LIPID PHOSPHATE PHOSPHATASES: PURIFICATION AND INVESTIGATION OF THEIR ROLE IN CELLULAR LIPID SIGNALLING

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ABBREVIATIONS

AC.....	Adenylyl cyclase
ADP.....	Adenosine 5' diphosphate
ARF.....	ADP-ribosylation factor
ASM.....	Airway smooth muscle
ATP.....	Adenosine 5' triphosphate
BK.....	Bradykinin
BSA.....	Bovine serum albumin
C1P.....	Ceramide 1-phosphate
cAMP.....	Cyclic adenosine 3',5' monophosphate
CAPK.....	Ceramide-activated protein kinase
CAPP.....	Ceramide activated protein phosphatase
CK2.....	Casein kinase II
DG.....	Diacylglycerol
DGK.....	Diacylglycerol kinase
DHS.....	Donor horse serum
DMSO.....	Dimethylsulfoxide
DNA.....	Deoxyribonucleic acid
DTT.....	Dithiothreitol
ECL.....	Enhanced chemiluminescence
EDTA.....	Ethylenediamine tetraacetic acid
EGF.....	Epidermal growth factor
EGTA.....	Ethyleneglycol-bis(β-aminoethylether)N,N,N',N'-tetraacetic acid
ERK-1/2.....	Extracellular signal-Regulated Kinase-1/2
EST.....	Expressed sequence tag
FCS.....	Foetal calf serum
fMPL.....	Formyl-Met-Leu-Phe
EFCS.....	European foetal calf serum
GPCR.....	G-protein coupled receptor
GAP.....	GTPase activating protein

GDP.....	Guanosine 5' diphosphate
GDI.....	GDP dissociation inhibitor
GIP.....	GTPase inhibitory protein
Grb2.....	Growth factor receptor binding protein-2
GTP.....	Guanosine 5' triphosphate
HEK.....	Human embryonic kidney
HRP.....	Horse radish peroxidase
ICA630.....	Igepal CA630
IL.....	Interleukin
IP_3.....	Inositol-1,4,5-triphosphate
JNK.....	c-Jun NH_2-terminal kinase
LPA.....	Lysophosphatidic acid
LPAAT.....	Lysophosphatidic acid acyl transferase
LPAP.....	Lysophosphatidic acid-specific phosphatase
LPC.....	Lysophosphatidylcholine
LPP.....	Lipid Phosphate Phosphatase
MAPK.....	Mitogen Activated Protein Kinase
MEK.....	MAP kinase kinase
MEKK.....	MAP kinase kinase kinase
MOG.....	1-mono-oleoyl-rac-glycerol
NEM.....	N-ethylmaleimide
PA.....	Phosphatidic acid
PAP.....	Phosphatidic acid phosphatase
PBS.....	Phosphate-buffered saline
PBSI.....	PBS/Igepal CA630
PDGF.....	Platelet-derived growth factor
PC.....	Phosphatidylcholine
PDE.....	Phosphodiesterase
PE.....	Phosphatidylethanlolamine
PI.....	Phosphatidylinositol
PIP_2.....	Phosphatidylinositol-4,5-bisphosphate
PKA.....	cAMP-dependent protein kinase

PKC.....	Protein kinase C
PLA_1.....	Phospholipase A_1
PLA_2.....	Phospholipase A_2
PLC.....	Phospholipase C
PLD.....	Phospholipase D
PMA.....	Phorbol 12-myristate 13-acetate
PP1.....	Protein phosphatase-1
PP2A.....	Protein phosphatase-2A
PS.....	Phosphatidylserine
PtdAlcs.....	Phosphatidylalcohols
PTX.....	Pertussis toxin
RNA.....	Ribonucleic acid
S1P.....	Sphingosine 1-phosphate
S1PP.....	Sphingosine 1-phosphate phosphatase
SDS-PAGE.....	Sodium dodecyl sulphate-polyacrylamide gel-electrophoresis
SM.....	Sphingomyelin
Sph.....	Sphingosine
SphK.....	Sphingosine Kinase
TBST.....	Tris buffered saline/Tween-20
TEMED.....	Tetramethylethylenediamine
TLC.....	Thin layer chromatography
TSM.....	Tracheal smooth muscle
TX-100.....	Triton X-100

PUBLICATIONS

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Pyne S., Rakhit S., Conway AM., McKie A., **Darroch P.**, Tate R., Pyne N. (1999): Extracellular actions of sphingosine I-phosphate through endothelial differentiation gene products in mammalian cells: role in regulating proliferation and apoptosis. *Biochem. Soc. Trans.* **27** (4): 404-409.

ABSTRACT

Several isoforms of LPP have now been identified and cloned but remain to be purified. In the present study, a bacterial expression system was established and hexa- and deca-histidine epitope tagged-LPP1 and LPP1a expressed in *E.coli*. In addition, a maltose binding protein (MBP) epitope tagged-LPP1a was expressed in *E. coli*. Hexa- and deca-histidine LPP1 and LPP1a, were partially purified using immobilised affinity chromatography. MBP-LPP1a was expressed to higher levels than hexa- and deca-histidine LPP1 and LPP1a in *E. coli*, most probably within insoluble inclusion bodies. In all cases, recovery of LPP activity was low.

Membranes derived from HEK293 cells that stably over-express LPP1, LPP1a, LPP2 or LPP3 were used to demonstrate the differential hydrolysis of several molecular species of PA, $LPA_{(18:1)}$, C8-C1P and S1P. Kinetic analysis using a multisubstrate assay system revealed that the LPP isoforms do not follow typical Michaelis-Menten kinetics towards most substrates under the assay conditions employed. The LPPs appear to show differential kinetics depending on the complement of substrates accessible to the enzymes.

Stable over-expression of LPP1, LPP1a, LPP2, but not LPP3, in HEK293 cells has previously been shown to attenuate the activation of ERK-1/2 by G-protein coupled receptors agonists such as S1P, LPA, PA and thrombin. The present study extended these observations by showing that basal growth rates were unaffected and that levels of mRNA transcript for the $S1P_1$/EDG1 receptor were reduced in the LPP stable cell lines but that this did not correlate with attenuation of the S1P-stimulated ERK-1/2 response. In addition, transient overexpression of LPP1, LPP1a and LPP2, but not LPP3 in HEK293 cells and GPASM cells also resulted in the attenuation of S1P-induced ERK-1/2 activation. Furthermore, transient transfection of a plasmid construct encoding the antisense sequence for LPP1 was also found to attenuate S1P-induced ERK-1/2 activation whereas the PMA-stimulated response was unaffected. Many questions remain to be fully answered in order to determine the physiological and pathophysiological roles of the LPPs and the reason for the molecular diversity of the enzyme family.

CHAPTER 1

GENERAL INTRODUCTION

1. General Introduction

An essential requirement for the survival and development of all living organisms is that of intercellular signalling. Central to intercellular signalling are many diverse and intricate signalling pathways which, when malfunctioning, have long been recognised to be the cause of disease (for reviews, see Levitzski 1996, Karin 1992). The signalling pathways of cells utilise a great variety of different signalling molecules ranging from chemical messengers, hormones, neurotransmitters and also many lipid molecules. Furthermore, diverse interactions between the different signalling pathways including co-operation, potentiation and synergism make these pathways extremely dynamic and complex (see Nishizuka 1992 for review).

1.1. Lipids and Signal Transduction

The classical view of phospholipids was that they were biologically inert entities which maintained the structure of cell membranes and provided permeability barriers between the compartments within and between cells. However, the past 20 years has seen a great change in our understanding of phospholipid function.

Glycerolipids and sphingolipids represent the two main classes of phospholipid in eukaryotic cell membranes (Ohanian et al., 1998) and in addition to their structural roles, serve to provide a rich reservoir of lipid mediators. The lipid mediators produced serve functions ranging from cell surface receptor ligands (primary messengers) and anchors for membrane associated proteins, to intracellular second messenger molecules (Merrill 1989, Ghosh et al., 1997).

Phosphatidylinositol (PI), phosphatidylcholine (PC) and sphingomyelin (SM) are the major phospholipids involved in signal transduction. The lipid mediators principally produced following hydrolysis of PI and PC are diacylglycerol (DG) and phosphatidic acid (PA) through the actions of phospholipases C and D (PLC, PLD) respectively (Figure 1.1a, 1.1b). Furthermore, PLD catalysed hydrolysis of phosphatidylethanolamine also leads to the production of PA (Figure 1.1b) [for

review, see Divecha & Irvine, 1995]. The hydrolysis of SM on the other hand occurs through the actions of PLC-like enzymes known as sphingomyelinases and results in the production of the lipid mediator ceramide (Figure 1.1c) [for reviews, see Perry & Hannun, 1999, Levade & Jaffrezou, 1999]. However, PA, DG and ceramide can be processed further to yield additional lipid signalling molecules (Figure 1.2). PA can be dephosphorylated by the actions of phosphatidic acid phosphatase (PAP) or lipid phosphate phosphatases (LPPs) to produce DG. Alternatively, PA can be hydrolysed by phospholipases A_1 and A_2 ($PLA_{1/2}$) to yield lysophosphatidic acid (LPA) [Figure 1.2a, 1.2b]. Furthermore, DG can be phosphorylated by diacylglycerol kinases (DGKs) to produce PA whereas LPA can be re-acylated by lysophosphatidic acid acyltransferase (LPAAT) to produce PA (Figure 1.2a, 1.2b). PA, DG and LPA are therefore, interchangeable through the actions of several enzyme groups (for reviews, see Ohanian et al., 1998, Divecha & Irvine, 1995).

In a similar manner, ceramide derived from the hydrolysis of SM can be further hydrolysed by ceramidases to produce sphingosine (Sph). Furthermore, ceramide can be phosphorylated by ceramide kinase resulting in the production of ceramide 1-phosphate (C1P) [Kolesnick & Hemer, 1990]. Additionally, Sph can be phosphorylated by sphingosine kinase (SphK) to produce sphingosine 1-phosphate (S1P) which is also a potent lipid mediator (Figure 1.2c, 1.2d) [for reviews, see Ohanian et al., 1998, Divecha & Irvine, 1995, Pyne & Pyne, 2000a]. Again, as was described for PA, DG and LPA, Sph and S1P as well as ceramide and C1P are interchangeable via the actions of a variety of enzymes. S1P can be dephosphorylated by the actions of sphingosine 1-phosphate phosphatase (S1PP) or LPP to produce Sph and C1P can be dephosphorylated by ceramide 1-phosphate phosphatase or LPP to produce ceramide (Figure 1.2c, 1.2d) [for reviews, see Ohanian et al., 1998, Pyne & Pyne, 2000a, 2000b].

The past ten years have witnessed a dramatic increase in interest in lipid signalling molecules, the mechanisms whereby these lipids are metabolised, and the identity of their molecular targets. Biologically active lipids are now known to exert a great

Figure 1.1: Diagram showing the major phospholipids involved in cell signalling and the potential signalling molecules produced from these phospholipids. phospholipase C (PLC), phospholipase D (PLD), sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), phosphatidylinositol-4,5-bisphosphate (PIP_2), phosphatidylinositol-3,4,5-trisphosphate (PIP_3), inositol-1,4,5-trisphosphate (IP_3).

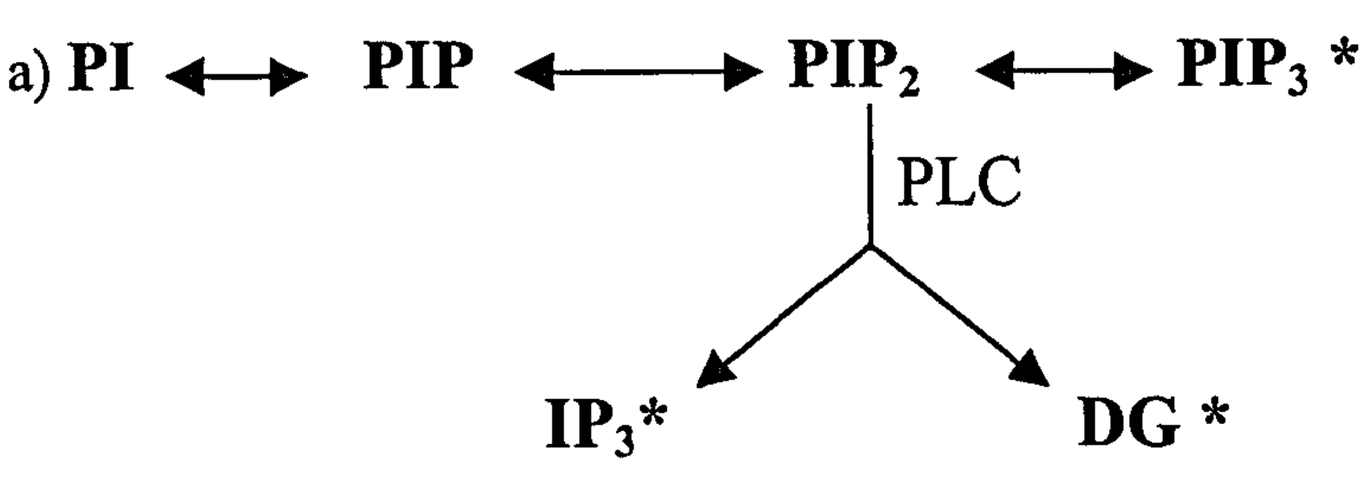
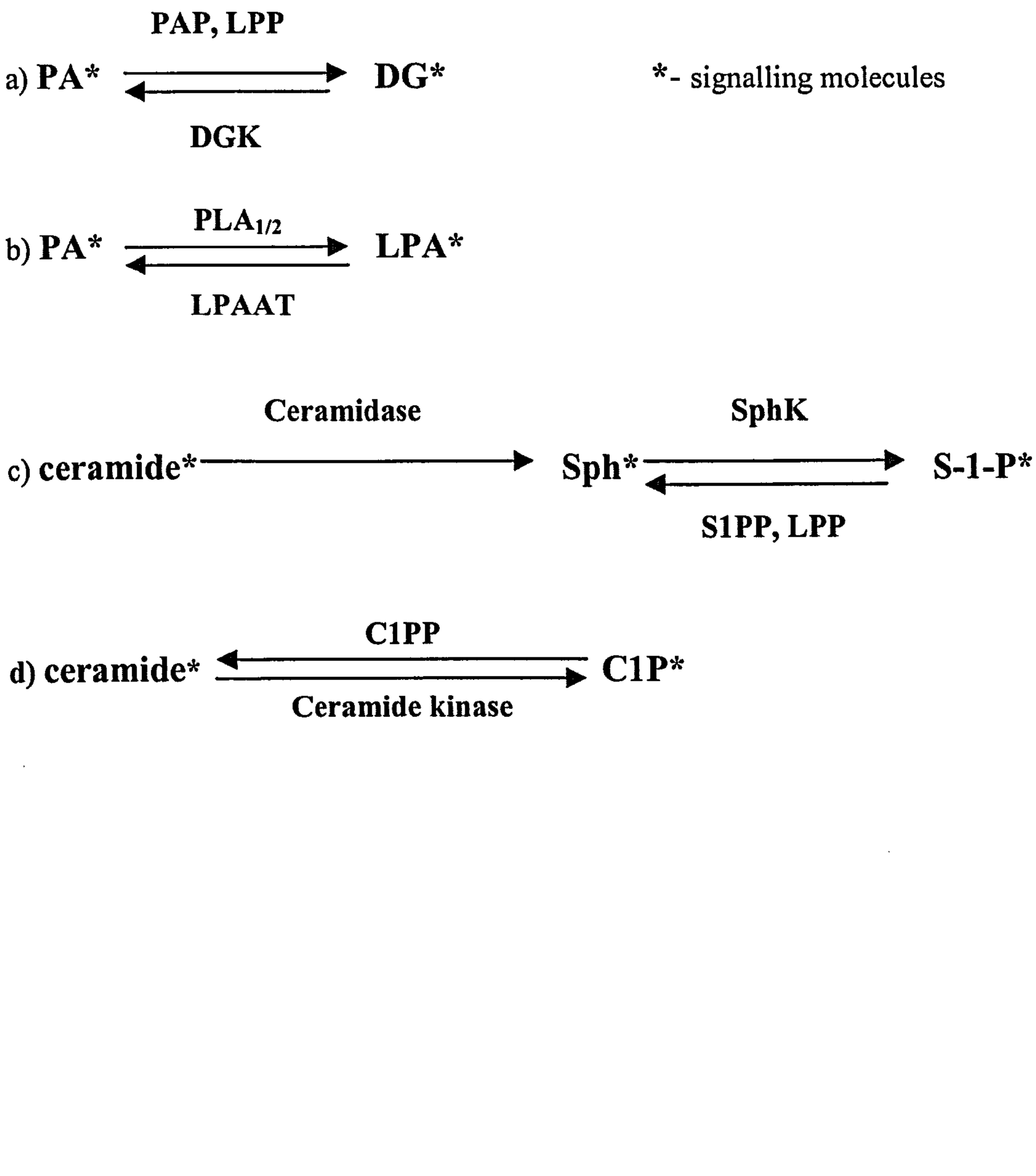


Figure 1.2: Diagram showing other lipid signalling molecules and how they are produced. phosphatidic acid phosphatase (PAP), lipid phosphate phosphatase (LPP), diacylglycerol Kinase (DGK), phospholipase A_2 (PLA_2), lysophosphatidic acid acyltransferase (LPAAT), sphingosine 1-phosphate phosphatase (S1PP), ceramide 1-phosphate phosphatase (C1PP), sphingosine kinase (SphK)



variety of profound cellular effects and have been shown to be involved in processes ranging from cell growth, survival, and differentiation, to cell death and apoptosis. Furthermore, several of the lipid mediators are produced or released by several cell types in both health and disease. For example, LPA, S1P, and PA have been shown to be released following activation of platelets and have been strongly implicated with roles in the angiogenesis observed in normal injury and wound repair as well as in disease states such as cancer (for review, see English et al., 2001). In addition, LPA has been shown to be released from ovarian cancer cells and has been found to promote the growth and progression of ovarian cancer (Erickson et al., 2001). Findings such as these have led to the recent focussing of the pharmaceutical industry on aspects of intracellular signalling in order to develop novel therapeutic targets. For example, PLD has been the recent interest of the pharmaceutical industry as a potential therapeutic target due to its role in the production of the potent lipid messenger PA (Steed & Chow, 2001).

