suggests that unlike CD69, expression of CD25 in Jurkat cells is dependent on MAP kinase activity.

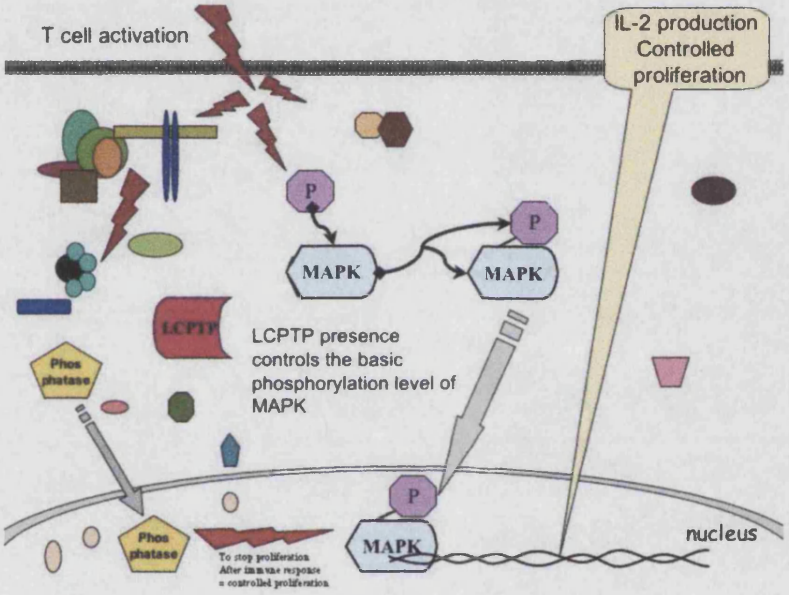
One final observation regarding the functional consequences of overexpressing LCPTP in Jurkat T cells was the tendency of cells to aggregate in culture. The increased homotypic aggregation seen when overexpressing LCPTP was integrin mediated, demonstrated by the fact that an antibody to CD18, the common beta chain of LFA-1, inhibited cell clumping. Addition of EDTA to cultures also inhibited cell aggregation, indicating that the effect was divalent cation dependent. This data supports previous studies in which overexpression of LCPTP altered fibroblast morphology. Transfection of LCPTP into fibroblasts (NIH 3T3) enabled cells to form colonies in soft agar (Zanke et al., 1994). The increased cell aggregation was not due to changes in LFA-1 or ICAM expression although it could reflect changes in integrin affinity. Thus LCPTP could increase binding integrin potential, via a MAPK independent pathway since downstream signalling of MAP kinase augments the affinity of integrins (O'Rourke et al., 1998).

There is still much to be learnt about the role of LCPTP in T cells. One obvious question for LCPTP is how and when its activity is regulated. We observed that the cell aggregation effect could be inhibited by plate bound anti-CD28 but not by plate bound anti-CD3. Anti-CD28 in solution was less effective at inhibiting the cell clumping. Taken together, these results suggest that LCPTP activity could in part be regulated by signalling via CD28 and requires crosslinking, which is mimicked by binding the antibody to plastic. CD28 is widely recognised as being an important costimulatory molecule for T cell activation and IL-2 production (June et al., 1990); (Schwartz, 1992); (Linsley and Ledbetter, 1993). It could be envisaged that CD28 signalling is important for 'switching off' negative signalling effects. This has been reported to be the case for another T cell phosphatase, Lyp. Lyp targets Cbl-b, a protein involved in the negative regulation of T cell signalling. Upon CD28 ligation, Lyp maintains Cbl-b in a dephosphorylated state and allows activation of signalling

pathways to proceed unimpeded which lead to IL-2 gene transcription (Cohen et al., 1999)[2].

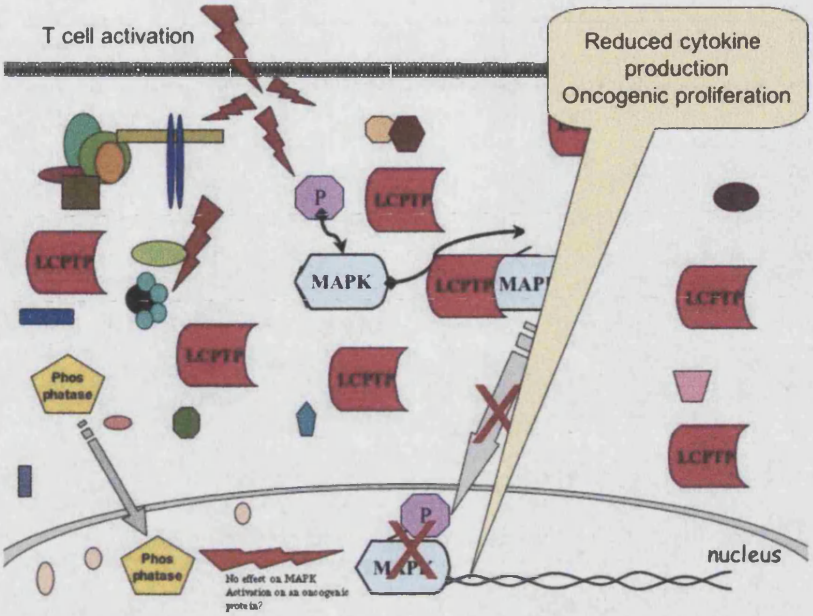
Studies have been initiated to determine how relevant the observations made in Jurkats are to what happens when LCPTP is overexpressed in primary T cells, these are described in the next chapter.

A



[2] The work described in this chapter has been submitted to Journal of Immunology.

B



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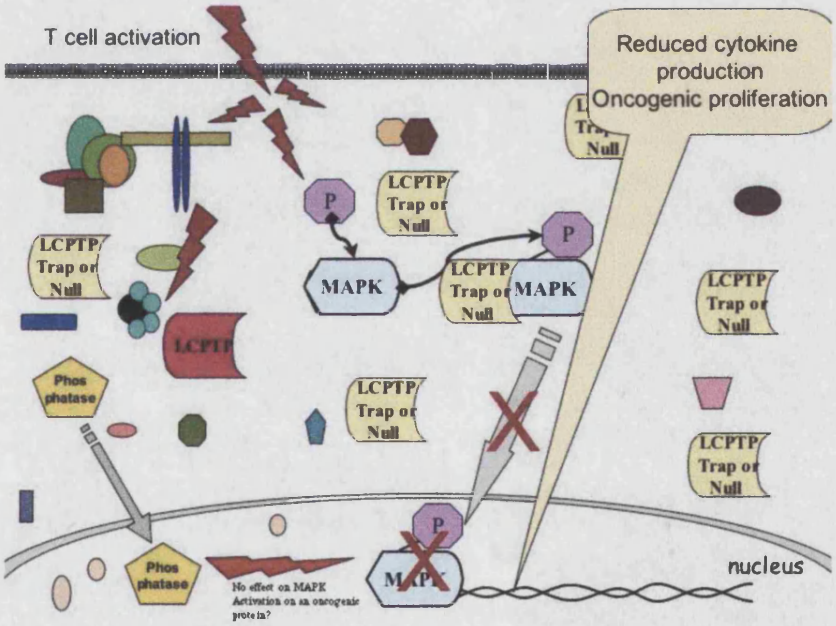

Figure 4.9 Model to show the effect of A) endogenous LCPTP, B) overexpression of LCPTP wild-type and C) overexpression of substrate-trap or null mutant LCPTP in Jurkat T cells, on MAP kinase translocation into the nucleus.

CHAPTER FIVE: RESULTS
Effect of overexpressing LCPTP in primary T cells

5.1 Introduction

In the previous two chapters (Chapters 3 and 4) Jurkat T cells have been used as a model to characterise the role of LCPTP in T cell signalling pathways. Jurkats have the benefits that they can be grown in large numbers and are relatively easy to transfect. However, there are some discrepancies between leukemic cell lines, such as Jurkat cells and nontransformed T cells, or primary T cells. One of the reasons why Jurkat T cells may behave differently to primary T cells is that Jurkat T cell lines do not express the PTEN or SHIP protein phosphatases (Shan et al., 2000); (Astoul et al., 2001) causing an imbalance in the PI3K pathway. This results in an abnormal and constitutive localisation of the tyrosine kinase, ITK, at the cell membrane and a deregulation of actin metabolism. Thus these factors should be considered when studying the TCR pathway using transformed cells and any conclusions drawn may differ in primary T cells. In order to address this, a relatively new strategy was adopted which facilitates transfection of primary T cells. The technique employed to overexpress LCPTP in $CD4^+$ T cells was to infect primary T cells using adenovirus for gene delivery.

Adenoviruses replicate in the host nucleus using their enzymes to synthesise DNA and mRNA. Adenoviruses are used in research to study mRNA synthesis and regulation, DNA replication and they are also used in cellular transformation (Feldman et al., 1997). The adenovirus used was engineered for safe use in the laboratory by depleting both the E1 1 gene essential for viral replication and the E3 gene, which is implicated in the evasion of the hosts immune response to the virus (Richards et al., 2000). The adenoviruses need a membrane receptor to attach and enter cells. This receptor is called CAR which stands for Coxsackie-adenovirus receptor (Bergelson et al., 1998). All human cells possess the receptor for adenovirus entry except skin fibroblasts, alveolar macrophages, smooth muscle cells and lymphocytes. James DeGregori's team engineered a transgenic mouse expressing the human CAR receptor on all cells. This allows the infection of primary T cells with adenovirus constructs expressing the gene of interest and enables the observation of the effects of overexpresssing the protein in cells. Unlike other systems used for

overexpressing proteins of interest in primary cells (eg. retrovirus), the adenoviral delivery system does not depend on cells being in cycle in order to infect and integrate into the host DNA. The adenovirus does not integrate into the host DNA. This overcomes another problem often encountered during transfection studies because there is no random integration of the transfected protein into the host cell's genome which could potentially leading to mutations in proteins key to signalling pathways of interest in the functional studies performed (Leon et al., 1998).

Utilising the adenoviral technology, studies have been done not only looking at effects of LCPTP on murine naïve T cells, but also on antigen differentiated Th1 and Th2 cells. These studies have made use of the double transgenic CAR-1-DO11.10 mice. In addition to the CAR receptor, all the T cells in the mice have been engineered to express a T cell receptor specific for an a ovalbumin peptide and can therefore be activated *in vitro* in the presence of APCs and peptide. Naïve $CD4^+$ T cells were purified from CAR-1-D011.10 and either used directly, or differentiated into Th1 and Th2 subsets using antigen and the appropriate cytokines, and infected with adenovirus containing LCPTP wild-type or substrate-trap proteins. The effect of overexpressing LCPTP in primary T cells was monitored by following the responses of cells to restimulation with anti-CD3 and anti-CD28 antibodies and measuring the effect of the phosphatase on MAP kinase activation, cytokine production and cell proliferation.

CHAPTER FIVE

5.2 Results

5.2.1 Cloning of LCPTP into adenovirus vector and transient transfection.

In order to transfect LCPTP into primary T cells, the phosphatase had to be cloned into the Admid system for generation of recombinant viruses (Richards et al., 2000). The first step involved cloning LCPTP into pCR259 vector. The genes cloned were LCPTP wild-type, substrate-trap and LCPTP wild-type cloned in backwards to produce anti-sense. Once cloned, the plasmid expression potential was verified by transiently transfecting Jurkat T cells and probing for LCPTP protein. LCPTP wild-type and substrate-trap were both highly expressed in Jurkat T cells. In contrast, there was no overexpression of proteins seen in cells overexpressing LCPTP anti-sense protein.

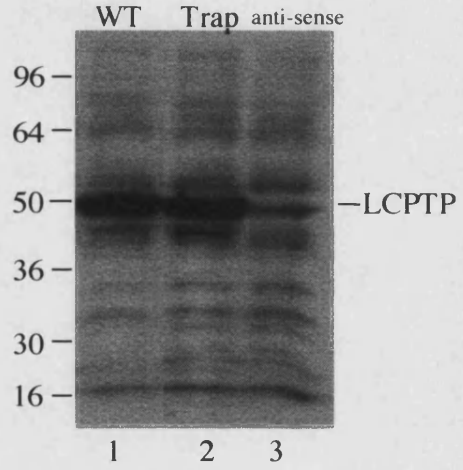

Figure 5.1 Transient transfection of pCR259-LCPTP wild-type, substrate-trap and anti-sense into Jurkat T cells.

Western blot of crude lysate of 5 x 10^5 cells probed with anti-LCPTP. 1) pCR259-LCPTP wild-type; 2) pCR259-LCPTP substrate-trap; 3) pCR259-LCPTP anti-sense. Data are representative of two separate experiments.

5.2.2 Concentration of viruses by caesium chloride gradient

In order to achieve efficient infection of T cells, it was necessary to obtain concentrated recombinant viral stocks. HEK cells were infected with virus and supernatants from cells collected. The virus was concentrated from culture supernatants using caesium chloride gradients. The first gradient was a step gradient formed with 1.2 and 1.4 M caesium chloride solutions and the second gradient was a

continuous gradient made with 1.2 and 1.4 M caesium chloride stock solutions. The gradients allow the separation of defective virus particles from the infectious viruses and concentrate the virus for infection of primary T cells. Figure 5.2 illustrates examples of each gradient and the separation of viruses.

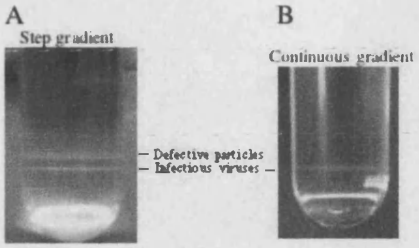

Figure 5.2. Photograph showing concentration of viruses using caesium chloride gradients.

A. Step gradient of 1.2 and 1.4 M caesium chloride. B. Continuous gradient of caesium chloride. Infectious viruses and defective particles are shown on the picture.

5.2.3 Th1/Th2 differentiation from primary CAR-1-D011.10 T cells

Primary T cells were isolated from mouse spleens, and differentiated into Th1 and Th2 cells using a cocktail of antibodies and appropriate cytokines and APCs presenting antigen, in the form of ovalbumin peptide. Intracellular cytokine staining was performed in order to characterise the phenotypes of the Th1 and Th2 cell populations generated. The CAR-1-D011.10 mice have been bred onto a BALB/c background. BALB/c mice T cells have a natural bias to differentiate towards a Th2 phenotype. The primary cells when pushed towards a Th2 phenotype produced IL-4 and no IFN-γ. In contrast, primary cells stimulated under Th1 differentiating conditions produced IFN-γ in addition to IL-4 (Ouyang et al., 2000); (Lee et al., 2000); (Ho et al., 1996); (Zheng and Flavell, 1997); (Feber, 1999). Figure 5.3 illustrates example of Th1 and Th2 phenotypes, unstimutated and stimulated with anti-CD3 and anti-CD28

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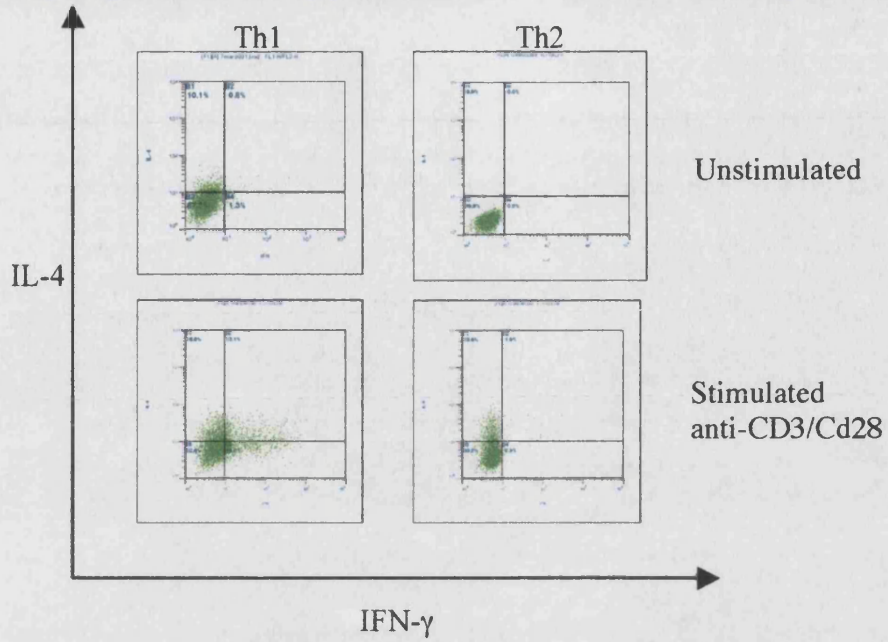


Figure 5.3. Th1/Th2 differentiation from primary T cells taken from CAR-1-D011.10 transgenic BALB/C mice.

Flow cytometry measurements showing intracellular staining of resting and activated Th1 and Th2 cells differentiated from primary CAR-1-D011.10 T cells. Cells are double stained with FITC conjugated antibody to IFN-γ and PE conjugated antibody to IL-4.

5.2.4 Optimisation of multiplicity of infection (m.o.i.) for overexpressing LCPTP in mouse primary T cells.

Conditions were evaluated in order to achieve overexpression of LCPTP in primary T cells and maintain a reasonable cell survival rate after infection. Different amounts of virus were added to equivalent cell numbers and cell lysates were probed for the presence of LCPTP. In addition, cell survival rate was monitored by counting viable cells following staining with Trypan blue. Figure 5.4A shows the increase in LCPTP protein expression in Th1 cells after infection with different m.o.i. of adenovirus containing wild-type LCPTP. 5 viruses per cell was sufficient to detect an increase in expression of LCPTP compared to the non-infected cells. Figure 5.4B shows the survival rate of Th1 and Th2 cells after infection with different m.o.i. of adenovirus containing either the wild-type LCPTP, the mutant substrate-trap form of LCPTP and the Th2 transcription factor Gata3 compared to non-infected cells (Zheng

and Flavell, 1997). The Gata3 adenoviral construct, was used as a positive control which had been shown previously in the laboratory to positively affect Th2 cytokine production (Nigel Sharp, personal communication). Similar survival was apparent between Th1 and Th2 cells infected with the same virus. In cells infected with LCPTP wild-type or substrate-trap protein there was a decrease in cell survival compared to infection with Gata3 protein. This decrease in survival correlated with the ability of cells to aggregate and form clumps in culture. Th1 and Th2 cells infected with LCPTP wild-type or substrate-trap protein formed clumps in the medium, as early as one hour after infection. This is shown in photomicrographs (x4 magnification) in Figure 5.4C. Gata3 infected cells did not form clumps and appeared similar to the uninfected control cells.