1.2. Phosphatidic acid and signal transduction

Phosphatidic acid (PA) is present in resting cells at very low levels where its lifetime is transient (English et al., 1996). However, following agonist stimulation of cells, membrane phospholipids are hydrolysed leading to a rise in the levels of a variety of lipid signalling molecules including PA. Agonist stimulated hydrolysis of membrane phospholipids such as PC, PI and PIP_2 occurs in response to agonists ranging from hormones and growth factors to tumour promoting agents and represents a ubiquitous cellular signalling mechanism (Exton, 1990, Billah & Anthes, 1990, Exton, 1994). As detailed, the PLD catalysed hydrolysis of membrane phospholipids such as PC, leads to the production of PA which has been implicated as a major signalling molecule for many years. A great number of studies have been conducted in an attempt to elucidate the physiological role of PA and identify the molecular targets of this lipid mediator (for reviews, see Billah & Anthes 1990, Exton 1990, 1994, Billah 1993, English et al., 1996, Bocckino & Exton 1996 and Hodgkin et al., 1998). The role of agonist stimulated rises in PA levels in signal transduction has been the

subject of intensive research and even though many targets for PA have been identified, the precise role of PA has yet to be fully established.

1.3. Phosphatidic acid production

As described previously (section 1.1), PA can be generated within cells through several different routes. The first of these is through its direct formation by the PLD-catalysed hydrolysis of membrane phospholipids, especially PC. Secondly, PA can be formed indirectly by the DGK catalysed phosphorylation of DG, derived from the PLC-catalysed hydrolysis of PIP_2 or PC. Finally, the acylation of LPA catalysed by LPAAT provides a further route for the generation of PA within cells. As will be discussed in more detail later, an important consequence of the fact that there are several routes for the production of PA is the potential for generation of distinct molecular species of PA depending on the precursor membrane phospholipid and the route of formation. Therefore, the fatty acyl side chain pairings found in PAs, and indeed any lipids subsequently derived from PA, are an important source of information on the phospholipid precursors for any PA species formed or accumulated within cells following cell stimulation.

1.3.1. Phospholipase D isoforms

Phospholipase D (PLD) catalyses the hydrolysis of the terminal diester bond of membrane glycerophospholipids to generate PA and choline. The mechanisms and factors involved in the regulation of PC-PLD and also PC-PLD signalling, along with the functional significance of these has been the subject of considerable interest over recent years (for reviews, see Exton, 1997, 2000, Gomez-Cambronero & Keire, 1998).

PLD activity has been detected in many cell types and tissues such as brain and lung, where activity is highest, but also in hepatocytes, endothelial cells, spermatozoa, platelets and neutrophils (Gomez-Cambronero & Keire, 1998). Furthermore, there have been a number of studies which have identified several isoforms of mammalian

PLD which differ in their subcellular localisation, dependence on cations, pH optima, effects of fatty acids, small molecular weight G-proteins and lipid co-factors (Exton, 1990, 1997, 2000, Billah, 1993, Liscovitch & Chalifa-Caspi, 1996).

Two mammalian PLD activities have now been cloned from human and mouse. The first of these, cloned from a HeLa cell library and named PLD1 (PLD1a) was found to be a 124kDa protein, which was membrane associated, PC-specific, magnesium-dependent and insensitive to calcium. Furthermore, hPLD1 is activated by PIP_2 and ARF and is inhibited by oleate (Hammond et al., 1995). Subsequently, an alternatively spliced form, PLD1b was identified and is closely related to PLD1a and has similar properties (Hammond et al., 1997). PLD1 is localised in perinuclear regions such as the endoplasmic reticulum, Golgi apparatus and late endosomes (for reviews, see Liscovitch et al., 2000, Exton, 1997, Gomez-Cambronero & Keire, 1998).

The second mammalian PLD was cloned from mouse cDNA and found to display only 50% homology with PLD1 (Colley et al., 1997). Three spliced variants of PLD2 have now been sequenced, named PLD2a, with two truncated forms being referred to as PLD2b and PLD2c. PLD2b lacks an 11 amino acid sequence and PLD2c consists solely of the first 335 amino acids of full length PLD2a (Liscovitch et al., 2000). PLD2 has been shown to exhibit high basal activity in contrast to PLD1 and is localised primarily to the plasma membrane (Cambronero & Keire, 1998).

The regulation of PLD enzymes continues to be the subject of considerable interest and remains to be fully clarified. The issues surrounding the regulation of PLD are extremely complex and reports addressing this issue are often contradictory in nature. For example, in some systems, activation of PLD is sensitive to phorbol 12-myristate 13-acetate (PMA) but in others it is not. Furthermore, other systems demonstrate PLD activation to be dependent on PKC, ras/ARF, PI, PIP_2, or even a combination of these factors (Gomez-Cambronero & Keire, 1998). More recent investigations using recombinantly expressed PLD enzymes have concluded that PLD1 is the major PLD isoform which is regulated by ARF, PKC and RhoA (for

review, see Liscovitch et al., 2000). Figure 1.3 outlines the major possible regulatory factors that exist for PLD and it can be seen that they range from G-proteins and kinases, to lipid molecules and ions (Gomez-Cambronero & Keire, 1998). It should be noted that each PLD isoform may be subject to different levels of regulation by each of the factors as has been demonstrated for mammalian PLD1 and PLD2.

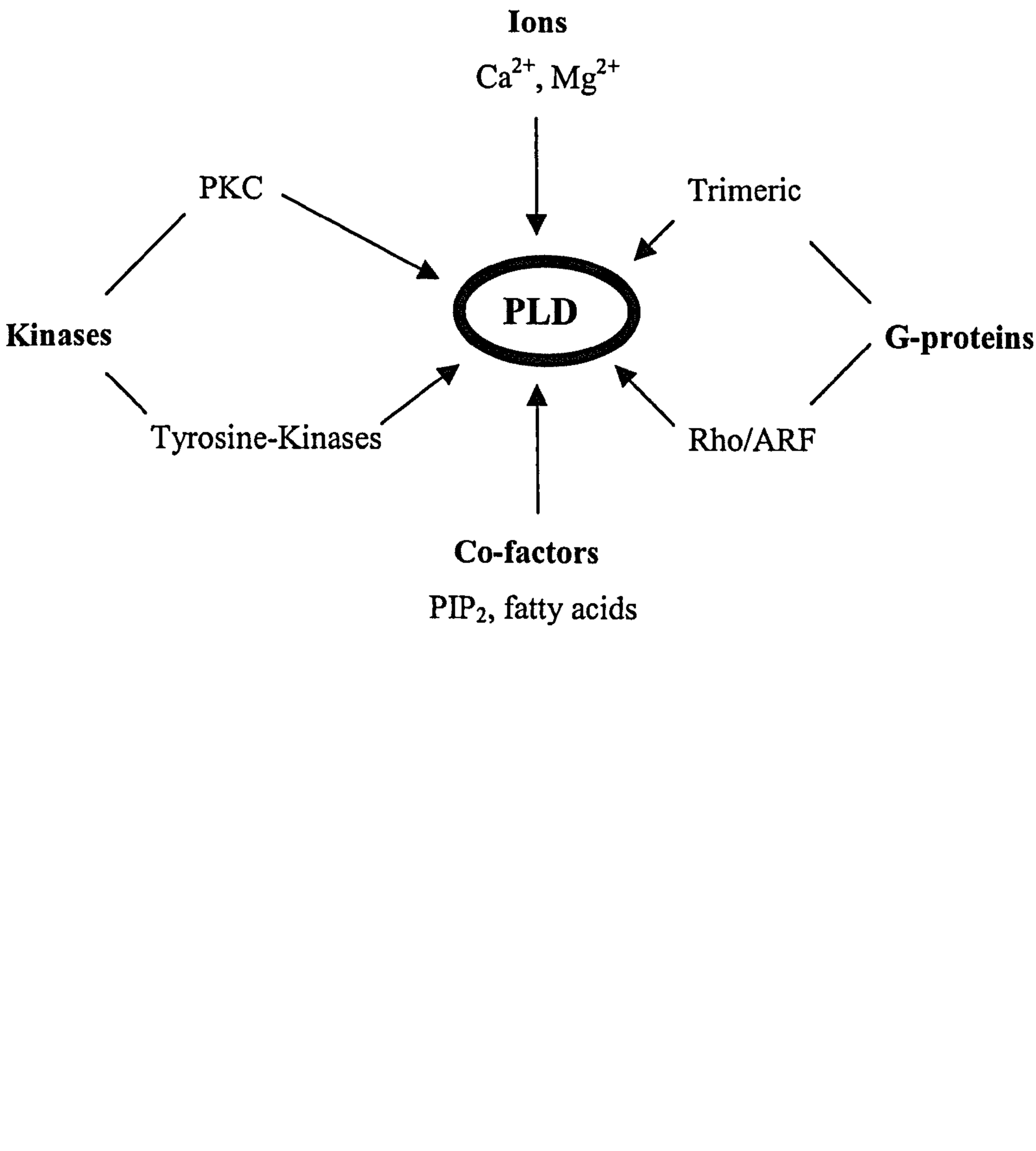
Reports have detailed the complex regulation of PLD1 by factors such as PKC, Rho and ARF. In contrast, PLD2 is insensitive to PKC and small G-proteins. PLD2 is generally believed to be regulated by inhibitory factors and α- and β-synucleins have recently been shown to inhibit PLD2 *in vitro* (Jenco et al., 1998, for reviews, see Exton, 2000, Liscovitch et al., 2000). The regulation of PLD activities continues to be investigated and recent evidence has shown PLD2 activity to be sensitive to tyrosine phosphorylation (Liscovitch et al., 2000). Furthermore, the mild activation of PLD2 activity by ARF has also been detailed (Sung et al., 1999).

1.3.2. Phospholipase C enzymes and the production of PA

1.3.2.1. Phosphatidylcholine hydrolysis by PLC enzymes and their regulation

As detailed previously, production of PA via the actions of PLC enzymes requires the sequential hydrolysis of membrane phospholipids by PLC enzymes producing DG, followed by the subsequent phosphorylation of DG by DGK. Hydrolysis of PC by PLC (PC-PLC) enzymes has been proposed in many cell types such as Swiss 3T3 fibroblasts (Cook & Wakelam, 1992), hepatocytes (Pittner & Fain, 1991), guinea pig tracheal smooth muscle cells (GPTSM) (Pyne & Pyne, 1994) and in intestinal smooth muscle cells (Murthy & Makhlouf, 1995). However, the regulation of PC-PLC enzymes has yet to be fully resolved. Pyne & Pyne (1994) demonstrated that bradykinin (BK) stimulated PC-PLC activation is not downstream of PIP_2 hydrolysis as a B2 receptor antagonist did not inhibit PC-PLC derived DG production but blocked IP_3 formation. In addition to this, a negative modulatory role for PKC on PC-PLC has been proposed based on the findings that in fibroblasts, PKC down-regulation inhibited PLD activity but increased the levels of PC-derived DG (Van

Figure 1.3: Outline of the possible upstream factors involved in PLD regulation (adapted from Gomez-Cambronero & Keire, 1998).



Blitterswijk et al., 1991). Consistent with this, down-regulation of PKC in GPTSM cells had no effect on BK stimulated PA and DG levels even though PLD activity was abolished (Pyne & Pyne, 1994). However, in contrast to these findings, Murthy & Makhlouf (1995) demonstrated that PC-PLC was calcium dependent and appeared to be G-protein coupled but was insensitive to PKC. In addition to this, a more recent study reported a bacterial PC-PLC activity that required calcium and zinc for activity (Preuss et al., 2001).

1.3.2.2. Phosphoinositide hydrolysis by PLC enzymes and their regulation

Multiple isoforms of phosphoinositide hydrolysing PLC (PI-PLC) enzymes have been described. These are divided into three groups, namely PLCβ of which there are four (PLCβ 1-4), PLCγ of which there are two (PLCγ1 & 2) and PLCδ of which there are four (PLCδ 1-4) isoforms (Rhee & Choi, 1992, Lee & Rhee, 1995 and Rhee & Bae, 1997). Furthermore, a fourth type, PLCα has been proposed (Cockcroft & Thomas, 1992). However, PLCα is apparently a proteolytic product of a PLCδ isoform (Exton, 1996, Exton, 1997). PLCβ, γ and δ isoforms have been shown to have two regions of homology, X and Y, and these are thought to be the catalytic domain of the enzymes (Lee & Rhee, 1995, Exton, 1996, Lee & Bae, 1997). Furthermore, all PI-PLC isoforms contain a pleckstrin homology (PH) domain, with PLCγ having two (Parker et al., 1994, Lee & Rhee, 1995). In addition, unlike the PLCβ and PLCγ isoforms, the PLCδ enzymes have three Src homology (SH) domains (two SH2 and one SH3) between the X and Y regions which participate in protein-protein interactions (Lee & Rhee, 1995, Exton, 1997).

The agonist stimulation of PI-PLC isoforms is regulated by two mechanisms. One of these involves G-proteins and the other tyrosine phosphorylation. The involvement of pertussis toxin-sensitive and –insensitive G-proteins in PI-PLC regulation was suggested to be due to pertussis toxin inhibiting PI-PLC in a cell type specific manner (Cockcroft & Thomas, 1992). However, it is now recognised that Gαq subunits (αq, α11, α14 and α16) are involved in the regulation of PLCβ isoforms only (Smrcka et al., 1991, Taylor et al., 1991) and that this occurs with an order of

responsiveness of PLCβ1 > β3 > β4 > β2 (Lee & Rhee, 1995, Rhee & Bae, 1997). Camps and colleagues (1992) were the first to demonstrate that the pertussis toxin-sensitive component of G-protein PI-PLC regulation was mediated by the βγ subunits of Gi and Go. It is now known that βγ subunits regulate PLCβ isoforms with a different order of potency from Gαq subunits such that the order of responsiveness is PLCβ3 > β2 > β1 and PLCβ4 is unresponsive (Lee et al., 1994). In addition to these regulatory mechanisms, other reports have shown the phosphorylation of several PLCβ isoforms by the Cyclic adenosine 3',5' monophosphate (cAMP)-dependent protein kinase (PKA) and also PKC (Liu & Simon, 1996, Ryu et al., 1990, for review, see Rebecchi & Pentyala, 2000).