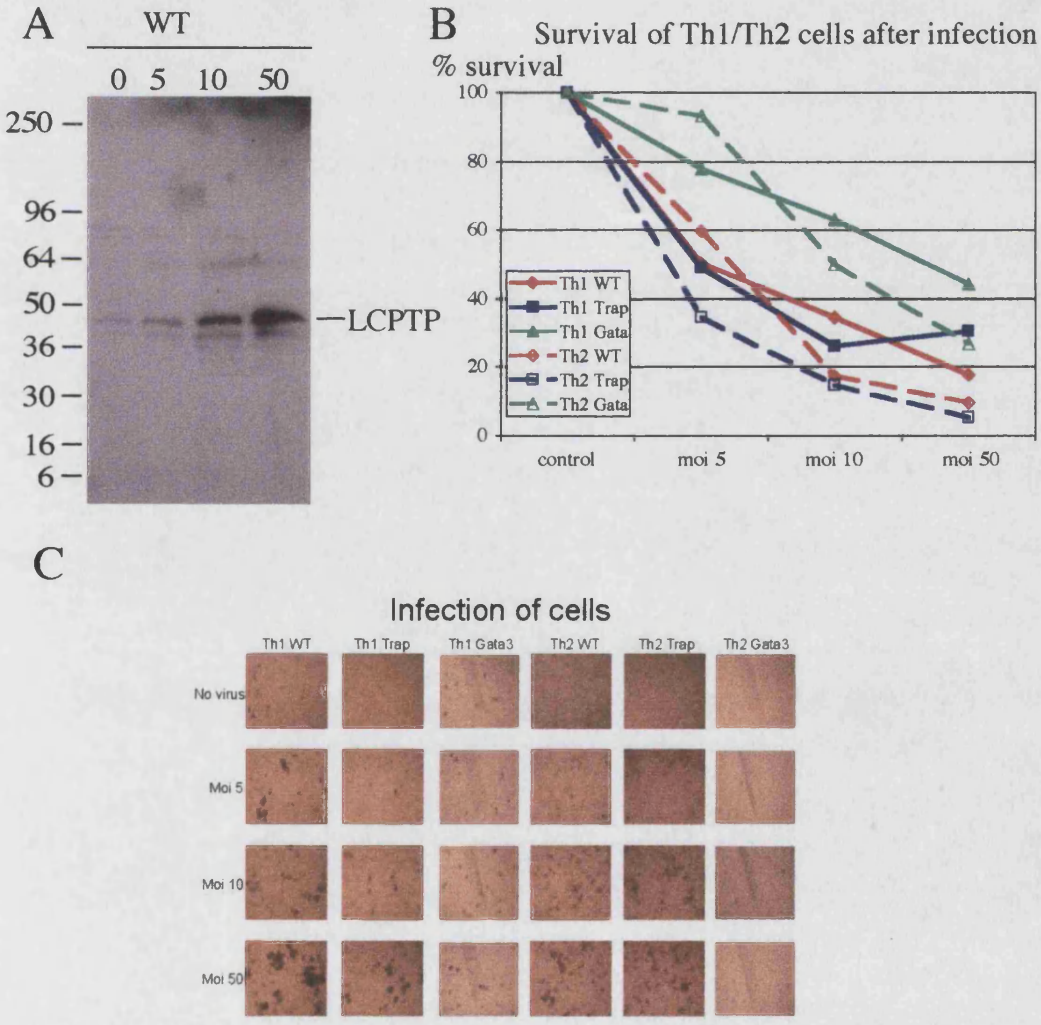


Figure 5.4. Effect of the multiplicity of infection on the expression of LCPTP

protein and growth characteristics of primary cells.

A. Western blot of cell lysates 24 hours after infection, probed for LCPTP. Th1 cells infected with different m.o.i. of LCPTP wild-type virus: 0, 5, 10, and 50. B. Survival rate of Th1 and Th2 cells at 24 hours following virus infection compared to non-infected cells. Cells were counted and data are given as a percentage of the viable uninfected control cell population. LCPTP wild-type (red); LCPTP substrate-trap (blue); Gata3 (green); solid line = Th1 cells, broken line = Th2 cells. C. Photomicrography (x4 magnification) of Th1 and Th2 cells either uninfected or infected with LCPTP wild-type, LCPTP substrate-trap and Gata3 viruses at m.o.i. of 5, 10 and 50. Data are representative of 2 separate experiments.

5.2.5 LCPTP wild-type and substrate-trap overexpression in Th1 and Th2 cells.

In order to have a significant amount of LCPTP expressed in cells and maintain a sufficient survival rate, a m.o.i. of 10 was selected as optimal for further experiments. Figure 5.5 shows a Western blot probed with LCPTP antibody from cell lysates of Th1 and Th2 cells infected with the LCPTP viruses at a m.o.i. of 10. The endogenous level of LCPTP in control uninfected Th1 and Th2 cells is shown in lanes 1 and 4 respectively. In comparison, there is an increase in the expression of LCPTP seen in cells infected with LCPTP wild-type (lanes 2 and 5) and substrate-trap proteins (lanes 3 and 6).

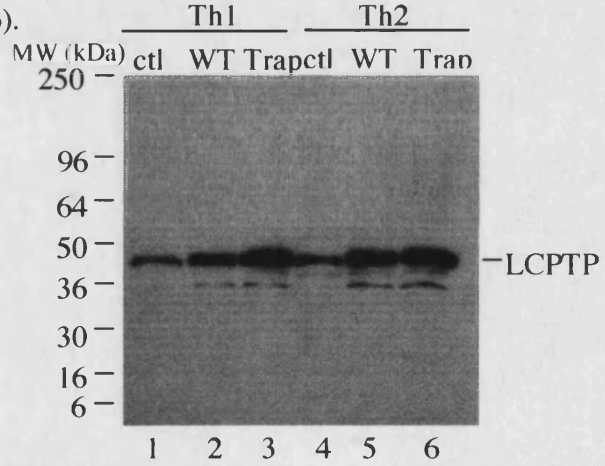


Figure 5.5. LCPTP wild-type and substrate-trap overexpression in Th1 and Th2 cells.

Western blot probed with LCPTP antibody. Uninfected Th1 and Th2 cells = control (ctl) and Th1 or Th2 cells infected with a m.o.i. of 10 for LCPTP wild-type and

substrate-trap viruses. Data are representative of two separate experiments

5.2.6 LCPTP overexpression affects MAPK phosphorylation in Th1 and Th2 cells.

In order to see if the effects seen in stable cell lines overexpressing LCPTP also occur in primary cell lines, Th1 and Th2 cells infected with LCPTP wild-type and substrate-trap were activated with anti-CD3 antibody, lysed and probed with a phospho-MAPK antibody. The endogenous level of MAP kinase phosphorylation in Th1 and Th2 cell lysates (Figure 5.6 lanes 1 and 5) was reduced when LCPTP wild-type is overexpressed in cells (lanes 2 and 6). No difference was observed when the substrate-trap mutant protein was overexpressed (lanes 3 and 7) which is contrary to the results observed in stable Jurkat T cell lines (Figure 4.3). The presence of overexpressed Gata3, as a control for cells infected with a virus gives a slight increase in MAP kinase phosphorylation (lanes 4 and 8).

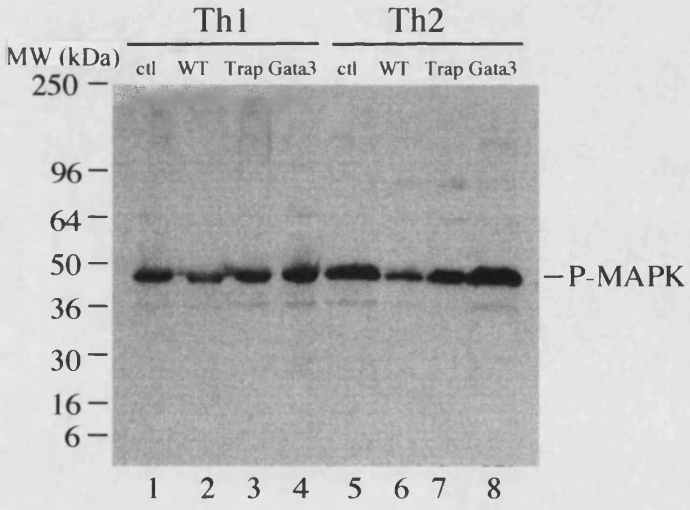


Figure 5.6. LCPTP overexpression affects MAPK phosphorylation in Th1 and Th2 cells.

Western blot probed with a phospho MAPK specific antibody. Cell lysate of anti-CD3 activated cells: Th1 and Th2, uninfected and infected with LCPTP wild-type, substrate-trap and Gata3.

5.2.7 LCPTP affects cytokine production in naïve, Th1 and Th2 cells.

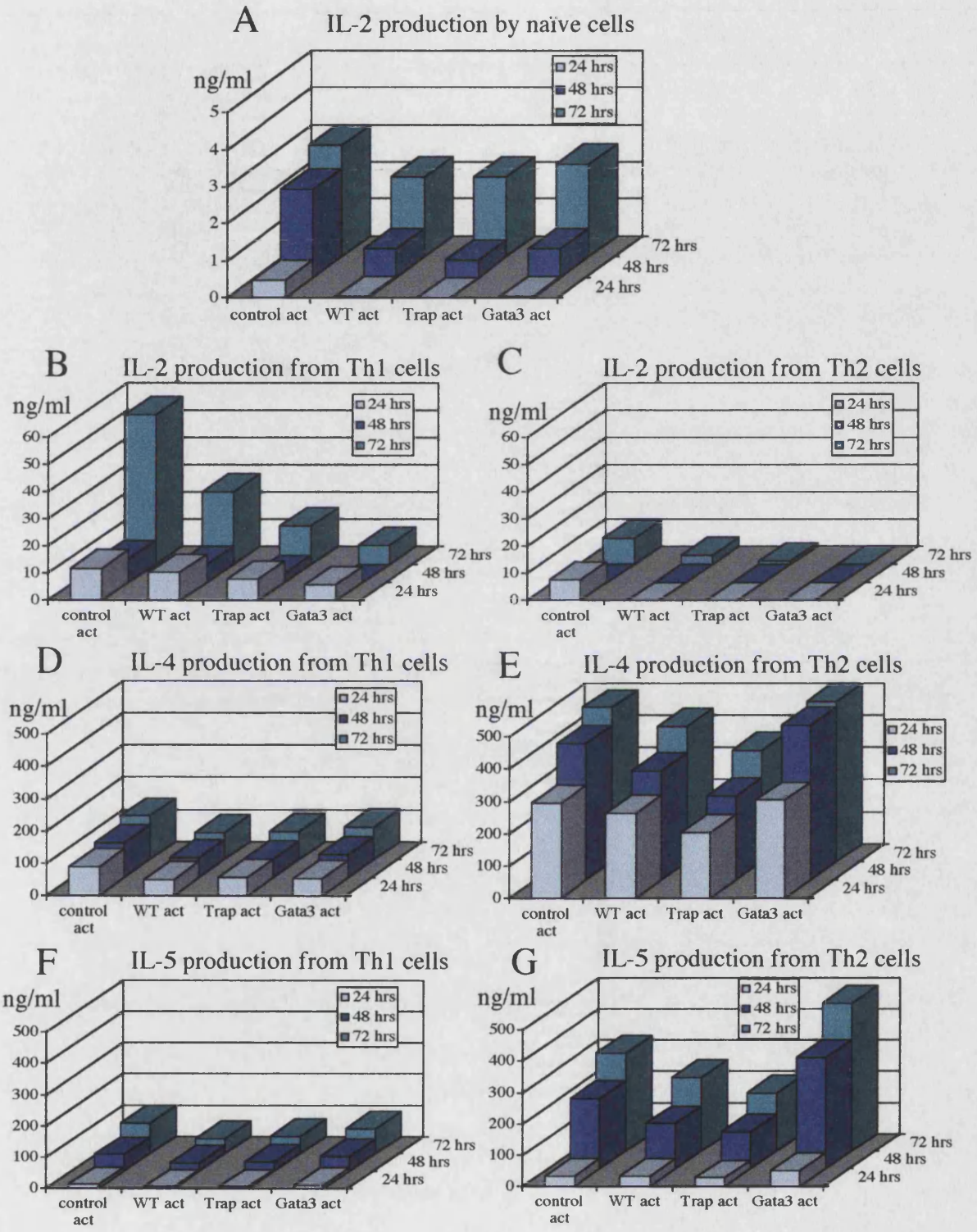
The stable Jurkat T cell lines overexpressing LCPTP wild-type,

substrate-trap and null phosphatase mutant had a reduced level of IL-2 production following anti-CD3 and anti-CD28 activation. This decrease in cytokine production correlated with a decrease in MAP kinase phosphorylation in the LCPTP wild-type overexpressing cell line. In order to see if the overexpression of LCPTP in primary T cells has the same effect, cytokine production following TCR and CD28 activation was monitored. Figure 5.7 shows the cytokine production measured at 24, 48 and 72 hours for IL-2, IL-4, IL-5, IL-13 and IFN-γ. In the case of IL-2, the overexpression of both LCPTP wild-type and substrate-trap, in naïve and both Th1 and Th2 cells, inhibited the production of IL-2 compared to the control uninfected cells (Figure 5.7 A, B and C). IL-2 production was also reduced in cells overexpressing Gata3. This result confirmed previous observations on the functional consequences of overexpressing Gata3 in primary murine cells (personal communication, Dr Andy Blanchard). As would be expected, the production of IL-2 is much lower in Th2 cells compared to Th1 cells (Ouyang et al., 2000); (Lee et al., 2000); (Ho et al., 1996); (Zheng and Flavell, 1997); (Feber, 1999).

The production of IL-4, IL-5 and IL-13 is characteristic of Th2 cells and well differentiated Th1 cells do not produce these cytokines. The CAR-1-D011.10 transgenic so called "Th1" cells produced a small quantity of IL-4, IL-5 and IL-13 due to the fact that the population is not homogeneous for Th1 cytokine secreting cells (see Figure 5.5). Overexpressed LCPTP had no effect on the production of IL-4 (Figure 5.7 D) and IL-5 (Figure 5.7 F) but lead to a decrease in IL-13 production from Th1 cell cultures (see discussion) (Figure 5.7 H). In Th2 cells, the presence of LCPTP wild-type had a small effect on the production of IL-4, IL-5 and IL-13 and a more pronounced effect was seen with overexpression of LCPTP substrate-trap protein (Figure 5.7 E, G and I). The presence of Gata3, an activator of transcription of Th2 cytokines, increased the production of IL-4, IL-5 and IL-13 from Th2 cells (Figure 5.7 E, G and I).

The production of IFN-γ is regarded as specific to Th1 cells (Mosmann et al., 1986); (O'Garra, 1998) and no IFN-γ production was detected from cells differentiated towards a Th2 phenotype (Figure 5.7 K). The overexpression of LCPTP wild-type, substrate-trap or Gata3 protein decreased the production of IFN-γ

from Th1 cells (Figure 5.7 J).



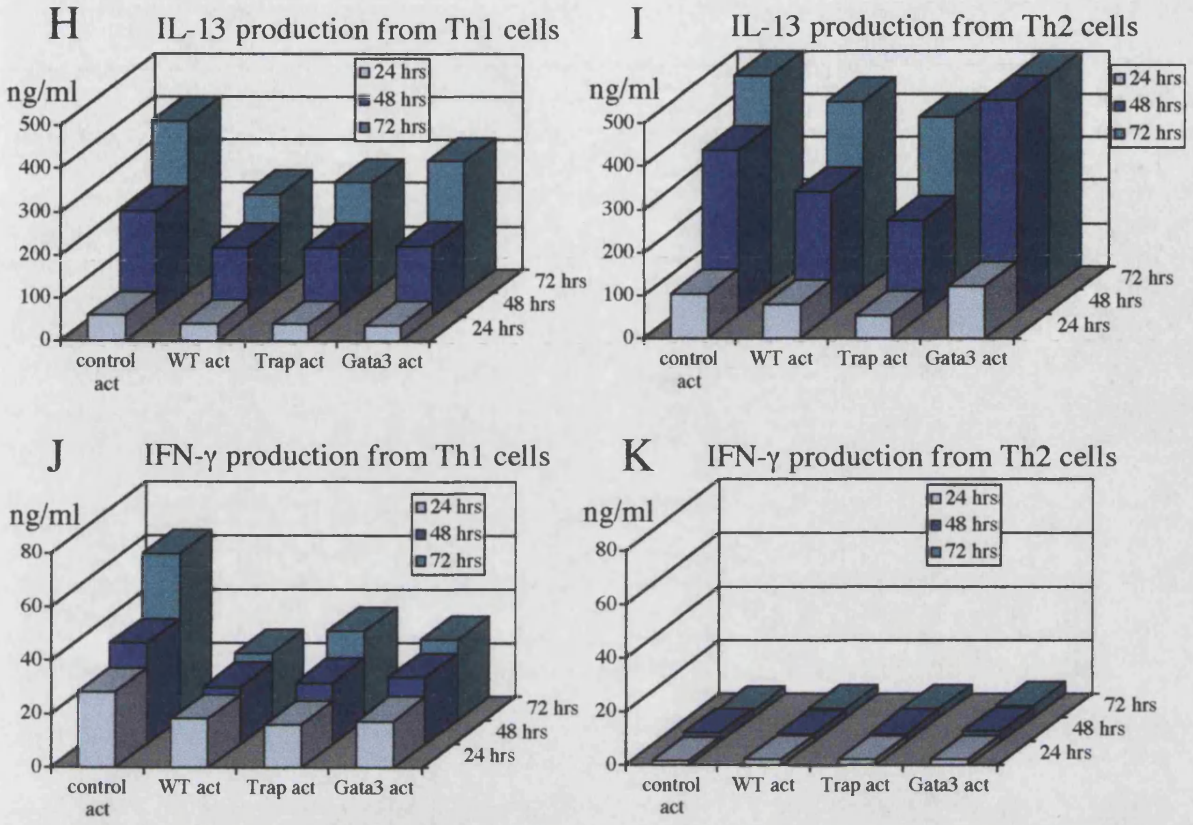


Figure 5.7. Effect of LCPTP overexpression on cytokine production from naïve, Th1 and Th2 cells.

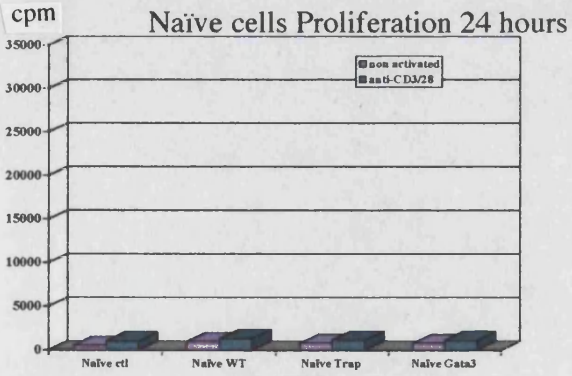
Th1 and Th2 cells, uninfected and infected with adenovirus-LCPTP wild-type, substrate-trap and adenovirus-Gata3 were activated with anti-CD3 and anti-CD28 antibodies. Supernatants were taken and analysed by ELISA for the presence of IL-2 (graphs A and B), IL-4 (graphs C and D), IL-5 (graphs E and F), IL-13 (graphs G and H) and IFN-γ (graphs I and J) cytokines. Cytokine measurements were done over three time points: 24 hours (light blue bars), 48 hours (dark blue bars) and 72 hours (green bars). Data are representative of three separate experiments and all error bars are within 5 percent range.