In contrast to the above regulation of PI-PLCβ isoforms, PLCγ is activated following growth factor binding to receptors through tyrosine phosphorylation (Nishibe et al., 1990, Foster, 1993, Lee & Rhee, 1995, Rhee & Bae, 1997). Several lines of evidence demonstrate the importance of tyrosine phosphorylation on PLCγ activation. For example, replacement of tyrosine^{783} of PLCγ by phenylalanine has shown that phosphorylation at this residue is important in the activation of this enzyme (Exton, 1996). Furthermore, tyrosine autophosphorylation of the platelet derived growth factor (PDGF) receptor at tyrosine^{1021} causes PLCγ to bind to the receptor through its SH2 domain (Kashishian & Cooper, 1993). Little is known about the regulation of PLCδ isoforms although calcium is believed to play an important role (Lee & Rhee, 1995).

1.3.3. Diacylglycerol Kinases

Diacylglycerol kinases (DGK's) constitute a family of lipid kinases which catalyse the phosphorylation of DG to PA. The DGK enzymes are believed to be important in the termination of PKC signalling through the removal of PIP_2 derived DG (Kanoh et al., 1993, Quest et al., 1996). Mammalian DGK activities have been reported within cytosolic, microsomal and nuclear fractions (Kanoh et al., 1993, Quest et al., 1996) and a number of DGK isoforms have been purified and cloned from several tissues. An 83 kDa, cytosolic DGK was initially purified and cloned from porcine

brain by Kanoh and colleagues (1983). Following this, a 90 kDa isoform was purified from rat brain (Goto & Kondo, 1993). Furthermore, Kato and Takenawa (1990) have purified 110 kDa cytosolic and 150 kDa membrane associated DGK activities. DGK activities have also been purified from the particulate fraction of bovine testes (Walsh et al., 1994) and porcine testicular membranes (Hodgkin et al., 1997). The wide distribution of DGK's in several cell types and tissues serves to demonstrate the functional significance of these enzymes. To date, nine DGK isoforms have been cloned and all isoforms contain a conserved catalytic domain and at least two cysteine-rich domains. However, DGK's δ and η have a bipartite catalytic domain and DGK θ has three cysteine-rich regions. In addition to these domains, most DGK's have structural motifs that form the basis of division into five subtypes as shown in Table 1.1 (see Topham & Prescott, 1999 for review).

Table 1.1: Summary of DGK isoforms cloned.

Subtype	**Isoforms**	**Defining Features**
Type I	α, β, γ	Calcium binding EF hand motifs at N-termini.
Type II	δ, η	Pleckstrin Homology (PH) domains at N-termini.
Type III	ε	No identifiable regulatory domains.
Type IV	ζ, ι	Ankyrin repeats at C-termini.
Type V	θ	Three cysteine rich domains and a PH domain.

The control of DGK's is essential due to their influence on cellular DG and PA levels. However, little is known about the regulation of the various DGK isoforms. Activation of Type I DGK's by calcium through the EF hand motifs represents a method by which increased DG levels could be countered through an increase in

DGK activity. Calcium normally increases in parallel with DG levels due to the concurrent actions of IP_3 within cells and so this signal could act to attenuate the DG signal (Topham & Prescott, 1999). Other potential regulatory mechanisms are associated with the regulatory domains detailed in Table 1.1. For example, the Type II and Type V DGKs possess PH domains which indicates possible involvement of other signalling molecules in the regulation of these isoforms (Sakane et al., 1996, Klauck et al., 1996, Houssa et al., 1997). Further possible regulatory mechanisms include phosphorylation by protein kinases. For example, DGKθ contains several potential PKC phosphorylation sites (Houssa et al., 1997). In addition, PKCα and PKCε have been shown to phosphorylate DGKα (Schaap et al., 1993).

1.3.4. Lyso-phosphatidic acid acyltransferase

Lyso-phosphatidic acid acyltransferase (LPAAT) catalyses the acylation of LPA to PA and is involved in the *de novo* synthesis of glycerolipids. LPAAT enzymes have been identified in a number of species including *Caernorhabditis elegans*, *Escherichia coli* and *Saccharomyces cerevisiae* (Eberhardt et al., 1997). However, two human LPAAT enzymes have also been cloned. The first of these was cloned by Eberhardt et al. (1997) and the second human LPAAT (LPAATα) was cloned by Aguado and Campbell (1998) and found to show 48% sequence homology to the first human LPAAT.

There is little evidence relating to the regulatory mechanisms involved in the control of these enzymes. However, Bursten et al. (1991) demonstrated activation of LPAAT in human mesangial cells (HMC) following interleukin-1 (IL-1) stimulation. Furthermore, EL-4 cells [a murine T cell (Thymoma) line with defective IL-1 receptors] failed to exhibit LPAAT activation following IL-1 stimulation and so demonstrates a requirement for intact IL-1 receptors for LPAAT activation. In addition, the apparent phosphorylation and activation of LPAAT *in vitro* using isolated pig parotid gland microsomes and the catalytic subunit of cAMP-dependent or calcium/calmodulin dependent protein kinases was demonstrated (Soling et al., 1989).

1.4. Routes of removal of Phosphatidic acid

1.4.1. Hydrolysis of PA by Phospholipase A enzymes

Phospholipase A (PLA) enzymes catalyse the hydrolysis of the fatty acid ester bonds of phospholipids. There have been several reports of PA-specific PLA activities. Higgs and colleagues reported the cloning and expression of a PA-preferring PLA_1 from bovine testis (Higgs et al., 1998). A more recent study by the same group reported this soluble PLA_1 to be phosphorylated *in vitro* by protein kinase CK2 (CK2) and extracellular-signal regulated kinase 2 (ERK-2) [Han et al., 2001]. Furthermore, protein phosphatase 2A (PP2A) was found to selectively hydrolyse these phosphorylations highlighting the possibility of regulation of PLA_1 by phosphorylation/dephosphorylation (Han et al., 2001).

In addition to the PA preferring PLA_1 activities, a specific PA-hydrolysing PLA_2 enzyme has been purified from rat brain (Thomson & Clark, 1995). Furthermore, a PA-specific, human secretory PLA_2 ($hsPLA_2$) has been identified and implicated in the conversion of PA to LPA in the physiological response to inflammatory stimuli through the hydrolysis of PA in microvesicles shed from activated cell types such as platelets (Snitko et al., 1997). In support of this, Kinkaid and colleagues also reported the hydrolysis of PA by $hsPLA_2$ and implicated this activity with a similar role in the hydrolysis of PA in microvesicles shed from activated cells during inflammatory disorders (Kinkaid et al., 1998). In addition, Kitatani and colleagues (2000) reported the hydrolysis of PA by a cytosolic PLA_2 ($cPLA_2$) activity that was regulated by PLD in the mast cell line RBL-2H3 following antigen stimulation.

1.4.2. Hydrolysis of PA by Phosphatidic acid phosphatases

Phosphatidic acid phosphatase (PAP) dephosphorylates PA into DG (Smith et al., 1957). It was identified as an important enzyme in glycerolipid biosynthesis by regulating the supply of DG which, in addition to its second messenger role, is an important intermediate in cellular lipid metabolism (Martin, 1988). PAP is now

regarded as a control point for the direction of *de novo* glycerolipid synthesis between neutral diglycerides and triglycerides, zwitterionic phospholipids e.g. PC, or to the acidic phospholipids such as PI (Aridor-Piterman et al., 1992).

Initial research demonstrated contrasting effects of Mg^{2+} ions on the activity of PAP in several systems (Jamdar & Fallon, 1973, Martin et al., 1987, Walton & Possmayer, 1989). This led to the discovery that two distinct PAP isoforms were present in rat liver (Jamal et.al., 1991). Three biochemical characteristics were used to discriminate the two distinct PAP activities and these were: **1** Subcellular localisation; **2** Inhibition by sulfhydryl reagents such as N-ethylmalaeimide (NEM); **3** Dependence on Mg^{2+} ions for activity (see Table 1.2). One activity was localised to the plasma membrane (PAP-2) and the other to microsomes and the cytosol (PAP-1). These two activities have subsequently been identified in several tissues including liver (Day & Yeaman, 1992), brain (Fleming & Yeaman, 1995a), adipose tissue (Jamdar & Cao, 1994), neutrophils (Boder et.al., 1994), cardiac myocytes (Swanton & Saggerson, 1997) and airway smooth muscle cells (Tolan & Pyne, 1995 a, b). Furthermore, similar PAP activities have been reported in a variety of cells including rat brain (Fleming & Yeaman, 1995a), rat lung (Walton & Possmayer, 1989), adipocytes (Jamdar & Cao, 1994), neural NG108-15 cells (Aridor-Piterman et al., 1992), myocytes (Swanton & Saggerson, 1997) and neutrophils (Taylor et al., 1993) (for reviews, see Kanoh et al., 1993, Brindley and Waggoner, 1996).

Table 1.2: The two types of PAP activity

	PAP-1	**PAP-2**
Subcellular localisation	Mainly cytosolic	Membrane
Inhibited by NEM	Yes	No
Dependent on Mg^{2+}	Yes	No

1.4.2.1. Phosphatidic acid phosphatase-1

Phosphatidic acid phosphatase-1 (PAP-1) is located in the cytosolic fraction of cells and is believed to play a crucial role in glycerolipid biosynthesis (Martin et al., 1994, Brindley & Waggoner, 1996). PAP-1 activity is characterised by an absolute requirement for Mg^{2+} and sensitivity to inhibition by NEM (Taylor et al., 1993) (Table 1.2) and has been extensively characterised in adipose and liver tissues (Martin et al., 1994, Brindley & Waggoner, 1996). However, the translocation of PAP-1 from the cytosol to the endoplasmic reticulum and mitochondria and subsequent activation has been observed in response to increases in the fatty acids oleate and palmitate, as well as their CoA esters (Freeman & Mangiapane, 1989, Martin-Sanz et al., 1984). However, in direct contrast to these findings, Elabbadi and colleagues have recently shown the inhibition of rat liver PAP-1 activity in all subcellular fractions by these fatty acids and their CoA esters (Elabbadi et al., 2002). These authors also reported the reversal of this inhibition by PA and albumin (Elabbadi et al., 2002). In addition, a role for protein phosphorylation /dephosphorylation in the translocation of PAP-1 has also been proposed (Butterwith et al., 1984). Furthermore, the translocation of PAP-1 to membrane compartments has been shown to require a negative charge on the membrane surface resulting in its sensitivity to inhibition by amphiphilic cations such as chlorpromazine (Brindley, 1988, Gomez-Munoz et al., 1992, Martin et al., 1994).

There is evidence to support a long-term hormonal regulation of PAP-1 activity which increases in response to glucocorticoids and glucagon, whereas insulin has the opposite effect (Pittner et al., 1985, Brindley, 1988). Furthermore, long-term incubation of rat hepatocyte cultures with transforming growth factor (TGF)-β was found to increase PAP-1 activity in a time and concentration dependent manner (Dixon et al., 1997). Other forms of regulation of PAP-1 activity have also been reported. For example, stimulation of polymorphonuclear leukocytes (PMNL) with formyl-Met-Leu-Phe (fMLP) was found to decrease soluble, Mg^{2+}-dependent PAP activity while increasing a similar activity in the particulate fraction (Truett et al., 1992). However, this was apparently not due to translocation of the enzyme (Truett

et al., 1992). More recent studies have detailed the regulation of PAP-1 activity in Rod outer segments by protein phosphorylation/dephosphorylation such that under PKC phosphorylation conditions, PAP-1 activity was inhibited in both darkness and light (Perez-Roque et al., 1998). Further evidence of co-ordinated regulation of PAP-1 in the eye was also provided by De Arriba Zerpa and colleagues who demonstrated the activation of PAP-1 by light exposure in retinal ganglion cells through a c-fos dependent mechanism (De Arriba Zerpa et al., 1999).

A role for PAP-1 in signal transduction following translocation to the plasma membrane can not therefore, be excluded. A Mg^{2+}-dependent, NEM-sensitive PAP activity has been shown to associate with the epidermal growth factor (EGF) receptor in A431 cells (Jiang et al., 1996). Furthermore, PAP-1 has been implicated with a role in regulating prostaglandin E2 (PGE2) production and signalling through the regulation of cyclooxegenase-2 expression in human amnionic WISH cells (Johnson et al., 1999).

1.4.2.2. Phosphatidic acid phosphatase-2 / Lipid phosphate phosphatases

PAP-2 was first identified as a distinct activity in rat liver (Jamal et al., 1991), where it was found to be localised predominantly to the plasma membrane (which was compatible with a role in signal transduction), insensitive to inhibition by NEM and independent of Mg^{2+} (Table 1.2). PAP-2 has been implicated with a role in signal transduction and specifically the PLD signalling pathway for many years (for review, see Brindley et al., 1996). Several attempts were made to purify the enzyme from various sources such as pig thymus (Kanoh et al., 1992, Kai et al., 1996) and rat liver (Fleming & Yeaman, 1995b, Waggoner et al., 1995) [see Chapter 3]. However, only partial purification was achieved with significant discrepancies in the molecular weights reported.

Subsequently, Kai and colleagues (1996) reported the first cDNA cloning of a PAP-2 isoform from mouse kidney (mPAP, GenBank™ accession number D84376) which encoded a protein with a predicted molecular weight of 31.89kDa. Following this,

the same group identified and cloned two human isoforms of PAP-2, hPAP-2a and hPAP-2b (47% homology: GenBank™ accession numbers A000888 and A000889 respectively) from HEPG2 cells (Kai et al., 1997). These two human PAP-2 genes were subsequently shown to have high sequence identity with several other members of a protein phosphatase family. hPAP-2a shares 84% nucleotide sequence identity with the original mPAP and hPAP-2b was found to share 94% nucleotide sequence identity with the rat *Dri42* gene which was identified as a protein localised to the endoplasmic reticulum (Barila et al., 1996). More recently, a third human PAP-2 isoform hPAP-2c was identified by two separate groups (GenBank™ accession numbers AF05683 and AF047760) [Roberts et al., 1998, Hooks et al., 1998 respectively]. The identification of hPAP-2c was then closely followed by the cloning and identification of a mouse gene with 86% amino acid sequence identity with hPAP-2c, termed the mouse *Ppap2c* gene (Zhang et al., 2000). It was therefore, apparent that several isoforms of PAP-2 existed within cells.

Early studies characterising PAP-2 activity utilised partially purified preparations (Waggoner et al., 1995, 1996, Fleming & Yeaman, 1995, Siess & Hofstetter, 1996, Kai et al., 1996, English et al., 1997). During these studies, Waggoner and colleagues demonstrated the ability of PAP-2 from rat liver to dephosphorylate PA, LPA, C1P and S1P (Waggoner et al., 1996). Furthermore, overexpression of recombinant PAP-2 isoforms confirmed their ability to dephosphorylate PA, LPA, C1P and S1P (Roberts et al., 1998, Kai et al., 1997, Hooks et al., 1998) [see Chapter 4]. This led to the suggested renaming of the PAP-2 enzyme family to the lipid phosphate phosphohydrolases or lipid phosphate phosphatases (LPPs) [Brindley & Waggoner, 1998, Waggoner et al., 1999]. Table 1.3 details the suggested nomenclature for the LPP enzyme family and also the original PAP-2 nomenclature.

Table 1.3: Nomenclature of the LPP enzyme family formerly known as PAP-2 (adapted from Brindley & Waggoner, 1998). m, mouse; r, rat; h, human; gp, guinea-pig.