5.2.8 LCPTP overexpression alters the proliferation capacity of naïve, Th1 and Th2 cells

As a consequence of T cell receptor and CD28 ligation, primary rested T cells undergo proliferation. In contrast, the Jurkat T cell line responded to anti-CD3 and anti-CD28 ligation by undergoing growth inhibition. LCPTP overexpression in Jurkats partially abrogated the CD3/CD28 mediated growth inhibition (Figure 4.6). It was therefore of interest to determine the effect of overexpressing LCPTP on

CD3/CD28 stimulated growth in primary T cells differentiated into Th1 and Th2 cells. Cell proliferation was monitored by measuring ^{3}H-thymidine incorporation (cpm) into cells over a time course up to 96 hours following stimulation with anti-CD3 and anti-CD28 antibodies. Results show the cell proliferation in unstimulated or CD3/CD28 stimulated cells in uninfected cells and cells infected with adenovirus-LCPTP wild-type, substrate-trap and Gata3 proteins (Figure 5.8). At 24 hours post-activation, the differences between the uninfected cells and the infected cells are minimal except for a higher basal proliferation in unstimulated Th2 cells (Figure 5.8D). The proliferation seen at 48 hours shows a higher basal proliferation for both Th1 and Th2 cells and consequently a higher proliferation following TCR and CD28 stimulation for Th1 cells infected with LCPTP (Figure 5.8E and I). The proliferation of Th2 cells was enhanced in cells infected with LCPTP compared to the unifected cells although this difference was small relative to the proliferation of unactivated cells (Figure 5.8E and J). The proliferation of activated naïve cells at 48 and 72 hours was reduced compared to the proliferation of uninfected cells (Figure 5.8B, C and H). The proliferation at 72 and 96 hours for Th1 and Th2 cells was similar with a higher proliferation rate for cells infected with LCPTP compared to uninfected cells and cells infected with Gata3 (Figure 5.8F, G, I and J).

A



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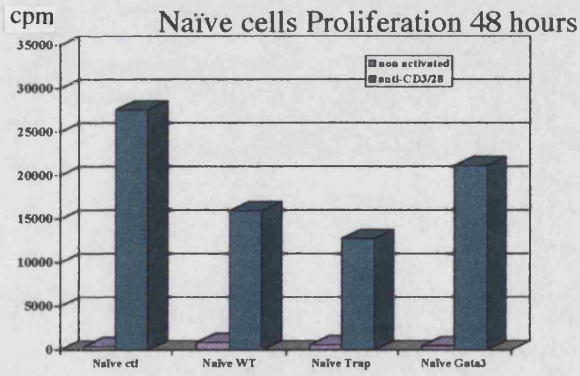

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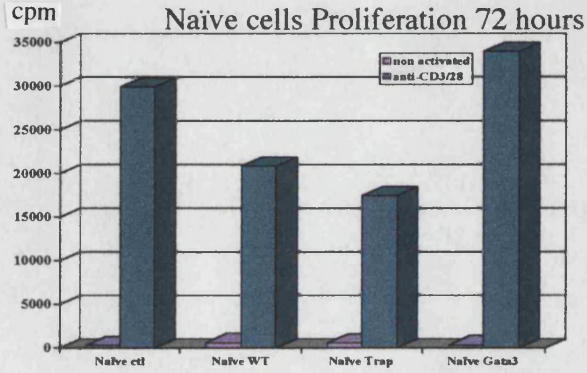

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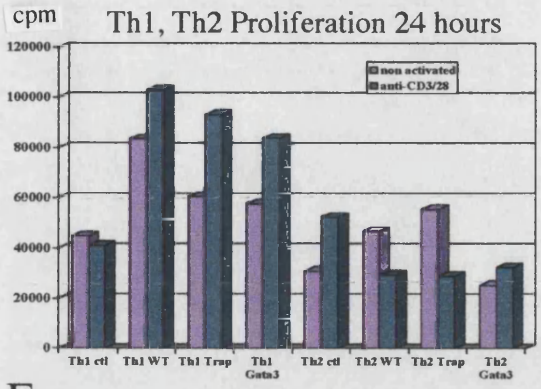
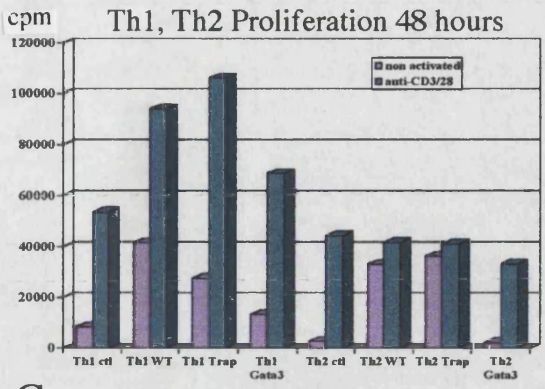

cpm
Th1, Th2 Proliferation 24 hours
non activated
anti-CD3/28
120000
100000
80000
60000
40000
20000
0
Th1 ctl
Th1 WT
Th1 Trap
Th1 Gata3
Th2 ctl
Th2 WT
Th2 Trap
Th2 Gata3

E


cpm
Th1, Th2 Proliferation 48 hours
non activated
anti-CD3/28
120000
100000
80000
60000
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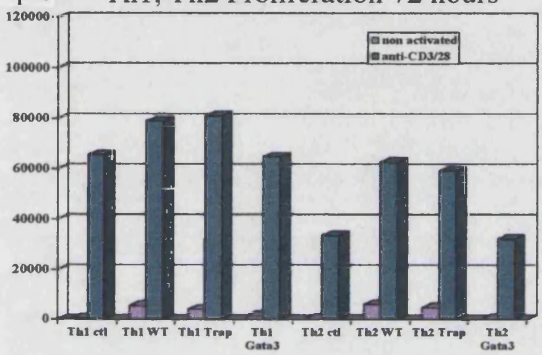
0
Th1 ctl
Th1 WT
Th1 Trap
Th1 Gata3
Th2 ctl
Th2 WT
Th2 Trap
Th2 Gata3

F


cpm
Th1, Th2 Proliferation 72 hours
non activated
anti-CD3/28
120000
100000
80000
60000
40000
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Th1 ctl
Th1 WT
Th1 Trap
Th1 Gata3
Th2 ctl
Th2 WT
Th2 Trap
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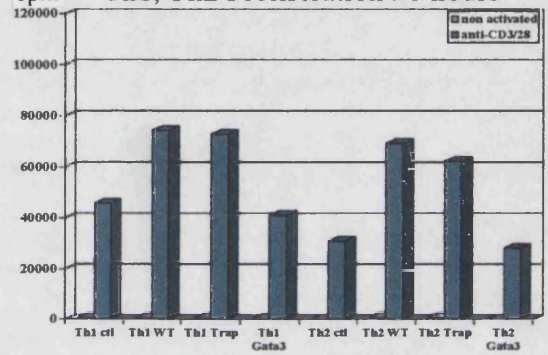
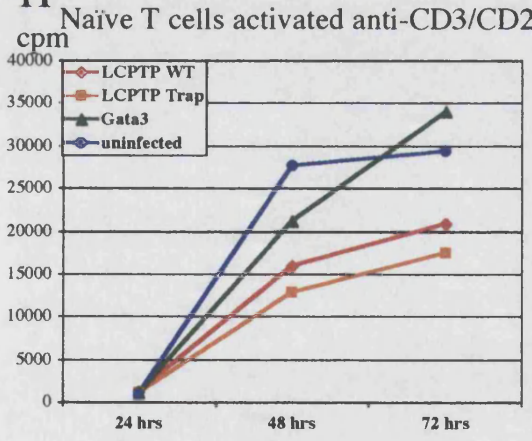
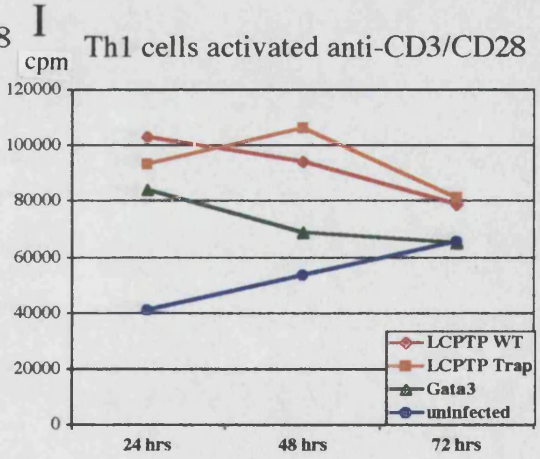
G


cpm
Th1, Th2 Proliferation 96 hours
non activated
anti-CD3/28
120000
100000
80000
60000
40000
20000
0
Th1 ctl
Th1 WT
Th1 Trap
Th1 Gata3
Th2 ctl
Th2 WT
Th2 Trap
Th2 Gata3

H


Naïve T cells activated anti-CD3/CD28
cpm
40000
35000
30000
25000
20000
15000
10000
5000
0
LCPTP WT
LCPTP Trap
Gata3
uninfected
24 hrs
48 hrs
72 hrs

I


cpm
Th1 cells activated anti-CD3/CD28
120000
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LCPTP WT
LCPTP Trap
Gata3
uninfected
24 hrs
48 hrs
72 hrs

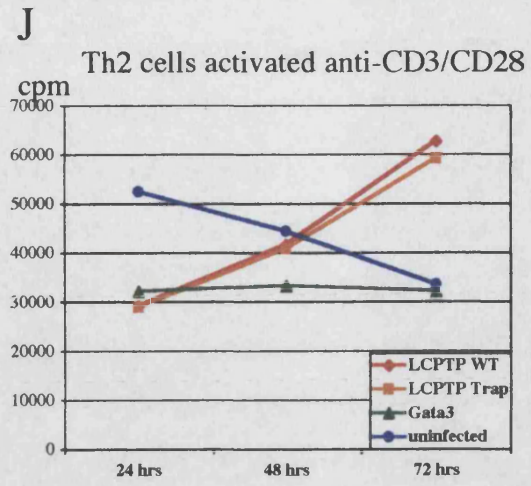

Figure 5.8. Effect of overexpressing LCPTP on CD3/CD28 stimulated proliferation in naïve, Th1 and Th2 cells.

Incorporation of ^{3}H-thymidine expressed as counts per minute (cpm) in Th1 and Th2 cells, either unstimulated or stimulated with anti-CD3 and anti-CD28 antibodies. Cells were either uninfected or infected with adenovirus LCPTP wild-type, substrate-trap and Gata3 proteins. Proliferation was measured at different time points ranging from 24 to 96 hours. Unstimulated cells = purple bars; CD3/CD28 stimulated cells = green bars. For graphs H, I, J, LCPTP wild-type infected cells: = red line, LCPTP substrate-trap infected cells = orange line, Gata3 infected cells = green line, uninfected cells = blue line. This is representative of 3 independent experiments and error bars are all within a 5 percent range.

CHAPTER FIVE

5.3 Discussion

In Chapter 4 stable transfectants were made in the Jurkat T cell line overexpressing the wild-type, substrate-trap and null mutant forms of LCPTP phosphatase. The conclusions on the phenotype of the Jurkat transfected cell lines were that LCPTP affects the rate of MAP kinase phosphorylation, the production of IL-2 following TCR and CD28 stimulation and finally the growth inhibition rate of Jurkat T cells following the same TCR and CD28 stimulation. These results provide important data towards the aim of characterising the function of LCPTP protein in a leukemic cell line. The question is: Does LCPTP produce the same functional effect in primary T cells? To answer this question, transient infections of primary $CD4^+$ T cells using adenovirus containing either LCPTP wild-type or substrate-trap proteins were carried out and experiments performed to determine whether the data obtained in Jurkats could be reproduced in primary T cells.

Unlike Jurkats, primary T cells are not easily transfected with foreign proteins using conventional techniques. To overcome this problem, it was decided to utilise a method dependent on viral infection to deliver LCPTP into cells. The CAR-1-DO11.10 transgenic mice, which express the adenoviral receptor, CAR, on the surface of T cells were used to maximise the chances of delivering LCPTP into primary cells. The expression of CAR on the surface of cells does not necessarily appear to be a prerequisite to achieve infection. This was highlighted in a recent report by a Finnish team who were able to infect primary T cells with recombinant adenovirus vector without the CAR receptor on the T cell surface (Chen et al., 2002). With a m.o.i. of 1000 up to 5000 viruses per cell, they were able to achieve nearly hundred per cent infection efficiency. The large number of viruses used per cell did not seem to affect the infected T cells capacities to produce IFN-γ for Th1 cells or IL-4 for Th2 cells (Chen et al., 2002). This has important implications for the studies discussed in this chapter because it shows that the adenovirus does not harm the cells and that they are able to react normally to TCR and CD28 stimulation. Expression of CAR-1 receptor on the surface of T cells, enable studies to be performed to evaluate the effect of overexpressing LCPTP using only 10 infective virus particles/cell. The fact that no effects were seen on cytokine production using one hundred times the number of viruses, gives one confidence that effects seen overexpressing LCPTP protein in

murine T cells were due to the presence of the phosphatase and not due to the adenovirus components.

The first hurdle to overcome was to produce adenovirus containing LCPTP phosphatase and to be able to express the phosphatase in cells. The primary step for the production of an altered adenovirus was to clone the gene of interest into a vector (pCR259), to make a recombinant plasmid which included the adenovirus genome (pCR249) (Richards et al., 2000). Transient transfections of pCR259 containing LCPTP wild-type or substrate-trap proteins overexpressed the phosphatase, indicating that the gene was in the correct reading frame in the vector and ready to be recombined into the viral genome. The concentration of the virus by caesium chloride gradient, shown in Figure 5.2, was essential for further studies infecting primary T cells because to achieve infection, the virus had to be combined with cells in a minimal volume (Dr Nigel Sharp personal communication).

Experiments were done infecting primary T cells with adenovirus constructs including two different forms of LCPTP, namely wild-type and substrate-trap. An anti-sense LCPTP construct was also made to act as a negative control. However, due to problems encountered during scale up, only limited amounts of the anti-sense containing virus were obtained. This was used to demonstrate that the overexpression of LCPTP seen in cells infected with wild-type or substrate-trap LCPTP protein was specific and not due to changes induced by viral proteins. All subsequent experiments were performed using an adenovirus construct containing the Gata3 gene instead of the anti-sense LCPTP in order to have a positive control for the adenovirus infection technique. Gata3 is a Th2 transcription factor and its effects in primary Th1 and Th2 cells have been studied previously in the department (Zheng and Flavell, 1997); (Zhang et al., 1997); (Drs Andrew Blanchard and Nigel Sharp, personal communication). Infection of primary T cells with adenovirus proved not to be a trivial technique. It was found that as the multiplicity of infection with LCPTP viral constructs increased, there was an inverse correlation with cell survival. In Jurkat T cells, we observed that overexpression of LCPTP wild-type did not disturb the growth potential of unactivated cells but overexpression of LCPTP substrate-trap appeared to be detrimental to cell growth. In primary Th1 and Th2 cells, the overexpression of

both wild-type and substrate-trap forms of LCPTP protein affected cell survival. Increasing the multiplicity of infection with LCPTP constructs, correlated with an increase in cell aggregation in cultures. In contrast to infection of cells with LCPTP, the growth of Th1 and Th2 cells overexpressing the Gata3 construct was less affected in terms of aggregates and cell death. This suggested that the effects seen on cell growth in cells overexpressing the LCPTP constructs were in some way linked to the increased level of phosphatase protein present. Due to time limitations, no further experiments were conducted in order to clarify this increase in cell toxicity. Instead, efforts were concentrated in obtaining functional data with the cells overexpressing the different forms of LCPTP protein.

The infection of cells with as few as five viruses per cell was enough to see an augmentation in LCPTP concentration in total protein extracts above the endogenous levels present. An m.o.i. of 10 viruses/cell was determined to be a reasonable compromise on being able to demonstrate overexpression and maintain enough viable cells in order to proceed with further functional studies. This is considerably less then the 1000 viruses per cell, used by Chen et al. (2002) (Chen et al., 2002). In order to determine whether LCPTP infection was equally efficient in Th1 and Th2 cells, western blots were performed on total protein extracts from cells, probed for the presence of LCPTP. Figure 5.5 shows that both cell types overexpressed LCPTP compared to the endogenous level seen in the non-infected cells. This result demonstrated clearly that we had achieved the aim of transiently overexpressing both LCPTP wild-type and substrate-trap proteins into primary Th1 and Th2 cells.

We reported that the phosphorylation of MAP kinase members ERK1 and EKR2 was reduced in the presence of overexpressed LCPTP in Jurkat T cells, following TCR and CD28 activation. The same experiment was done in differentiated Th1 and Th2 cells. As expected, the overexpression of LCPTP wild-type reduced the amount of phosphorylated MAP kinase members. Contrary to the result obtained in Jurkat T cells, the overexpression of LCPTP substrate-trap did not increase the level of MAP kinase phosphorylation which remained equivalent to that seen in the uninfected control. A possible explanation for this is that the substrate-trap phosphatase is not able to trap the MAP kinase thus maintaining it in a

phosphorylated form. However, the gene cloned into the adenoviral vector was sequenced and does contain the designated mutations. An alternative explanation, is that there is a difference in the ratio of the substrates in Th1 and Th2 cells compared to Jurkats. Some phosphorylated MAP kinase protein could be trapped by the mutant LCPTP protein, but enough remains free in the cytoplasm to become phosphorylated. A third explanation, is that the LCPTP used for the adenoviral construct is human. It is possible that although transfecting wild-type LCPTP into murine cells, clearly has an effect, that there are differences between the way human LCPTP interacts with human and mouse MAP kinases. These differences become apparent when transfecting murine cells with the substrate-trap LCPTP protein. The presence of overexpressed Gata3 increased the MAP kinase phosphorylation supporting the theory that the reduction of MAPK phosphorylation observed in cells overexpressing LCPTP wild-type is due to the presence of the phosphatase and not due to an adenovirus component. An unexpected observation was the increase seen in MAP kinase phosphorylation in cells overexpressing Gata3. A possible explanation is based on the fact that it is known that MAP kinases phosphorylate Gata3, which induces the release of Th2 cytokines, including IL-4. IL-4 acts in an autocrine loop binding back onto receptors and activating MAP kinase signalling pathways (Chen et al., 2000); (Day et al., 2002); (Moore et al., 2002). There was an apparent greater increase in MAP kinase phosphorylation in Th2 cells overexpressing Gata3 compared to Th1 cells relative to respective controls. The above explanation would fit with the observation that the "Th1" population was shown to include some IL-4 secreting cells in addition to ones secreting IFN-γ.

The overexpression of LCPTP wild-type and substrate-trap proteins in naïve and both Th1 and Th2 cells reduced the production of specific cytokines (Figure 5.7). As previously shown in Jurkat T cells, overexpression of LCPTP in primary T cells resulted in a reduction in the production of IL-2 following stimulation with anti-CD3/CD28 antibodies. In addition, overexpression of LCPTP protein in primary T cells also reduced the production of IL-4, IL-5 and IL-13 in Th2 cells and IFN-γ in Th1. This reduction was not due to increase in cell death since the same number of viable cells were taken to measure the cytokine production. This is in contrast to overexpressing Gata3 which lead to an increase in the production of IL-4, IL-5 and

IL-13 cytokines in Th2 cells. One could expect an increase in the production of Th2 cytokines in Th1 cells transfected with Gata3 protein as reported by Lee et al. (Lee et al., 2000). The fact that overexpressed Gata3 in Th1 cells does not raise the amount of Th2 cytokines in our experiments, may suggest the need of a post-translational modification on Gata3 in order to bind to the promotor regions of the Th2 cytokine genes as proposed by Yamagata et al. and Cousins et al. (Yamagata et al., 2000); (Cousins et al., 2002). The reduction of Th2 cytokines by overexpressing LCPTP is unexpected because it has previously been shown that the ERK inhibitor PD98059, inhibits the MEK and ERK signalling pathways and this leads to enhanced production of Th2 cytokines (IL-4, IL-5, IL-13) following cell activation with a combination of PMA/anti-CD3 or by anti-CD3/CD28 antibodies (Dumont et al., 1998). The differences could indicate that PD98059 hits other targets in addition to ERK. Badou et al. showed that the MAPK signalling pathway can be dispensable for the production of IL-4. In contrast, IFN-γ synthesis is dependent on both the calcium response and the MAPK activation (Badou et al., 2001). Reported increases in IL-4 synthesis could merely reflect a bias in cultures following Th2 cytokines in the absence of regulating Th1 cytokines. There is controversy in the literature regarding the role of the different MAP kinase signalling pathways in secretion of the different cytokines. For instance, in one study it was shown that IL-2, IL-4 and IFN-γ production was reduced when p38 activity was inhibited (Zhang et al., 1999). In contrast, another study reported that blocking p38 activity in Th1 cells affected IFN-γ production but not Th2 cytokine production (Rincon et al., 1998). From our results, it is concluded that the reduction in MAPK activity in naïve, primary Th1 and Th2 cells has a direct effect on the production of IL-2 in both naïve and Th1 cells, on the production of IFN-γ in Th1 cells and, on the production of IL-4, IL-5 and IL-13 in Th2 cells.