Suggested LPP name	Previous PAP-2 name	GenBank™ accession number
mLPP1	mPAP-2	D84376
rLPP1	rPAP-2	U90556
hLPP1	hPAP-2a hPAP2α1 PAP-2a	AB000888 AF014402 AF017116
gpLPP1	gpPAP-2a1	AF088283
hLPP1a	hPAP-2α2	AF014403
gpLPP1a	gpPAP-2a2	AF088284
hLPP2	PAP-2γ PAP-2c PAP-2c	AF035959 AF056083 AF047760
hLPP3	hPAP-2b hPAP-2β hPAP-2b	AB000889 AF043329 AF017786

Amino acid sequence analysis of the LPP isoforms predicts that they possess six putative transmembrane domains with three extracellular loops, two intracellular loops and intracellular N- and C-terminal domains (Kai et al., 1996, Barila et al., 1996, Kai et al., 1997, Hooks et al., 1998, Roberts et al., 1998, Tate et al., 1999, Nanjundan & Possmayer, 2001). Notably, they contain a novel sequence motif [$KX_6RP(X_{12-54})PSGH(X_{31-54})SRX_5HX_3D$] found in a superfamily of phosphatases (Stukey & Carman, 1997) [Figure 1.4]. Furthermore, recent mutational studies have demonstrated that the three conserved domains of LPP1 represent the active sites of the enzyme as mutation of seven conserved residues within the domains was found to abolish LPP1 activity (Zhang et al., 2000). The superfamily of phosphatases contains among others, the Dri42 protein of rat (Barila et al., 1996), the Drosophila protein wunen (Zhang et al., 1997), mammalian HIC-53 (Egawa et al., 1995), as well as a number of other enzymes such as bacterial acid phosphatases (for review, see Brindley & Waggoner, 1998). Spliced variant forms of LPP1 have also been identified in human and guinea-pig, termed hLPP1a (Leung et al., 1998) and gpLPP1a (Tate et al., 1999) respectively.

Further investigation into the molecular structure of the LPP enzymes revealed a single N-glycosylation site on the predicted second extracellular loop. This has been confirmed experimentally for the LPP1 from porcine thymus (Kai et al., 1996), mouse (Kai et al., 1996) and both human LPP1 and LPP3 (Kai et al., 1997, for review, see Kanoh et al., 1999). Furthermore, a glycosylation site in the predicted second extracellular loop of mLPP1 was recently demonstrated by mutation of Asn^{142} (N142Q mutation) and was found to decrease the molecular weight of the protein by 4 kDa on SDS-PAGE, as expected for the unglycosylated form of the enzyme (Zhang et al., 2000). It is possible to enter the amino acid sequence of enzymes into the online program PROSITE© (http://www.expasy.org) [Hofmann et al., 1999] which searches a protein sequence for specific domains or biologically important sequences. Analysis of gpLPP1, gpLPP1a, hLPP2 and hLPP3 (Figure 1.4) revealed a number of N-myristoylation sites within each LPP sequence. Furthermore, the four LPP isoforms were found to possess a number of phosphorylation consensus sites for several protein kinases (see Section 1.4.2.3.).

LPP activity against PA has been detected in all tissues examined to date (Brindley & Waggoner, 1998). However, Waggoner and colleagues showed that LPP activity in rats was low in skeletal muscle and heart tissue with the highest specific activity being detected in brain, lung, spleen and kidney (Waggoner et al., 1995). More recent studies have shown a widespread but varying distribution of the individual LPP isoforms. LPP1 and LPP1a mRNAs have been found to be expressed in heart, lung, kidney, bladder, prostate, uterus and aorta (Kai et al., 1997, Leung et al., 1998, Ulrix et al., 1998). However, these reports suggest a tissue dependent variation in the levels of mRNA expressed such that hLPP1 was found to be abundant in prostate but undetectable in the thymus, placenta and leukocytes (Kai et al., 1997). In contrast however, Leung and colleagues reported the detection of hLPP1 as the predominant LPP1 isoform in kidney, lung, liver, but also in placenta (Leung et al., 1998). Furthermore, this study reported that hLPP1a was the predominant isoform in heart and pancreas. LPP2 mRNA has been found to be less widely expressed, being found mainly in brain, pancreas and placenta (Hooks et al., 1998). Conversely, LPP3

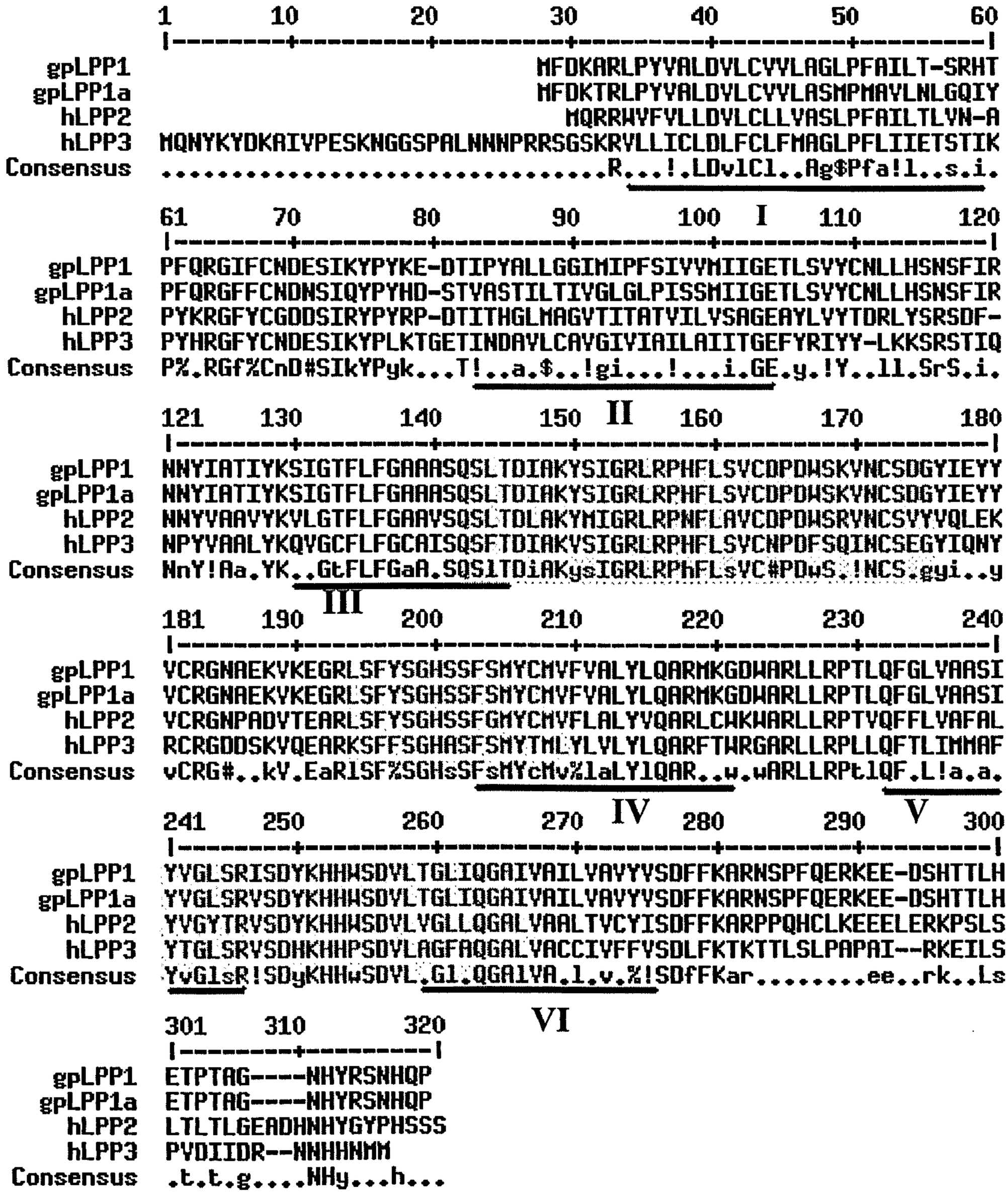


Figure 1.4: Amino acid sequence comparison of gpLPP1, gpLPP1a, hLPP2, and hLPP3.

The deduced amino acid sequences of gpLPP1, gpLPP1a, hLPP2 and hLPP3 are shown with a consensus sequence of conserved amino acids are indicated (! is anyone of IV, $ is anyone of LM, % is anyone of FY, # is anyone of NDQEBZ). Shaded regions encompas the three conserved domains which are conserved among the phosphatase superfamily (see text). Predicted transmembrane domains, denoted I – VI are underlined.

mRNA has been found to be ubiquitously expressed in all human tissue to comparable levels (Kai et al., 1997).

1.4.2.3. Regulation of Lipid phosphate phosphatases

The LPPs represent a family of enzymes which lie at a pivotal position for the regulation of the levels of several potent lipid mediators. However, there is very little known about the regulation of LPP activity or expression. As detailed previously, there are several potential phosphorylation sites on each of the LPPs. Analysis of the amino acid sequences of gpLPP1, gpLPP1a, hLPP2 and hLPP3 using the PROSITE© database (Hofmann et al., 1999) revealed the presence of potential phosphorlyation sites for Casein Kinase II (CK2) in all four LPP isoforms. Furthermore, there were also phosphorylation consensus sequences for PKC in all LPP isoforms tested except gpLPP1a and interestingly, a phosphorylation site for PKA in hLPP2 alone and a cell attachment sequence in hLPP3 alone. These findings are consistent with evidence reported by Waggoner and colleagues who demonstrated LPP from rat liver to be a phosphoprotein (Waggoner et al., 1995). Furthermore, rLPP1 has been detailed to have phosphorylation sites for PKC and CK2 (Waggoner et al., 1999). Thus, potential exists for the differing post-translational modification of the various LPP isoforms although direct evidence remains to be provided.

Agonist stimulation of LPP has been reported, in *ras* or the non-receptor tyrosine kinase fps transformed fibroblasts, where a role in cell growth has been suggested. The specific activity of LPP is decreased in both cell types and they fail to exhibit contact inhibition (Martin et al., 1993). In agreement with the above, levels of PA in *ras*-transformed fibroblasts have been shown to increase in a time dependent manner in contrast to that of control fibroblasts (Martin et al., 1997). In addition, the regulation of LPP activity by Sph and S1P has been demonstrated in *Saccharomyces cerevisiae* (Wu et al., 1993), neutrophils (Mullmann et al., 1991) and NG108-15 neuroblastoma cells (Lavie et al., 1990). Furthermore, the precursor of sphingosine, namely ceramide has been shown to apparently stimulate LPP activity (Gomez-

Munoz et al., 1994). This therefore, represents a further dimension to the possible regulatory mechanisms involved in the control of LPP activity. A more recent study has detailed the regulation of LPP activity in rod outer segments of the bovine eye via a transducin-mediated mechanism suggesting the possibility of G-protein regulation of certain LPP isoforms (Pasquare et al., 2000).

In addition to the above reports of possible regulatory mechanisms for LPP activity, *LPP1* has been previously identified as an androgen regulated gene in the human prostatic adenocarcinoma cell line LNCaP (Ulrix et al., 1998). Furthermore, mRNA levels for LPP3 but not LPP1 were found to increase up to 3-fold following treatment of HeLa cells with epidermal growth factor (EGF) indicating a possible difference in the regulation of LPP isoforms (Kai et al., 1997). The expression of the rat LPP homolog Dri42 has been found to increase during the differentiation of rat intestinal mucosa suggesting highly regulated expression of this protein during this process (Barila et al., 1996).

There are therefore, many indications for complex regulatory mechanisms controlling LPP expression and activity in many diverse cell types. However, the actual regulatory mechanisms *in vivo* remain poorly understood.

1.5. PA and its role in cellular signalling

1.5.1. *In vitro* effects of PA

Early research supported a role for PA in mediating calcium influx in cells. For example, PA was proposed to act as a calcium ionophore due to its ability to cause the movement of radioactive calcium across the phases of a suspending media (Putney et al., 1980). However, Holmes & Yoss demonstrated that the ionophoretic properties of PA were in fact due to contaminating oxidised fatty acids (Holmes & Yoss, 1983). Many cell free systems have been used to investigate the actions of PA. For example, PA was found to augment the guanine nucleotide-dependent and receptor agonist stimulation of PLC (Jackowski & Rock, 1989). Other studies

investigating the effects of PA on PLC enzymes detailed a two-fold stimulation in *Xenopus laevis* oocytes (for review, see English et al., 1996) and also that PA acts as an allosteric modifier which activates PLC-γ1 immunoprecipitated from A431 cells (Jones & Carpenter, 1993). More recent studies have now demonstrated PA to be involved in the regulation of several PLC isoforms, such as PLC-δ3 (Pawelczyk & Matecki, 1999) and PLC-β1 (Litosch 2000, Litosch 2002). The regulation of PLC-β1 by PA was found to be relatively independent of acyl chain length and was proposed to involve both a direct stimulation of PLC-β1 by PA and a dual regulation of G-protein stimulation of PLC-β1 (Litosch, 2002). However, the exact mechanism of PA regulation of G-protein stimulation of PLC-β1 could not be determined (Litosch, 2002). In contrast, PA was found to stimulate PLC-δ3 activity in a concentration and calcium dependent manner. The stimulation was proposed to require the calcium dependent interaction of PA with the C2 domain of PLC-δ3. Furthermore, PA was found to promote the binding of PLC-δ3 to phospholipid membranes most likely through interaction with the PH domain of the enzyme (Pawelczyk & Matecki, 1999).

PA has also been reported to modulate the activity of many other enzymes. These include a rolipram sensitive cyclic nucleotide phosphodiesterase (PDE) [Marcoz et al., 1993, Nemoz et al., 1997] which is activated upon PA binding (Grange et al., 2000). A specific PA binding site was identified by deletion of amino acids 31 to 59 of PDE4D3 and was characterised by the presence of a 13 amino acid fragment which included 4 basic residues and 5 hydrophobic residues (Grange et al., 2000). PA has also been shown to regulate protein tyrosine phosphatase 1C (PTP1C) [Tomic et al., 1995] and activate bacterial PLD (Geng et al., 1998), PDE4A5 (El Bawab et al., 1997), as well as secretory PLA_2 (Kinkaid et al., 1998). Furthermore, a recent study by Jones and colleagues has identified the γ-isoforms of the catalytic subunit of protein phosphatase-1 (PP1cγ) as a specific target for PA *in vitro* (Jones & Hannun, 2002). In this study, the authors demonstrate the non-competitive inhibition of PP1 through the binding of PA to PP1cγ (Jones & Hannun, 2002).

The activities of several protein kinases have also been shown to be regulated by PA such as phosphatidylinositol 4-phosphate kinase (Moritz et al., 1992), phosphatidylinositol 3-kinase (Lauener et al., 1995), the insulin receptor tyrosine kinase (Arnold & Newton, 1996) and the neutrophil, non-receptor tyrosine kinase Fgr (Sergeant et al., 2001). Several studies have detailed the activation of PKC by PA. Indeed, Senisterra and colleagues demonstrated that PA could replace phosphatidylserine (PS) to activate PKC (Senisterra et al., 1993). Furthermore, Limatola and colleagues demonstrated the calcium dependent activation of two PKC isoforms in a cell free assay by PA (Limatola et al., 1994). In this system, PA was found to be a strong activator of the DG-insensitive PKCξ isoform in the absence of calcium, implicating PA with a role in the activation of PKCξ in cells where intracellular calcium is at basal levels (Limatola et al., 1994). In addition to the studies described above, PA has been found to activate a novel, calcium-independent protein kinase distinct from PKC isoforms, in Triton extracts of human platelets (Khan et al., 1994). More recent studies have also shown the PA-mediated activation of a novel protein kinase in neutrophils which is involved in NADPH oxidase activation during the respiratory bust of neutrophils and this is discussed in more detail below (Waite et al., 1997, McPhail et al., 1999).

1.5.2. Intracellular effects of PA

The most extensively studied physiological role of PA is that of activation of NADPH oxidase and subsequent superoxide generation during the respiratory burst of neutrophils. NADPH oxidase activation has been found to be a complex event that involves several components including Rac2, a small molecular weight G-protein. During cell stimulation, Rac2 dissociates from a complex with GDP dissociation inhibitor (GDI), allowing binding of GTP to Rac2 and its subsequent translocation to the plasma membrane for involvement in NADPH oxidase activation (Park, 1996, Waite et al., 1997, McPhail et al., 1999, Erickson et al., 1999, for review, see English et al., 1996). PA has been found to disrupt the complex between Rac2 and GDI and also to cause a conformational change in p47Phox which is also involved in NADPH oxidase activation upon translocation to the plasma membrane

(Park, 1996). Later studies have demonstarted the phosphorylation of p47Phox by a novel PA-dependent cytosolic protein kinase (Waite et al., 1997, McPhail et al., 1999). McPhail and colleagues also report the partial purification of the PA-dependent protein kinase and demonstrate it to be distinct from other protein kinases known to phosphorylate p47Phox such as PKC and ERK and to be widely distributed in a variety of haematopoietic cell lines and in rat brain (McPhail et al., 1999). In addition to these proposed roles for PA in NADPH oxidase activation, Erickson and colleagues detailed the direct activation of NADPH oxidase by PA in a cell free system (Erickson et al., 1999).

Further intracellular effects of PA include the induction of mitogenesis. For example, PA has been shown to be mitogenic in fibroblasts (Yu et al.,1988, Van Corven et al., 1992) and mesangial cells (Knauss et al., 1990). Furthermore, in Balb/c 3T3 cells, PLD-derived PA has been linked with the mitogenic response of PDGF (Fukami & Takenawa, 1992). PA has also been implicated in mediating the proliferation associated with other receptor agonists including epidermal growth factor (EGF) [Kaszkin et al., 1992] as well as Interleukins 1, 2 and 11 (Bursten & Harris, 1994, Cano et al., 1992, Siddiqui & Yang, 1995). In addition, in Swiss 3T3 fibroblasts, the mitogenic effects of sphingosine (Sph) and sphingosine 1-phosphate (S1P) have been suggested to involve a PA-mediated effect (Zhang et al., 1990).

It is suggested that the mitogenic response to PA is mediated through the PA-dependent activation of the ERK-1/2 signalling cascade. This is based on reports that PA inhibits p21RasGTPase activating protein (GAP) [Tsai et al., 1989] and also activates the p21RasGTPase inhibitory protein (GIP) [Tsai et al., 1990]. The combination of these two actions would result in the prolonged lifetime of active GTP-bound Ras which is important in the activation of ERK-1/2 and the proliferative responses of cells. In support of this, PA-induced mitogenesis was blocked by micro-injection of an anti-Ras antibody in NIH3T3 fibroblasts (Yu et al., 1988). In addition to the effects of PA on GAP and GIP, PA can integrate into the ERK-1/2 signalling cascade at the level of Raf-1 kinase. For example, Ghosh and colleagues reported the direct interaction and subsequent activation of Raf-1 kinase by PA

(Ghosh et al., 1996). More recently however, PA has been demonstrated to mediate the translocation of Raf-1 to the plasma membrane leading to subsequent activation of Raf-1 and the ERK-1/2 signalling cascade (Rizzo et al., 1999). This group also reported that PA was required, but not sufficient alone for the activation of Raf-1 and concluded that the main role of PA was in the induction of Raf-1 translocation to the plasma membrane (Rizzo et al., 1999). In addition to the effects of PAs on NADPH oxidase activation and mitogenesis, PA has also been implicated in many other cellular responses such as actin polymerisation (Ha & Exton, 1993), platelet activation (Kroll et al., 1989) and hormone release (Metz & Dunlop, 1990).

1.5.3. Extracellular effects of PA

The addition of exogenous PA has been shown to cause calcium influx in many biological tissues including cultured hepatocytes (Osugi et al., 1984). Furthermore, exogenous PA has been shown to cause mitogenesis in normal mouse mammary epithelial cells (Imagawa et al., 1989). However, LPA has been attributed with many of the proposed signalling functions of exogenously added PA (reviewed by Moolenaar, 1995). For example, LPA which is derived from PA has been shown to cause calcium influx in fibroblasts (Moolenaar et al., 1992). Furthermore, both LPA and PA inhibit adenylyl cyclase (Murayama & Ui, 1987) and are potent mitogens in fibrobalasts (Van Corven et al., 1992) and endothelial cells (Imagawa et al., 1989). However, LPA and PA are found to be equipotent mitogens in fibroblasts and Van Corven and colleagues suggest this to be an argument against LPA mediating the effects of PA (Van Corven et al., 1992).

Several groups have investigated the involvement of a PA receptor in mediating these effects of PA. Murayama and Ui suggested that the adenylyl cyclase inhibition resulting from exposure of fibroblasts to PA was mediated by membrane receptors as pre-treatment of these cells with pertussis toxin (PTX) decreased these effects suggesting the presence of a Gαi/o coupled G-protein coupled receptor (GPCR) system (Murayama & Ui, 1987). In addition to this, Ferguson and Hanley reported the induction of calcium-dependent electrical responses in *Xenopus laevis* oocytes by

both dioleoyl $PA_{(C18:1,18:1)}$ and oleoyl $LPA_{(C18:1)}$ through a mechanism which was not intracellular, thereby implicating a surface receptor (Ferguson & Hanley, 1992). More recently Sliva and colleagues suggest that the PA-mediated migration of human breast cancer cells is mediated via a cell surface GPCR specific for PA (Sliva et al., 2000). In addition, a recent report by Alderton and colleagues demonstrated the receptor mediated activation of ERK-1/2 by various molecular species of PA (Alderton et al., 2001). In this study, the rank order of potency for stimulation of ERK-1/2 by the different molecular species of PA tested was dioctanoyl $PA_{(C8:0,8:0)}$ > dipalmitoyl $PA_{(C16:0,16:0)}$ > stearoyl/arachidonyl $PA_{(C18:0,20:4)}$ ≈ dioleoyl $PA_{(C18:1,18:1)}$ (Alderton et al., 2001). Furthermore, these responses were found to be PTX sensitive suggesting that PA binds to a GPCR coupled to Gαi/o G-proteins (Alderton et al., 2001).

1.5.4. Molecular species of PA produced during cell stimulation

Following cell stimulation, phospholipids are hydrolysed resulting in the production of a variety of lipid signalling molecules. PA is rapidly generated via several different pathways involving the hydrolysis of different parent phospholipids such as PI and PC by the actions of phospholipases C and D or through the phosphorylation of PIP_2-derived DGs by DGK. The fatty acid side chain composition of the parent phospholipid molecules involved in the production of PA are known to vary considerably (Pessin & Raben, 1989, Pettitt & Wakelam, 1993, Heung & Postle, 1995, Hodgkin et al., 1998). In relation to this, the differential hydrolysis of particular parent phospholipids can lead to the production of distinct PA species depending on the source parent phospholipid (Ohanian et al., 1998). Accordingly, to selectively identify PLD-generated products, an experimental strategy utilising the phosphatidyltransferase activity of PLD has been developed. In the presence of a primary alcohol such as propan-1-ol or ethanol, PLD generates more slowly metabolised phosphatidylalcohols (PtdAlcs) instead of PA. The structural analysis of the PtdAlcs produced following cell stimulation allows the selective sampling and investigation of PLD-generated products (Hodgkin et al., 1998). Investigations using this technique have revealed that PtdAlcs generated following cell stimulation

contain a combination of saturated, and mono-unsaturated fatty acids which were similar to the molecular species of PC present (Heung & Postle, 1995, Pettitt et al., 1997). In addition, others have analysed the PA species accumulating in various cell types following agonist stimulation. Divecha and colleagues demonstrated that, in Swiss 3T3 fibrobalasts stimulated with bombesin, polyunsaturated, mono-unsaturated and saturated PA species accumulated due to the activation of both PIP_2-PLC and PLD (Divecha et al., 1991). The accumulation of several different PA species was also reported following muscarinic receptor activation of human SK-N-SH neuroblastoma cells (Lee et al., 1991). In contrast, analysis of the PA species produced following stimulation of rat thymocytes with concanavalin-A revealed an accumulation of only polyunsaturated PA species (El Bawab et al., 1997). Furthermore, LPA stimulation of porcine aortic endothelial cells led to an increase in mono-unsaturated and saturated PA species (Pettitt et al., 1997). Interestingly, during this study, only PLD was reported to be activated.

Even though several reports have shown signalling roles for various PA species, the specific functions of the different molecular species of PA remain unclear. Murayama & Ui reported that, in Swiss 3T3 fibroblasts, PA species containing two saturated fatty acid side chains were less potent mitogens than PAs with unsaturated fatty acid side chains (Murayama & Ui, 1987). In contrast, Imagawa and colleagues demonstrated that the mitogenic effect of PA in mouse mammary epithelial cells required a polyunsaturated fatty acid at the sn-2 position of the glycerol moiety (Imagawa et al., 1989). Alternatively, $PA_{(16:0,16:0)}$ species have been shown to be the most potent stimulator of ERK-2 activity in mouse 3T3-L1 cells (Siddiqui & Yang, 1995). In another study, Tokumura and colleagues reported superoxide anion release from polymorphonuclear leukocytes induced by PA species with saturated, short chain fatty acid side chains such as $PA_{(6:0,6:0)}$, $PA_{(8:0,8:0)}$ and $PA_{(10:0,10:0)}$ (Tokumura et al., 1997). Furthermore, the actin stress fibre formation stimulated by PLD derived PA has been shown to be mimicked to a greater extent by mono-unsaturated PA species compared to poly-unsaturated PA species (Cross et al., 1996, Hodgkin et al., 1996). The latter studies led to the suggestion that the PA species with a role in

signal transduction were of the mono-unsaturated and saturated forms (Hodgkin et al., 1998).

It is believed by many that signalling PA species, including those which contain saturated, mono-unsaturated and possibly di-unsaturated fatty acid side chains are derived from the PLD catalysed hydrolysis of parent phospholipids such as PC (Hodgkin et al., 1998, Pettitt et al., 2001). Accordingly, biologically active DG species are considered to possess predominantly poly-unsaturated fatty acid side chains (three or more double bonds) and be derived via the PLC catalysed hydrolysis of inositol phospholipids (Jones et al., 1999). This therefore, allows the termination of an incoming PA signal via the dephosphorylation by PAPs or LPPs to inactive DG species or, conversely, the phosphorylation of a signalling DG species by DGK, to an inactive PA species. Recent work on PLD1b and PLD2a enzymes has revealed that both PLD isoforms produce structurally identical PA species following stimulation of mammalian cells (Pettit et al., 2001). The authors report that both PLD isoforms hydrolyse a common phospholipid pool to generate PA species rich in mono- and di-unsaturated fatty acid side chains (Pettitt et al., 2001). However, several studies have reported signalling roles for PA species with saturated, mono-unsaturated, di-unsaturated but also poly-unsaturated fatty acid side chains (Murayama & Ui, 1987, Imagawa et al., 1989, Siddiqui & Yang, 1995, Cross et al., 1996, Hodgkin et al., 1996). There is therefore, still much work to be done to discover if certain molecular species of PA have a signalling role while others do not and also by what route these signalling PA species are produced following cell stimulation.

1.6. Other phosphorylated lipids with a signalling role – Lysophosphatidic acid, Sphingosine 1-phosphate and Ceramide 1-phosphate

As described previously, phosphatidic acid (PA) is recognised as a potent signalling molecule. However, in addition to PA, the LPP substrates, lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), and ceramide 1-phosphate (C1P) have also been shown to have diverse signalling roles in health and disease. LPA and S1P are the most studied of these three phosphorylated lipids and research over recent years

has resulted in dramatic progress in the understanding of the biological roles of these two lysophospholipid mediators.

LPA and S1P are produced by a number of different cell types including platelets, macrophages, other leukocytes, adipocytes, some epithelial cells, as well as some tumour cells (for review, see Goetzl, 2001). Furthermore, once released or produced by these cells, LPA and S1P have been shown to be involved in processes such as cell growth, differentiation, cell survival and apoptosis (for reviews, see Pyne & Pyne, 2000; Tigyi, 2001).

In contrast to LPA and S1P, very little is known about the physiological functions of C1P. Gomez-Munoz and colleagues reported the stimulation of DNA synthesis by synthetic, short chain C1P (Gomez-Munoz et al., 1995a). In addition, ceramide kinase activity has been reported in HL-60 cells (Kolesnick & Hemer, 1990) and more recently in human polymorphonuclear leukocytes (PMNs) [Hinkovska-Galcheva et al., 1998]. Hinkovska-Galcheva and colleagues report that C1P is formed during phagocytosis through the activation of ceramide kinase and suggest that C1P could be involved in promoting phagolysosome formation (Hinkovska-Galcheva et al., 1998). Furthermore, the first cDNA cloning of a ceramide kinase was recently reported, based on sequence homology with sphingosine kinase type 1 (SphK1) by Sugiura and colleagues (Sugiura et al., 2002). Overexpression of the cDNA in HEK293 cells was found to produce significant amounts of ATP dependent, ceramide phosphorylating activity but very little activity towards sphingosine and diacylglycerol (Sugiura et al., 2002). However, for the purposes of this thesis, the discussion will focus on LPA and S1P and in particular, their signalling roles mediated via the recently identified LPA-specific and S1P-specific families of G-protein-coupled receptors (GPCRs).

1.7. Lysophosphatidic acid metabolism

1.7.1. Lysophosphatidic acid production

Lysophosphatidic acid (LPA) represents the simplest phospholipid which, apart from being the first intermediate in glycerolipid biosynthesis, has been attributed with a plethora of biological signalling functions. LPA consists of a glycerol backbone with a phosphate group at the *sn*-3 position, an hydroxyl group at either the *sn*-2 (or *sn*-1) position, and a fatty acid chain of predominantly long chain saturated ($C_{18:0}$, $C_{16:0}$) or unsaturated ($C_{18:1}$, $C_{20:4}$) at the *sn*-1 (or *sn*-2) position. LPA levels are extremely tightly controlled in cells, although the mechanisms of this control remain unclear. In addition, as with other lipid signalling molecules such as PA and DG, the relationship between LPA as a metabolic intermediate in the biosynthesis of glycerolipids and as a molecule involved in signal transduction remains to be determined. It is now clear that LPA is released from a variety of cell types such as platelets, adipocytes and ovarian cancer cells in response to agonist stimulation. Once released, LPA binds to a family of G-protein coupled receptors (GPCRs) known as the Endothelial differentiation gene (EDG) family of receptors which are now known as the LPA receptors (Section 1.8.) in order to elicit a multitude of biological responses (Erickson et al., 2001).

As described previously (Section 1.4.1.) LPA can be produced through the actions of phospholipases A_1 and A_2 on PA. However, in addition to this route of production, direct hydrolysis of lysophosphatidylcholine (LPC) by a lysoPLD activity has been reported to produce bioactive LPA (Van Dijk et al., 1998). In support of this, Tokumura and colleagues have also reported the production of LPA through a lysoPLD activity in rat plasma and human follicular fluids (Tokumura et al., 1996 & 1999). In addition, the lysoPLD activity in rat plasma was found to be selective for LPC containing unsaturated fatty acyl chains (Tokumura et al., 1999).

1.7.2. Routes of removal of LPA

Lysophosphatidic acid metabolism can occur via several different mechanisms. As described in Section 1.3.4., lysophosphatidic acid acyl transferases (LPAAT) catalyses the acylation of LPA into PA. In addition to this, multiple Lipid Phosphate phosphatase (LPP) isoforms exist which have been shown to dephosphorylate LPA to monoacylglycerol (MAG) (Section 1.4.2.2).

In addition to the two routes of hydrolysis described above, a lysophospholipase activity which converts LPA into glycerol 3-phosphate, has been described in rat brain (Thompson & Clark, 1994). This lysophospholipase activity was found to hydrolyse LPA at differing rates depending on the species of acyl group at the sn-1 position (1-oleoyl-LPA ≈ 1-stearoyl-LPA > 1-palmitoyl-LPA > 1-myristoyl-LPA) and was proposed to have a potential role in terminating the cellular responses mediated by LPA (Thompson & Clark, 1994). Furthermore, an LPA-specific phosphatase (LPAP) was recently purified from bovine brain cytosol and found to have little activity towards PA and no activity towards C1P and S1P (Hiroyama & Takenawa, 1998). Subsequently, Hiroyama and Takenawa reported the cDNA cloning of LPAP from a human brain library (Hiroyama & Takenawa, 1999). Transient overexpression of the cloned LPAP in COS-7 cells resulted in LPA-specific enzyme activity which showed little activity towards PA, S1P and C1P (Hiroyama & Takenawa, 1999).