In Jurkat T cells overexpressing LCPTP protein, the growth inhibition seen following TCR and CD28 stimulation was reduced. In primary Th1 and Th2 cells, there was an increase in basal proliferation following recent stimulation in cells overexpressing LCPTP wild-type or substrate-trap proteins. Thus LCPTP appears to have a role in regulating cell cycle progression. A plausible explanation for the higher basal proliferation is provided by the study of Zanke (1994), where he

demonstrated that when the LCPTP gene was present in cells in a multiple copy number, the growth of cells became unregulated and establishment of clonal dominance was also seen (Zanke et al., 1994). Zanke's studies and the results in the Jurkats and primary murine naïve, Th1 and Th2 cells where LCPTP apparently favours cell growth under certain conditions suggests that a disregulation in LCPTP activity renders it to have the potential to become an oncogene. The fact that the difference in the proliferation rate between the control uninfected cells and the infected cells became less obvious could be explained by the transient nature of adenovirus infection, losing copies of the virus from mother to daughter cells.

In conclusion, the phenotypes observed in transformed cell lines where LCPTP affects the rate of MAP kinase phosphorylation, the production of IL-2 following TCR and CD28 stimulation and finally the rate of growth inhibition following cell activation can be extrapolated to primary $CD4^+$ T cells where an overexpression of LCPTP also affects MAP kinase phosphorylation, cytokine production and proliferation[3].

[3] Part of the work described in this chapter will be submitted to Journal of Immunology.

CHAPTER SIX: GENERAL DISCUSSION

The role of phosphatases in T cell signalling has been the focus of many investigations in recent years. The present work contributes to further the knowledge and understanding on LCPTP, a hematopoietic restricted phosphatase. Previously, several studies have looked at the biochemical function of LCPTP, but few have investigated the biological function of LCPTP. The characterisation of the role of LCPTP in T cell signalling was expanded with a number of approaches. Firstly, we looked at protein interactions of endogenous LCPTP in Jurkat T cells and peripheral $CD4^+$ T cells (Chapter 3). Secondly, the functional consequences of overexpressing stable LCPTP wild-type, substrate-trap and null mutant proteins in Jurkat cells were investigated (Chapter 4). The final results chapter describes the consequences of overexpressing LCPTP in primary naïve, Th1 and Th2 murine T cells infected with wild-type or substrate-trap LCPTP protein (Chapter 5).

6.1 LCPTP – The Present

6.1.1 The function of LCPTP in cells

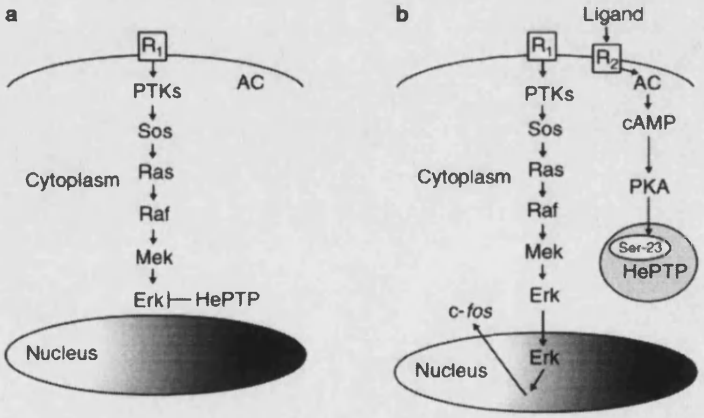

Figure 6.1: Model for LCPTP (HePTP) in T cells signalling

Figure from Saxena et al., 1999. "A. In the absence of external stimuli, the classical pathway of MAP kinase activation (originating at a receptor, R1) through Ras, Raf and Mek has a low basal activity, which is counteracted by HePTP. B. Stimulation of receptors (R2) that couple to adenylate cyclase (AC) turn on the new pathway that involves phosphorylation of HePTP at Ser 23 by PKA, leading to dissociation of ERK from HePTP. This release from inhibition results in MAP-kinase activation and subsequent c-fos induction." (Saxena et al., 1999).

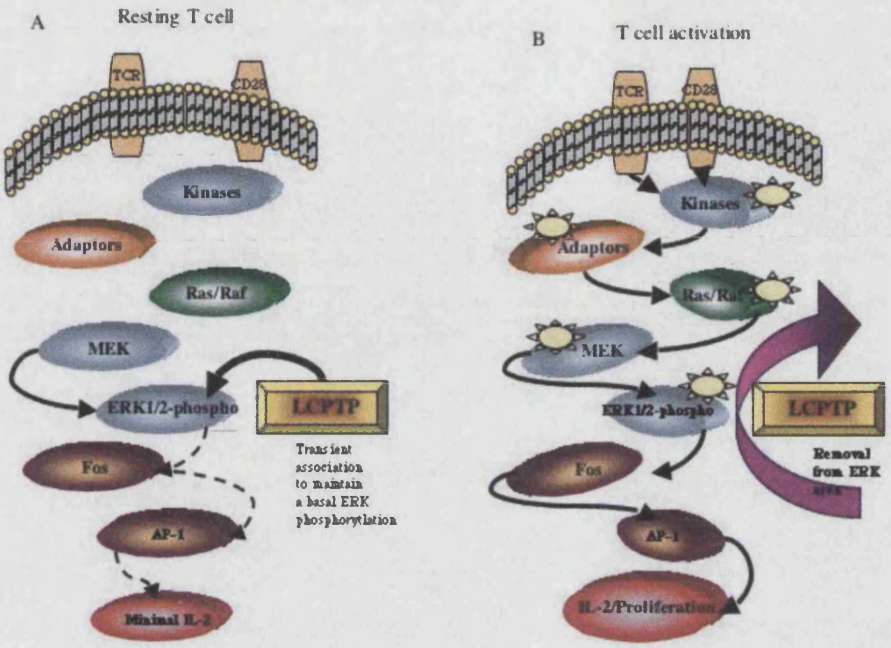

Figure 6.2: Model for LCPTP role in early T cells signalling

A. In resting cells, the TCR pathway is not engaged. LCPTP maintains a basal phosphorylation level of MAPK family members through transient associations. In controlling MAPK phosphorylation, LCPTP prevents unregulated cell proliferation. B. In cells activated by both TCR and CD28 signals, LCPTP is removed from the MAPK family members which allows full MAPK phosphorylation leading to IL-2 production and proliferation.

T cells have evolved to undergo proliferation and clonal expansion on recognition of specific peptide and appropriate costimulatory and accessory signals. Only a very small percentage of T cells possess a TCR capable of binding to a given peptide with subsequent initiation of signalling pathways which drive cell proliferation. It is important that the whole T cell repertoire is not activated simultaneously as the consequences of every single T cell undergoing clonal expansion would lead to a severe effect on haematopoietic cell homeostasis. To guard against such an event, there must be tight regulation at multiple steps in signalling pathways to prevent inadvertent triggering of cell proliferation. On such

step is the regulation of the MAP kinase family members and a candidate protein for achieving this is LCPTP. We have shown that LCPTP forms transient associations with ERK1 and ERK2 and that overexpressing LCPTP in cells reduces MAPK phosphorylation. Thus LCPTP stops MAPK family members translocating into the nucleus and prevents unregulated proliferation. This role of LCPTP is suggested by the fact that overexpressing LCPTP leads to the phosphatase interacting with ERK1 and ERK2 in both unstimulated and stimulated cells. Data published by Pettiford and Herbst (2003) confirm this role. After transfecting HePTP anti-sense into K562 cells, they observed enhanced nuclear translocation of ERK, ultimately leading to uncontrolled differentiation and proliferation (Pettiford and Herbst, 2003).

Studies by Zhong et al. (2000) also support a role for LCPTP in termination of MAPK signalling. Specific monoclonal phospho-antibodies were used to demonstrate that the dephosphorylation of ERK operates differentially dependent on its localisation either in the nucleus, or in the cytoplasm. Switching off signalling via MAP kinases involves dephosphorylation in the nucleus by a Ser/Thr phosphatase and a tyrosine phosphatase while the completion and maintenance of its inactivation is carried out by a PTP in the cytoplasm. LCPTP mRNA has been shown to increase several fold following T cell stimulation (Zanke et al., 1992) although protein levels increased only moderately compared to resting cells. It is possible that upon termination of signalling, stabilisation of mRNA leading to increased LCPTP protein expression then allows LCPTP to fulfil a role in returning to and maintaining MAP kinases in an inactive state. The T cell returns to a rested state.