1.8.Lysophosphatidic acid receptors

1.8.1. Lysophosphatidic acid receptor identification

There are currently three GPCRs which are believed to be specific for extracellular signalling by LPA. These receptors belong to the Endothelial differentiation gene (EDG) product receptor family and have recently been renamed as LPA_1, LPA_2 and LPA_3 (Table 1.4) (Chun et al., 2002).

Table 1.4 – Current nomenclature of the LPA and S1P receptor families previously known as the EDG receptor family (Adapted from Chun et al., 2002).

Agonist Ligand	IUPHAR-recommended name	'EDG' name	Previous names
LPA	LPA_1	EDG2	lp_{A1}, vzg-1, rec1.3
LPA	LPA_2	EDG4	lp_{A2}
LPA	LPA_3	EDG7	lp_{A3}
S1P	$S1P_1$	EDG1	lp_{B1}
S1P	$S1P_2$	EDG5	lp_{B2}, AGR16, H218
S1P	$S1P_3$	EDG3	lp_{B3}
S1P	$S1P_4$	EDG6	lp_{B4}
S1P	$S1P_5$	EDG8	lp_{B5}, nrg-1

LPA_1/EDG2 was the first LPA receptor to be identified and shown to be a high affinity LPA receptor. The cDNA for the receptor was identified from mouse cerebral cortical neuroblasts and was named *ventricular zone gene-1* (*vzg-1*) (Hecht et al., 1996). This was closely followed by the identification and cloning of human *LPA_1/EDG2*, as a functional LPA receptor (An et al., 1997a). Further support for the role of LPA_1/EDG2 as a functional LPA receptor was provided in yeast where LPA stimulated the pheromone response pathway (Erickson et al., 1998).

Subsequently, the *LPA_2/EDG4* gene was identified by two separate groups through the use of genomic cloning and analysis of expressed sequence tags (ESTs) in the GenBank database. An and colleagues identified an EST of high similarity to LPA_1/EDG2 using homology searches of mouse tumour cell libraries, which they named LPA_2/EDG4. Functional studies demonstrated that the protein encoded by the cDNA was an LPA receptor (An et al., 1998). At the same time, Contos & Chun identified a human genome sequence with 60% amino acid similarity to the LPA_1/EDG2, which they termed *lp_{A2}* (Contos & Chun, 1998). Subsequent work by Contos and Chun showed that the mouse gene *LPA_2/Edg4* identified by An and colleagues (1998), actually encoded a frame shift mutant which was distinct from that encoded by the human *lp_{A2}* gene (Contos & Chun, 2000).

The third LPA receptor (LPA_3/EDG7) was identified from human Jurkat T cells and was shown to be a functional LPA receptor (Bandoh et al., 1999). The amino acid sequence of the LPA_3/EDG7 receptor was detailed to be 53.7% and 48.8% identical to the other identified human LPA receptors LPA_1/EDG2 and LPA_2/EDG4 respectively (Bandoh et al., 1999). LPA_3/EDG7 was subsequently isolated from Human Embryonic Kidney 293 cells (HEK293) as further confirmation of its function as an LPA receptor (Im et al., 2000a).

1.8.2. LPA receptors – cellular effects, G-protein coupling and effector mechanisms

The first report of a cellular effect evoked by LPA was by Tokumura and colleagues who reported a vasopressor phospholipid, which they identified as LPA by Gas chromatography-Mass spectrometry (Tokumura et al., 1978). Additional cellular effects of LPA were then documented in the mid to late 1980's when LPA was found to increase [^{3}H]-thymidine incorporation, inhibit adenylyl cyclase (AC), increase PKC activity and increase inositol phosphates, intracellular calcium and arachidonic acid release in cultured cells (for review, see Contos et al., 2000). Interestingly, the proliferative and AC effects were abolished by the actions of pertussis toxin (PTX) which inactivates G-proteins of the Gαi/o-type and so implicated G-protein coupled receptor involvement. LPA induced effects on cell morphology were then reported in the early 1990's by several groups. For example, Ridley and co-workers reported an LPA-induced, Rho-dependent, rearrangement of the actin cytoskeleton through actin stress fibre formation in 3T3 fibroblasts (Ridley & Hall, 1992). Furthermore, several groups reported cell rounding and neurite retraction induced by LPA in neural cell lines such as PC12 cells (Dyer et al., 1992) as well as NIE-115 and NG108-15 neuronal cells (Jalink et al., 1993). The number of cell lines responding to LPA, as well as the variety of cellular and biochemical responses evoked by LPA have increased dramatically since these initial studies and include effects on differentiation, neurotransmitter release and smooth muscle contraction (for reviews, see Moolenaar et al., 1997, Chun et al., 1999, Contos et al., 2000).

The initial cloning of vzg-1/LPA_1/EDG2 by Hecht et al. (1996) represented one of the most important developments in the study of the functional responses evoked by LPA. This coupled with the subsequent cloning of LPA_2/EDG4 and LPA_3/EDG7 allowed the investigation of the G-protein coupling and downstream effectors involved in the biological responses evoked by LPA and its GPCRs. Heterotrimeric G-proteins are divided into four principle classes, namely Gαi/o, $G\alpha_{12/13}$, Gαs and Gαq. The LPA receptors have been shown to couple to members of the Gαi/o, $G\alpha_{12/13}$ and Gαq family, but not the Gαs family, under physiological conditions (Contos et al., 2000).

LPA_1/EDG2 is widely expressed in cerebrovascular tissue, gastrointestinal tissue, gonadal tissue and the central nervous tissue and has been shown to couple to G-proteins of the Gαi/o, $G\alpha_{12/13}$ and Gαq families (English et al., 2001, Contos et al., 2000). Responses evoked by LPA via LPA_1/EDG2 include the Gαi/o mediated intracellular calcium mobilisation, activation of the ERK-1/2 pathway and inhibition of adenylyl cyclase (An et al., 1998, Ishii et al., 2000). Furthermore, LPA_1/EDG2 has been shown to mediate arachidonic acid release via a combined Gαi and Gαq-mediated mechanism. Other Gαq mediated effects reported for LPA_1/EDG2 include inositol phosphate production via the Gαq-mediated activation of PLC (An et al., 1998, Ishii et al., 2000). In addition to the Gαi and Gαq-mediated effects, LPA_1/EDG2 has been demonstrated to cause cell rounding in B103 neuroblastoma cells through a Rho GTPase dependent mechanism (Fukushima et al., 1998). These effects are most likely mediated via $G\alpha_{12/13}$ type G-proteins which have been shown to interact with the Rho guanine nucleotide exchange factor (p115RhoGEF) [Kozasa et al., 1998].

LPA_2/EDG4 has been found to be less widely expressed than LPA_1/EDG2 being found principally in leukocytes and testicular tissue but has been shown, like LPA_1, to couple to G-proteins of the Gαi/o, $G\alpha_{12/13}$ and Gαq families (English et al., 2001, Contos et al., 2000). Due to the similar G-protein coupling of LPA_2/EDG4 to LPA_1/EDG2, the downstream signalling events mediated by the two receptors are similar. For example, stimulation of LPA_2/EDG4 has been shown to mobilise

intracellular calcium, activate ERK-1/2 and inhibit adenylyl cyclase via a Gαi dependent mechanism. Furthermore, LPA_2/EDG4 has been shown to cause the Gαq-mediated activation of PLC and inositol phosphate production, as well as the $G\alpha_{12/13}$ induced, Rho-mediated cell rounding also observed for LPA_1/EDG2 (An et al., 1998, Ishii et al., 2000, Bandoh et al., 1999). A difference between LPA_1/EDG2 and LPA_2/EDG4 mediated responses is that LPA_2/EDG4 has been shown to stimulate adenylyl cyclase in Sf9 insect cells although the mechanism of this stimulation remains unclear (Bandoh et al., 1999).

LPA_3/EDG7 has been reported in three independent studies (Bandoh et al., 1999, Im et al., 2000a, Ishii et al., 2000) and shown to be expressed in cerbrovascular, gastrointestinal, testicular and prostate tissues (English et al., 2001). Bandoh and colleagues showed that when overexpressed in Sf9 insect cells, human LPA_3/EDG7 led to increases in intracellular calcium and a stimulation of adenylyl cyclase in a manner similar to LPA_2/EDG4 (Bandoh et al., 1999). However, Im and co-workers demonstrated that overexpression of human LPA_3/EDG7 in RH7777 cells produced the same observed intracellular calcium mobilisation but did not result in modulation of cAMP levels (Im et al., 2000a). The study by Ishii and colleagues showed that overexpression of LPA_3/EDG7 in B103 cells led to PLC activation and inositol phosphate release, as well as activation of ERK-1/2, arachidonic acid release and inhibition of adenylyl cyclase (Ishii et al., 2000). Interestingly, Ishii and colleagues also reported that the LPA_3/EDG7 overepressed in B103 cells failed to induce cell rounding suggesting that LPA_3/EDG7 couples to G-proteins of the Gαi/o and Gαq families but not to members of the $G\alpha_{12/13}$ or Gαs family. (Ishii et al., 2000).

1.9. Sphingolipid metabolism

In addition to the more extensively characterised glycerolipid signalling pathways, it has recently been appreciated that, in a similar manner to the 'phosphatidyl inositol (PI) cycle', the 'sphingomyelin (SM) cycle' is an evolutionary conserved signalling pathway which can produce several potent lipid messengers. The initial observations of SM turnover having a role in signal transduction were made by Hannun and Bell

(1989). It is now recognised that agonist-stimulated metabolism of SM represents an important cellular signalling pathway.

SM is hydrolysed by specific PLC-like enzymes known as sphingomyelinases, resulting in the production of ceramide and phosphocholine. Ceramide, through the addition of a phosphocholine head-group from PC by SM synthase, can be converted back to SM (Mathias et al., 1998). Five distinct sphingomyelinases have been identified and distinguished by their differing pH optima, cellular localisation and cation dependence. Sphingomyelinase activation has been observed in response to agonists such as growth factors, cytokines and arachidonic acid (Levade & Jaffrezou, 1999, Perry & Hannun, 1999) although the precise mechanism involved remains to be clarified. Following the production of ceramide, ceramidases and ceramide kinases lead to the formation of sphingosine (Sph) and ceramide 1-phosphate (C1P) respectively (Mathias et al., 1998). Furthermore, the sphingosine kinase (SphK) mediated phosphorylation of Sph, leads to the production of sphingosine 1-phosphate (S1P) (Spiegel et al., 1996). The past ten years have seen S1P become recognised as a potent lipid signalling molecule which may function as both an intracellular second messenger and as an extracellular agonist. Indeed, S1P acts as a ligand for a family of S1P-specific G-protein coupled receptors to mediate a plethora of cellular responses (for reviews, see Pyne & Pyne, 2000a, 2000b, Hla, 2001, Yatomi et al., 2001).

1.10. Sphingosine 1-phosphate metabolism

1.10.1. Sphingosine 1-phosphate production

S1P production in cells is via the SphK catalysed phosphorylation of Sph. SphK activity has been shown to increase in response to various growth factor including Platelet-derived growth factor (PDGF) (Olivera & Spiegel, 1993, Pyne et al., 1996) and Nerve growth factor (Edsall et al., 1997). The first purification of SphK was by Olivera and colleagues who purified SphK from rat kidney to apparent homogeneity and showed it to be a protein of 49kDa (Olivera et al., 1998). This was followed by

the subsequent cloning and characterisation of two mammalian SphK's, murine SphK1a and SphK1b which were found to differ by only 10 amino acids (Kohama et al., 1998). The first human SphK was identified and cloned by Nava and colleagues using sequence homology to the murine SphK1a and was found to be ubiquitously expressed but with high levels in liver, kidney and skeletal muscle (Nava et al., 2000). In addition to the SphK type 1 enzymes described above, Liu and colleagues recently reported the cloning and characterisation of a second type of mammalian SphK activity, SphK type 2 (SphK2) (Liu et al., 2000). SphK2 is considerably larger than SphK1, containing an extra 236 amino acids. Furthermore, even though expression of SphK2 was found to be ubiquitous, the level of expression was highest in liver and heart, but in contrast to SphK1, was virtually undetectable in skeletal muscle and spleen (Liu et al., 2000). The regulation of SphK activity is critical in determining the levels of S1P in cells and so in the regulation of the many biological reponses to S1P.

1.10.2. Sphingosine 1-phosphate hydrolysis

Cellular levels of S1P are tightly regulated through the balance between it production and hydrolysis. There are currently two identified pathways by which S1P can be degraded within cells. The first of these is through the enzyme S1P lyase which catalyses the cleavage of the C2-C3 bond of S1P, forming trans-2-hexadecanal and ethanolamine phosphate (Saba et al., 1997). S1P lyase is a membrane bound enzyme which has been shown to require the coenzyme pyridoxal 5'-phosphate for full activity (Saba et al., 1997). Furthermore, S1P lyase expression has been found to be ubiquitous with respect to species and tissues, with the exception of platelets which do not express the enzyme (Pyne & Pyne, 2000a). The first cloning of S1P lyase was by Saba and colleagues who cloned the enzyme from the *yeast Saccharomyces cerevisiae* (Saba et al., 1997). However, a mammalian S1P lyase has subsequently been identified and cloned in mouse (Zhou & Saba, 1998).

The second route for the degradation of S1P is via it dephosphorylation to Sph. The dephosphorylation of S1P to Sph can be catalysed by either the Lipid phosphate

phosphatases (LPP's) or by S1P phosphatase (S1PP). As described in Sections 1.4.2.2. and 1.4.2.3., there are several LPP enzymes which exhibit a broad substrate specificity which includes S1P. In addition to the LPPs, a S1P phosphatase has been identified which is distinct from LPP activity. S1P phosphatase was originally cloned from yeast as a long chain base phosphatase (LBP) [Mandala et al., 1998, Mao et al., 1997]. Subsequently, mammalian S1PPs have been cloned and shown to have high specificity for S1P (Mandala et al., 2000, Le Stunff et al., 2002). Le Stunff and colleagues demonstrated that overexpression of murine or human S1PP in HEK293 cells or Chinese hamster ovary (CHO) cells resulted in an increase in S1PP activity that was, unlike the known LPPs, specific for S1P (Le Stunff et al., 2002).

1.11. Sphingosine 1-phosphate: An intracellular second messenger and an extracellular agonist!

Sphingosine 1-phosphate (S1P) is a recently discovered lipid signalling molecule which has been recognised as a key regulator of cell survival, proliferation and differentiation. For example, S1P has been shown to regulate cell growth in a variety of cell types such as airway smooth muscle cells (Rakhit et al., 1999), rat-1 fibroblasts (Gomez-Munoz et al., 1995b), Thyroid FRTL-5 cells (Tornquist et al., 1997) and Swiss 3T3 fibroblasts (Su et al., 1994, Van Brocklyn et al., 1998). The pathways involved in the physiological responses evoked by S1P have therefore, become the subject of intensive research. These include the activation of PLD (Desai et al., 1992, Natarajan et al., 1994), activation of the ERK-1/2 signalling cascade (Pyne & Pyne, 1996, Wu et al., 1995), followed by subsequent activation of the transcription factor Activator protein-1 (AP-1) (Su et al., 1994) as well as mobilisation of intracellular calcium (Mattie et al., 1994) and inhibition of adenylyl cyclase (Van Koppen et al., 1996). However, the mechanisms whereby S1P elicits its plethora of biological responses remains the subject of much controversy. Many studies have shown the agonist stimulation of SphK and subsequent increase in intracellular S1P (Olivera & Spiegel, 1993, Pyne et al., 1996, Xia et al., 1998, for review, see Pyne & Pyne, 2000a). However, the fate of this intracellular S1P remains unclear such that it may be degraded, may act on unidentified intracellular

receptors, or may be released from cells such that it could act in an autocrine manner on extracellular receptors (Hla et al., 1999). Furthermore, a family of S1P specific G-protein coupled receptors have now been identified and characterised by several groups (for review, see Hla, 2001).

1.11.1. Intracellular actions of sphingosine 1-phosphate

The intracellular actions of S1P were proposed following investigations using inhibitors of SphK activity such as D,L-threo-dihydrosphingosine and N,N-dimethylsphingosine. For example, Rani and co-workers reported that the S1P formed following PDGF stimulation of Swiss 3T3 fibroblasts was responsible for the activation of ERK-1/2 (Rani et al., 1997). They also showed that the SphK inhibitor, D,L-*threo*-dihydrosphingosine inhibited this effect and so implicated S1P with a 'second messenger' function (Rani et al., 1997). Tolan and colleagues subsequently demonstrated similar results for the PDGF activation of airway smooth muscle (ASM) cells (Tolan et al., 1999). However, in contrast to the conclusions reached by Rani and colleagues, Tolan and colleagues detailed D,L-*threo*-dihydrosphingosine as an inhibitor of protein kinase C (PKC) in addition to SphK and suggested this to be an alternative route for the inhibition of ERK-1/2 activation as PDGF has been shown to use PKC as an intermediate in the regulation of ERK-1/2 (Tolan et al., 1999). Furthermore, the epidermal growth factor (EGF) mediated activation of ERK-1/2 in ASMs was also tested due to the EGF response being independent of PKC activation and was found to be unaffected by the inhibitors of SphK (Tolan et al., 1999). However, Van Brocklyn and colleagues reported that in HEK293 cells, the PLD activation, calcium mobilisation and tyrosine phosphorylation of focal adhesion kinase mediated by extracellular S1P were unrelated to $S1P_1$/EDG1 receptor expression (Van Brocklyn et al., 1998). Furthermore, it was demonstrated that the mitogenesis and inhibition of apoptosis induced by exogenous S1P was also unrelated to $S1P_1$/EDG1 expression in Swiss 3T3 fibroblasts and that microinjection of S1P into these cells increased DNA synthesis (van Brocklyn et al., 1998). However, the study by Van Brocklyn and colleagues (1998) does not rule out the

presence of other S1P receptors in their system as there are now known to be five independent S1P receptors (Table 1.4).

Other recent investigations have also proposed a role for intracellular S1P in formyl peptide receptor signalling in HL-60 cells (Alemany et al., 1999) and also in the calcium mobilisation induced by LPA in SH-SY5Y neuroblastoma cells (Young et al., 1999). In the study by Young and colleagues, the LPA mediated calcium mobilisation was found to be independent of the inositol 1,4,5-trisphosphate and ryanodine receptor pathways, but was sensitive to inhibitors of SphK (Young et al., 1999). In a later study by the same group, the LPA mediated calcium mobilisation was reported to be associated with an endogenous LPA_2/EDG4 receptor which utilised the production of intracellular S1P in order to stimulate calcium mobilisation in the SH-SY5Y cells (Young et al., 2000). Young and colleagues also showed that LPA stimulated cells preloaded with [^{3}H]Sph displayed elevated production of [^{3}H]S1P and also that the calcium mobilisation induced by Sph was sensitive to removal of adenosine 5' triphosphate (ATP) which suggested that Sph must first be converted to S1P by SphK before causing calcium release (Young et al., 2000).

There is therefore, still much controversy with regards to the intracellular 'second messenger' role of S1P. Furthermore, the identities of the intracellular targets of S1P remain to be fully defined.

1.11.2. Extracellular actions of sphingosine 1-phosphate: The sphingosine 1-phosphate receptors

Exogenous S1P has been shown to mediate a great number of diverse biological functions ranging from neurite retraction (Postma et al., 1996), activation of an atrial cardiomyocyte I(K.ACh) potassium channel (Himmel et al., 2000), vascular endothelial cell migration and cell spreading (Okamoto et al., 2000) to the modulation of the growth and adhesion of ovarian cancer cells (Hong et al., 1999). Much attention has therefore, been directed towards the recently identified family of G-protein coupled receptors (GPCRs) for S1P which have now been shown to

mediate most, if not all of the biological effects reported for S1P. The next section therefore, deals with the identification and role of the S1P receptors in mediating the biological responses evoked by S1P.

1.11.3. Sphingosine 1-phosphate receptor identification

At present there are five identified, high affinity S1P receptors which are part of the recently renamed endothelial differentiation gene (EDG) family of GPCRs (Chun et al., 2002) (Table 1.4). These include EDG1 ($S1P_1$), EDG3 ($S1P_3$), EDG5/ARG16/H218 ($S1P_2$), EDG6 ($S1P_4$) and EDG8/nrg-1 ($S1P_5$) [Table 1.4].

The first S1P receptor to be cloned was $S1P_1$/EDG1 which was originally recognised as an immediate-early gene product in human umbilical vein endothelial cells (HUVEC), proposed to have a role in the differentiation of the endothelial cells into capillary-like tubules (Hla & Maciag, 1990). It was not until 1996 however, that the identity of S1P receptors began to be explicitly defined. Lee and colleagues demonstrated that overexpression of $S1P_1$/EDG1 in HEK293 cells led to activation of ERK-1/2 (Lee et al., 1996) and later characterisation by the same group confirmed $S1P_1$/EDG1 as a high affinity S1P receptor (Lee et al., 1998). In addition, An and colleagues reported the identification of cDNAs for two GPCRs, rat H218 ($S1P_2$) and human $S1P_3$/EDG3 which when overexpressed in Jurkat T cells or *Xenopus oocytes* were found to encode functional S1P receptors (An et al., 1997b). The identification of these functional S1P receptors was quickly followed by the identification of $S1P_4$/EDG6, the expression of which, was found in lymphoid and haematopoietic tissue as well as the lung (Graler et al., 1998). The identification of $S1P_4$/EDG6 as a functional S1P receptor was further confirmed by reports that expression of $S1P_4$/EDG6 in Chinese hamster ovary (CHO) cells and K562 cells resulted in S1P dependent signalling (Yamazaki et al., 2000). The final S1P receptor to be identified to date is that of $S1P_5$/EDG8 which was identified in the white matter and spleen of rats (Im et al., 2000b). In this report, expression of $S1P_5$/EDG8 in several cell lines including HEK293 and Rh7777 (rat hepatoma cell line) cells resulted in expression of a functional S1P receptor (Im et al., 2000b).

1.11.4. Sphingosine 1-phosphate receptors: G-protein coupling and effector mechanisms

S1P has been shown to bind to all the known S1P receptors with low nM affinity (for review, see Pyne & Pyne, 2000a). However, following the binding of S1P to each of the S1P/EDG receptors, the G-protein coupling and effector mechanisms for each of the S1P receptors have been found to be somewhat distinct (for reviews, see Hla, 2001, Takuwa et al., 2001).

$S1P_1$/EDG1 has been demonstrated to couple to multiple effector pathways such as PLC activation, ERK-1/2 activation, calcium mobilisation and adenylyl cyclase inhibition via the PTX sensitive Gαi/o G-proteins (Okamoto et al., 1998, Lee et al., 1996). In addition, *in vitro* binding studies have demonstrated that $S1P_1$/EDG1 does not interact with G-proteins of the Gαq, $G\alpha_{12/13}$ or Gαs families (Windh et al., 1999). However, there has been a report detailing that $S1P_1$/EDG1 signalling is essential for events that require activation of the small GTPase Rac but not the activity of Gαi, such as the formation of lamellipodia and asembly of cortical actin structures (Lee et al., 1999). This data therefore, suggests that $S1P_1$/EDG1 receptor activation couples to Rac activation via an unidentified mechanism which is distinct from Gαi (Lee et al., 1999). Interestingly, $S1P_1$/EDG1 has been demonstrated to undergo receptor-mediated endocytosis in response to S1P binding (Liu et al., 1999). This conclusion was reached in part by the finding that $S1P_1$/EDG1 was found to co-localise with the transferrin receptor which has been shown to be internalised via clathrin coated pits (receptor-mediated endocytosis). The authors also suggest that this process could be important in mediating the $S1P_1$/EDG1 intracellular signalling events (Liu et al., 1999).

In contrast to $S1P_1$/EDG1, $S1P_2$/EDG5 has been shown to couple via a variety of G-proteins in response to S1P binding. For example, S1P induced serum response element (SRE)-driven transcription and calcium mobilisation when $S1P_2$/EDG5 receptors were overexpressed in Jurkat T cells and *Xenopus oocytes* (An et al., 1997b

& 2000). Other overexpression studies have also shown stimulation of $S1P_2$/EDG5 to result in PLC activation, ERK-1/2 activation, and inhibition of cell migration via $G\alpha i/o$, $G\alpha q/_{11}$ and $G\alpha_{12/13}$ dependent mechanisms (Windh et al., 1999, Gonda et al., 1999). In addition to these effector pathways, S1P stimulation of $S1P_2$/EDG5 has been demonstrated to result in activation of c-Jun NH_2-terminal kinase (JNK), p38 MAPK and a Rho GTPase dependent pathway most likely through $G\alpha q/_{11}$ or $G\alpha_{12/13}$ G-proteins (Gonda et al., 1999). Furthermore, S1P activation of $S1P_2$/EDG5 was shown to result in an increase in cAMP suggesting possible coupling to $G\alpha s$ G-proteins although no direct evidence of this mechanism of this action was found (Kon et al., 1999, for review, see Takuwa et al., 2001).

In a manner similar to $S1P_2$/EDG5, $S1P_3$/EDG3 receptors have also been shown to couple to multiple G-proteins including $G\alpha i/o$, $G\alpha q$ and $G\alpha_{12/13}$ (Windh et al., 1999). This varied coupling allows $S1P_3$/EDG3 receptors to signal to similar effector systems as described for $S1P_2$/EDG5 receptors such as SRE-driven transcription, ERK-1/2 activation, PLC and Rho through $G\alpha i$, $G\alpha q/_{11}$ and $G\alpha_{12/13}$ mediated pathways (An et al., 2000). However, in contrast to $S1P_2$/EDG5, $S1P_3$/EDG3, like $S1P_1$/EDG1 has been shown to inhibit adenylyl cyclase via a $G\alpha i$ dependent mechanism (Gonda et al., 1999, for review, see Takuwa et al., 2001).

Less information is available on the effector mechanisms and G-protein coupling of the $S1P_4$/EDG6 and $S1P_5$/EDG8 receptors. However, $S1P_4$/EDG6, when overexpressed in CHO cells, has been shown to couple via PTX sensitive G-proteins (probably $G\alpha i/o$) to PLC and calcium mobilisation (Yamazaki et al., 2000). Similarly, $S1P_5$/EDG8, when overexpressed in Rh7777 cells was found to couple via $G\alpha i/o$ G-proteins to mediate inhibition of forskolin driven cAMP formation (Im et al., 2000b).

An important feature of the signalling by phosphorylated lipids such as PA, LPA and S1P is their ability to activate the ubiquitous Extracellular-signal regulated protein kinase (ERK) pathway. For LPA and S1P, this may be key to their well recognised proliferative properties. The next section therefore, deals with the Extracellular-

signal regulated protein kinases and the mechanisms of activation of this conserved signalling pathway.

1.12. Extracellular-signal regulated protein kinase pathway

All eukaryotic cells express the highly conserved MAP kinase (MAPK) signalling cascades which are activated by stimuli as diverse as cytokines, growth factors, neurotransmitters, hormones, cellular stress and cell adherence (for reviews, see Widmann et al., 1999; Seger & Krebs, 1995; Robinson & Cobb 1997; Force & Bonventre, 1998 and Kolch, 2000).

Currently, six MAPK signalling modules can be distinguished (Kolch, 2000). The best characterised of these are the Extracellular-signal regulated kinase (ERK1/2) pathway, the c-jun NH_2-terminal kinase (JNK) or stress activated protein kinase (SAPK) pathway and the p38 MAPK pathway. The MAPK pathways share structurally related components which mediate fundamental cellular processes such differentiation, adaptation to stressful stimuli, survival and apoptosis in addition to cell proliferation (Widmann et al., 1999 and Kolch, 2000). One important feature of the MAPK pathways is that they are composed of a three kinase module often referred to as the MAPK module (Cobb & Goldsmith, 2000). This basic MAPK module contains a MAPK (ERK1/2, JNK, p38) which is activated by a MAPK kinase (MAPKK), which itself is activated by a MAPKK kinase (MAPKKK) as shown in figure 1.5.

It should be noted that in each MAPK module, there are several MAPKKK, MAPKK and MAPK found and these are commonly interchangeable. The reason for this complexity is not fully appreciated but co-ordination of many signals may lead to specific cellular responses.

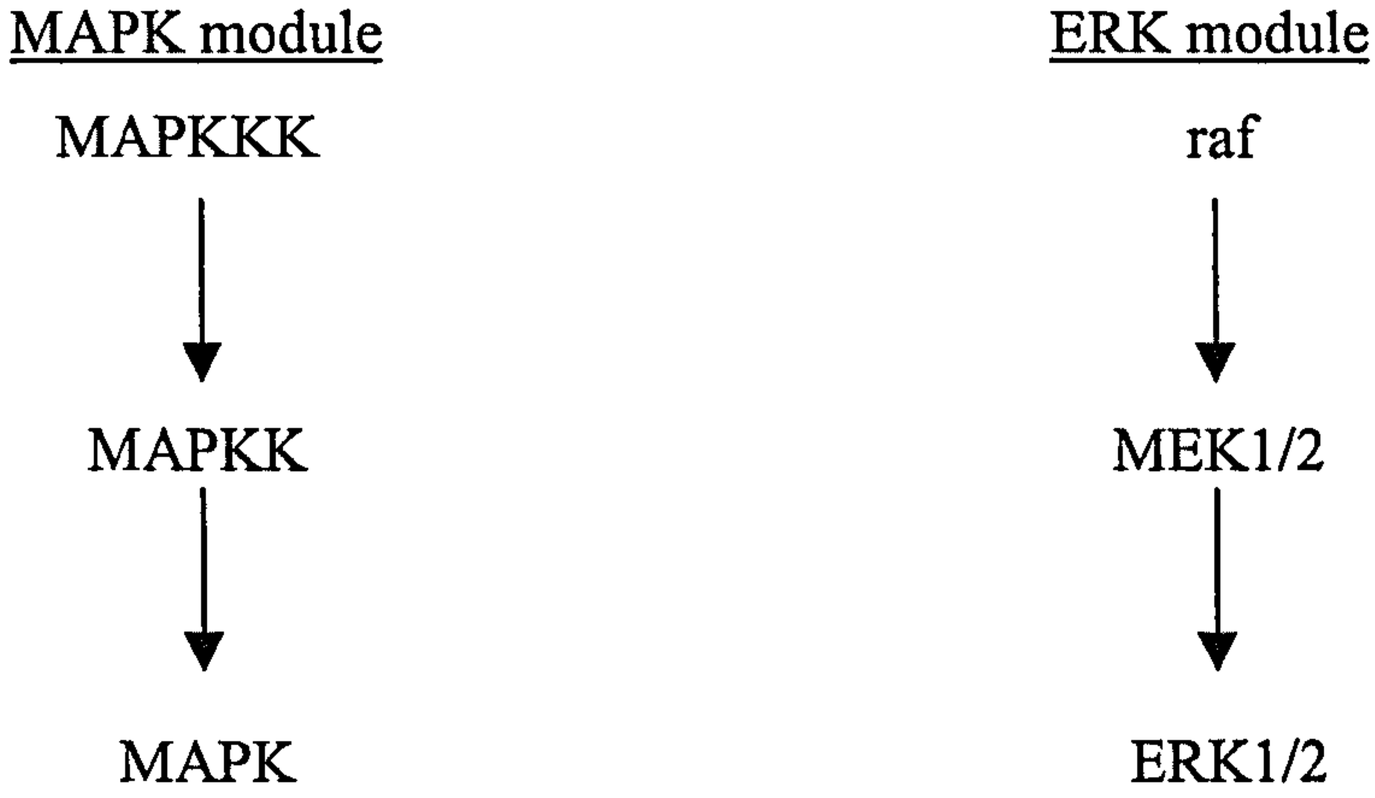


Figure 1.5: The minimal MAPK signalling module (adapted from Widmann et al., 1999) is composed of three kinases, MAPKKK, MAPKK and MAPK. Specific kinases of the ERK signalling module are also detailed (right).