6.1.2 Regulation of LCPTP

It would be predicted that upon recognition of antigenic peptide by a T cell bearing the appropriate TCR and costimulatory and accessory signals, the activity of LCPTP changes. We do not have sufficient data from our studies to determine exactly how and when LCPTP itself is regulated, although we do have data suggesting that this could be mediated in part via CD28 ligation. Alterations in LCPTP activity would mean that the balance of phosphatase and kinase activities regulating MAP kinase activity is shifted in favour of kinases. Upstream kinases phosphorylate and activate MAP kinases leading to nuclear translocation and

downstream activation of transcription factors. Munoz et al. (2003) suggested in a recent publication that intracellular redox conditions can play a role in the modulation of the activity and subcellular location of the MAPK family members by regulating the association of phosphatases including LCPTP with their substrates (Munoz et al., 2003). Interestingly, a synergism between CD28 costimulation and changes in intracellular redox conditions has been shown to enhance transcription from the IL-2 promotor (Hehner et al., 2000).

Other clues as to how and when LCPTP is regulated, include studies by Wang et al. (2003) who identified a complex containing the two phosphatases, PP2A and LCPTP, working cooperatively on phosphorylated forms of ERK1 and ERK2 in a cholesterol-regulated way (Wang et al., 2003b). They observed a higher proportion of the phosphorylated form of MAPK family members in lipid rafts when cells were stressed by acute cholesterol depletion. This was as a consequence of the disassembly of the PP2A and LCPTP complex. This data supports the fact that LCPTP is present in cells to control the basal phosphorylation level of MAPK family members and when the cell is stimulated, the phosphatases are removed from the MAPK area in order to promote signalling.

There are a large number of phosphatases in addition to LCPTP which have MAP kinase family members as substrate. Homologs of human LCPTP have been found in rat, mouse and also in the budding yeast *Saccharomyces cerevisiae* (Ptp2 and Ptp3). Both Ptp2 and Ptp3 are global regulators of MAPK signalling in yeast. Ptp2 is a nuclear protein and Ptp3 is a cytoplasmic protein (Mattison and Ota, 2000). The existence of homologs in a primitive eukaryote may suggest a highly important function for LCPTP, since it has been conserved through evolution. Resting T cells express only two phosphatases which have been shown to specifically dephosphorylate MAP kinase family members, namely LCPTP and VH1-related phosphatase. The other phosphatases which have the same substrate specificity need to be transcribed following TCR stimulation (Mustelin et al., 2002); (Alonso et al., 2003). VH1-related phosphatase is under the regulation of ZAP-70, which phosphorylates VH1, and thereby, inactivates VH1 (Alonso et al., 2003). An unpublished result from our group demonstrated a potential interaction between

LCPTP protein and TCR zeta protein. It is possible that like VH1, LCPTP is regulated by a TCR proximal kinase, or even by TCR zeta itself. Studies were performed to look for increases in LCPTP tyrosine phosphorylation following TCR ligation to see if any change could be detected analogous to the increase in LCPTP tyrosine phosphorylation seen in rat mast cells following ligation of surface IgE receptors (Swieter et al., 1995). No changes in LCPTP tyrosine phosphorylation were detected which could reflect differences between regulation of LCPTP in rat mast cells and human T cells (data not shown). This aspect of regulation of LCPTP in T cells requires further investigation.

The first model of the role of LCPTP in T cell signalling, proposed by Saxena and collaborators, placed LCPTP directly downstream of the cAMP pathway linking the TCR and cAMP pathways (Figure 6.1). This model depends on the activation of the cAMP pathway in order to inactivate LCPTP. In the cell systems our studies were performed in, we have shown that the amount of cAMP in cells is usually low and thus LCPTP inactivation is unlikely to be caused by the accumulation of cAMP since the TCR signal is known to be associated with a decrease of the cAMP pathway (Tamir et al., 1996); (Ramstad et al., 2000). On the basis of the present study and previous reports in the literature, a possible model for the role LCPTP plays in T cell signalling and activation is presented (Figure 6.2).

We have shown that the functional consequences of overexpressing LCPTP in cells, include inhibition of TCR stimulated IL-2 production and T cell proliferation which would fit with LCPTP playing a negative role in MAP kinase activation.

6.2 LCPTP - The future

There is still much to learnt about LCPTP – How and when it is regulated, possible as yet unidentified substrates, the full extent of its role in T cell signalling and activation.

An LCPTP knockout mouse has been generated with no associated phenotype except for a comment about raised ERK phosphorylation in spleen cells (Gronda et al., 2001). This implies that LCPTP plays a redundant role in regulating MAP kinase

phosphorylation. However, the problem with knocking out a gene in mice, is that it can cause an imbalance in the expression of other proteins (Okkenhaug and Vanhaesebroeck, 2001). For example, in cells from PI-3 kinase p85α knockout mice, p85β expression were upregulated and p110α, p110β and p110δ was downregulated (Fruman et al., 1999); (Suzuki et al., 1999); (Lu-Kuo et al., 2000). In the LCPTP knockout, the expression of other proteins regulating MAP kinase activity, such as VH1 was not examined. In attempting to define a biological function for a protein in vivo, a more physiological approach might be to replace the endogenous protein with equivalent levels of a defective protein. Okkenhaug et al. have taken this approach for examining the role of p110δ in TCR signalling, by generating a point mutation in the p110δ gene (Okkenhaug et al., 2002). Expression of p110δ and the other P1-3 kinase subunits was equivalent to wildtype mice. Studies were performed looking at the effects of a mutant enzyme without gross changes on other P1-3 kinase subunits. Another example for cautionary interpretation of data obtained from in vivo experiments, is studies which differences have been observed between knock-out cells and cells expressing a dominant negative form of protein. Lck provides an example. Studies in Lck-/- cells showed an inability to mobilise calcium after TCR stimulation, whereas cells expressing the dominant negative form of the protein respond normally (Trobridge and Levin, 2001). Thus, elimination of a particular protein can push another protein to take up another role and replace the function of the eliminated protein, resulting in a poor phenotype from the knock-out mouse. On the other hand, overexpression of a dominant negative protein can create effects outside the normal physiological function of the protein (Trobridge and Levin, 2001). The use of a null mutant LCPTP phosphatase, as a replacement for endogenous LCPTP in the mouse, could lead to a more extended phenotype than seen in the LCPTP knock-out mouse published. We observed that overexpression of the null LCPTP phosphatase in Jurkat T cells, reduced the production of IL-2 nearly as much as the wild-type protein. This suggests that LCPTP might have functions in cells independent of its phosphatase activity and the expression of a negative mutant could reveal those functions.

Other approaches which could be used to study the roles of signalling proteins in cells, include the use of RNA anti-sense technology, where a mRNA encoding for

the anti-sense protein is transfected into cells leading to an association between the sense and anti-sense mRNA and a block in protein translation (Bettaieb et al., 2003); (Troussard et al., 2003); (Wang et al., 2003a). Alternatively, RNA interference on LCPTP in hematopoietic cells could be performed (Elbashir et al., 2001). Another way to create a knock-out of LCPTP in differentiated cells would be to use antibody against LCPTP protein transfected into cells, making complexes with the protein, and abolishing its function, as has been done with PKC theta (Szamel et al., 1998). An alternative approach is to create a conditional knock-out by flanking the LCPTP gene containing the antibiotic resistance with sequence targeting the expression of the protein to a specific type of cell or organ which would allow the effects of the knock-out to be studied in restricted cell types/tissue (Metzger and Chambon, 2001); (Xu et al., 2001); (Troussard et al., 2003). Inducible knockouts have been useful in cases where knocking out a protein has proved to be embryonically lethal, or where expression of a protein is key for cell development. An example is provided again by studies on Lck. In the absence of Lck, T cells fail to mature. By creating an inducible knockout, Zamoyska et al., (2000) were able to study the effects of Lck on mature T cell function, by switching off gene expression post thymic development (Seddon et al., 2000).

The only identified substrates of LCPTP to date, are members of the MAP kinase family. It is possible that additional substrates await identification, and almost certainly, there are other proteins with which LCPTP interacts within signalling complexes, controlling and regulating LCPTP activity. Proteomics technology has advanced considerably in recent years, enabling detection and identification of members of signalling complexes from ever decreasing cell numbers. Whereas in our studies, cell numbers restricted us to using cell lines for proteomic work, progress should facilitate the use of primary cells (Nyman et al., 2001). Likewise, advances in protein microarray technology will enable detailed studies in the future, looking at tissue distribution/expression levels of proteins, protein-protein interactions and enzyme-substrate interactions which could give further clues as to what stages in T cell activation/differentiation, LCPTP is involved (Kumble, 2003).

Many diseases are thought to be due to an inbalance in the immune system,

including autoimmune diseases, such as rheumatoid arthritis and juvenile diabetes, as well as asthma. Understanding how proteins function in cells can provide targets which could attenuate the reactivity of cells and help reduce the effect of disease. As demonstrated in this study, the overexpression of LCPTP in primary cells reduces the production of IL-2 and effector cytokines, contributing to lower the level of T cell reaction to an antigen. Anergy is a situation where cells fail to proliferate upon restimulation because they cannot produce IL-2 (DeSilva et al., 1991); (Beverly et al., 1992). In situations where there is inappropriate T cell activation, overexpression of LCPTP could promote a state of anergy in cells because they produce much less IL-2 than normal cells following TCR and CD28 stimulation. Li et al. found reduced ERK and JNK kinase activities in anergic cells (Li et al., 1996). They proposed that the reduced ERK function could result from a defective phosphorylation by an upstream kinase or from an increase activity of a kinase specific phosphatase namely PAC. LCPTP protein could also be implicated in this process.

In conclusion, we propose that LCPTP is a regulator of MAP kinase phosphorylation at the basal level, adjusting the balance between overreaction and anergy and further studies understanding the biology of LCPTP could present it as a future therapeutic target.

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