The Extracellular-signal Regulated protein Kinases (ERK-1/2) or p42/p44 MAPK represent the most extensively studied MAPK signalling pathway. ERK1 and ERK2 are 90% homologous and are believed to be functionally identical (Boulton et al., 1991; Seger & Krebs, 1995). Stimulation of a wide variety of growth factor receptors and G-protein linked receptors leads to the activation of ERK1 and ERK2. Following activation, a great number of nuclear, cytoplasmic and cytoskeletal substrates are phosphorylated in a proline directed manner, with Pro-Leu-Ser/Thr-Pro representing the most notable consensus sequence (Gonzalez et al.,1991). The nuclear transcription factors Elk-1, c-myc and c-fos and the nuclear proteins RNA polII represent some of the nuclear targets for ERK-1/2 phosphorylation (Seger & Krebs, 1995, reviewed in Treisman, 1996). Cytoplasmic substrates for ERK-1/2 include upstream components of the ERK cascade such as the NGF and EGF receptor, Raf-1 and MEK and may represent autoregulatory mechanisms (Seger & Krebs, 1995). Other cytoplasmic substrates include $cPLA_2$, p90 ribosomal S6 kinase and phospholipase Cγ (Davis, 1993; Lin et al., 1993; Seger & Krebs, 1995; Bornfeldt & Krebs, 1999). In addition to the nuclear and cytoplasmic substrates for ERK-1/2, cytoskeletal elements such as MAP-1, MAP-2, MAP-4 and Tau have been shown to be substrates for ERK-1/2 (Minshull et al., 1994). The diverse array of substrates for ERK-1/2 within several compartments of a cell demonstrates the importance of the ERK-1/2 signalling cascade in the control of cell function. Indeed, dysfunction of components of the ERK pathway can cause cells to transform and constitutive activation of components has been shown to be sufficient for tumorigenesis (Murga et al., 1999; Mansour et al., 1994; Force & Bonventre, 1998).

1.12.1. Growth Factor activation of ERK-1/2

The most extensively studied pathway for activation of the ERK-1/2, MAPK cascade is that of growth factors such as PDGF and EGF activating ERK-1/2 via their respective receptor tyrosine kinases (for reviews, see Widmann et al., 1999; Force & Bonventre, 1998). Figure 1.6 details the pathway for receptor tyrosine kinase activation of ERK-1/2.

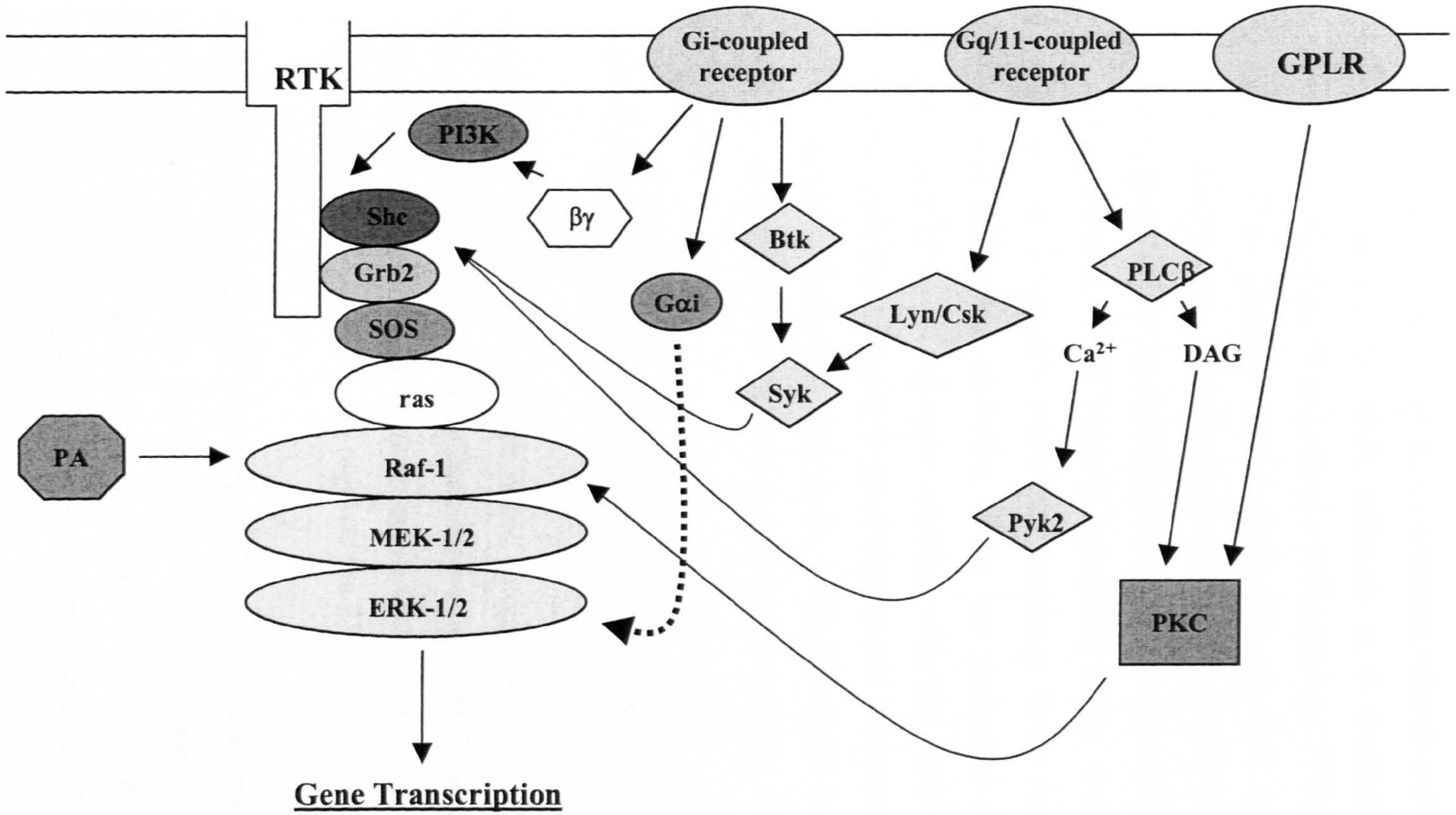


Figure 1.6: Schematic representation of the pathways for activation of the ERK-1/2 signalling cascade by receptor tyrosine kinases and G-protein coupled receptors (Adapted from Widmann et al., 1999).

Following the binding of a growth factor to its receptor tyrosine kinase, the intrinsic tyrosine kinase domains of the receptor are activated, resulting in 'autophosphorylation' and a multistep cascade leading to ERK-1/2 activation (Murga et al., 1999; Widmann et al., 1999). The cascade begins with several adapter proteins such as Shc (SH2 domain-containing α2-collagen related) and Grb2 (Growth factor receptor bound protein 2) which associate at a receptor. Shc contains a phosphotyrosine binding (PTB) domain, a Src homology 2 (SH2) domain, and a Src homology 3 (SH3) domain, whereas Grb2 has one SH2 domain and two SH3 domains (Widmann et al., 1999, Sasaoka et al., 1994). Grb2 is known to bind to Shc via its SH2 domain and is constitutively bound to Sos (Son of Sevenless) via its SH3 domain (Sasaoka et al., 1994; Force & Bonventre, 1998). Sos, when localised to a receptor, acts as a guanine nucleotide exchange factor and causes GDP-GTP exchange and subsequent activation of the low molecular weight G-protein ras. Activated ras-GTP then interacts directly with the amino-terminal of Raf-1 (MAPKKK), contributing partly to its activation and subsequent engagement of the ERK-1/2 signalling module (Vojtek et al., 1993; Moodie et al., 1993). It should be noted that the regulation of Raf-1 is a complex process that requires several factors including ras.

1.12.2. G-protein coupled receptor activation of ERK-1/2

In addition to the classical growth factor receptor tyrosine kinase activation of the ERK-1/2 pathway, it has recently been shown that GPCR agonists can also regulate ERK-1/2. Furthermore, it is now appreciated that there are several different mechanisms whereby GPCRs can regulate the ERK-1/2 pathway.

Following the findings that a number of GPCR ligands activated ERK-1/2, research was directed at finding the mechanisms involved (for reviews, see Gutkind, 1998; Murga et al., 1999 & Gudermann et al., 2000). The easiest method of explaining the known mechanisms is by dividing them into regulation by G-protein βγ dimers and G-protein α subunits.

Regulation of ERK-1/2 by G-protein βγ dimers

Initial experiments demonstrated the ability of ligand bound Gαi and Gαq-coupled receptors to stimulate ERK's in a PTX-sensitive and –insensitive manner. However, subsequent experiments demonstrated the inability of activated Gα subunits to stimulate ERK-1/2 (Gutkind, 1998). This, coupled with growing evidence of a role for G-protein βγ dimers in signal transduction led to the investigation of βγ signalling to the ERK pathway. In fact, many findings now suggest that virtually all mitogenic signals from Gi-coupled receptors are mediated by Gβγ dimers (Van Biesen et al., 1996).

Early experiments demonstrated that genistein (a tyrosine kinase inhibitor) blocked LPA induced ERK activation thereby implicating tyrosine kinases in the response (Hordijk et al., 1994). In addition, the rapid phosphorylation of Shc and also Shc-Grb2 complex formation following GPCR activation was reported by several groups and led to the investigation of possible tyrosine kinases involved in the GPCR activation of the ERK pathway (reviewed in Gutkind, 1998; Gudermann et al., 2000). It is now appreciated that many growth factor receptors such as the PDGF and EGF receptors, as well as non-receptor tyrosine kinases, such as Src, Lyn, Syk and the Ca^{2+} and PKC dependent tyrosine kinase Pyk2 are involved in the pathway linking GPCRs to ERK activation (Figure 1.6) (for review, see Murga et al., 1999). Several other proteins have also been implicated in the Gβγ regulation of ERK-1/2 including PI3Kγ which is activated by Gβγ dimers acting upstream of Src-like tyrosine kinases (Lopez-Ilasaca, 1998).

Regulation of ERK-1/2 by G-protein α subunits

The ability of G-protein α subunits to activate ERK-1/2 has been demonstrated for a number of years. A great number of G-protein α subunits have now been discovered through molecular cloning and these are divided into four subfamilies: Gi, Gq/11, Gs, and $G_{12/13}$ (Morris & Malbon, 1999; Lopez-Ilasaca, 1998).

As detailed previously, it is suggested that Gβγ dimers, mediate the regulation of ERK-1/2 by Gαi-coupled receptors. However, an indirect mechanism whereby Gαi can regulate ERK-1/2 is through the inhibition of certain adenylyl cyclase (AC) isoforms (for review, see Simonds, 1999). Inhibition of adenylyl cyclase would lead to decreased levels of cAMP and so decreased protein kinase A (PKA) activation. PKA has been shown to phosphorylate Raf-1 kinase and lead to a decrease in ERK activation (Cook & McCormick, 1993). Therefore, Gαi may indirectly regulate ERK-1/2 activation.

In addition to Gαi-mediated regulation of ERK-1/2, the PTX-insensitive Gαq/11 family of G-proteins have also been shown to activate ERK-1/2. Two mechanisms link Gαq/11 to ERK-1/2 activation and these involve PLCβ and PKC. The PLCβ mediated activation of ERK-1/2 involves both the production of diacylglycerol (DAG) and the stimulation of intracellular Ca^{2+} production. DAG is known to activate protein kinase C (PKC) which has been shown to directly phosphorylate and activate Raf-1 *in vitro* and also in an NIH3T3 cell clone (figure 1.6) (Kolch et al., 1993). PKC isoforms represent crucial enzymes coupling Gαq/11 G-proteins to the ERK-1/2 pathway. However, there are conflicting reports regarding the mechanisms whereby this coupling occurs. For example, Hawes and colleagues (1995) report a fully PKC-dependent mechanism, whereas, Berts and colleagues (1999) reported a PKC-independent coupling. In contrast however, Crespo and co-workers (1994) reported a partially PKC-dependent coupling of Gαq/11-coupled receptors to the ERK-1/2 pathway. Therefore, the coupling of Gαq/11 G-proteins to the ERK-1/2 via PKC remains to be fully appreciated. The second mechanism linking Gαq/11 G-protein subunits to the ERK-1/2 pathway is that of an increase in intracellular Ca^{2+}. This mechanism involves the Ca^{2+}-mediated activation of the non-receptor tyrosine kinase Pyk2 which activates ERK-1/2 via a ras-dependent mechanism similar to receptor tyrosine kinases (Figure 1.6), (Widmann et al., 1999; Lopez-Ilasaca, 1998).

As mentioned above, indirect regulation of the ERK-1/2 pathway can occur through modulation of intracellular cAMP levels and PKA activation. G-protein subunits of the Gαs family are known to stimulate all cloned AC isoforms (Simonds, 1999) and

so provide a further indirect mechanism for negative regulation of ERK-1/2 by G-protein α-subunits through the modulation of cAMP levels. Accordingly, in NIH3T3 cells, increased cAMP levels was shown to increase PKA-mediated inhibition of Raf-1 kinase and thereby inhibit ERK-1/2 activation (Cook & McCormick, 1993).

1.12.3. Growth factor receptor and G-protein coupled receptor transactivation and signal integration in the regulation of ERK-1/2 signalling

The cell proliferation mediated by mitogenic stimuli occurs following the activation of the ERK-1/2 signalling cascade via different classes of cell surface receptors. These include the growth factor receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs). However, certain GPCR agonists and growth factors have been known for some time to function together as co-mitogens to stimulate DNA synthesis (Alderton et al., 2001). The mechanisms underlying this co-mitogenicity remain to be fully defined but there is evidence now for two plausible mechanisms for this action. These are receptor transactivation as is the case with EGF receptors (EGFR) (Daub et al., 1996) and receptor signal integration as has been shown for the platelet-derived growth factor receptor (PDGFR) [Alderton et al., 2001].

Daub and colleagues were the first to suggest transactivation of the EGFR following stimulation of Rat-1 fibroblasts with the GPCR agonists endothelin-1, LPA and thrombin (Daub et al., 1996). A later study by the same group demonstrated the initiation of EGFR tyrosine phosphorylation by various GPCR agonists in several different cell lines including keratinocytes, COS-7 cells and primary mouse astrocytes (Daub et al., 1997). This transactivation was later found to be mediated through the GPCR-mediated activation of a metalloproteinase resulting in the cleavage of proHB-EGF and subsequent release of EGF to act as a ligand at the EGFR (Prenzel et al., 1999). Abrogation of the EGFR receptor transactivation and also downstream signalling events occurs when proHB-EGF processing is inhibited (Prenzel et al., 1999).

Several groups have reported other examples of possible interactions between RTKs and GPCRs. For example, Hallak and colleagues as well as Dalle and co-workers have demonstrated the interaction of Insulin and Insulin-like Growth factor receptors with components of G-protein coupled receptor signalling pathways (Hallak et al., 2000, Dalle et al., 2001). Furthermore, Wang and colleagues reported that the activation of ERK-1/2 by insulin was amplified by β-adrenergic receptor expression indicating the presence of 'cross-talk' between the insulin receptor tyrosine kinase and the β-adrenergic GPCR (Wang et al., 2000).

Of relevance to S1P signalling, Alderton and colleagues reported the tethering of the PDGFR tyrosine kinase to G-protein coupled receptors as a means of signal integration and so to allow more efficient regulation of downstream effector pathways such as ERK-1/2 (Alderton et al., 2001). In this study, PTX was found to reduce PDGF stimulation of ERK-1/2. Furthermore, the authors report that transfection of recombinant Gαi into HEK293 cells led to an increased activation of ERK-1/2 by PDGF and also phosphorylation of Gαi (Alderton et al., 2001). In addition to this evidence, the study demonstrated the co-immunoprecipitation of overexpressed, recombinant, Myc-tagged $S1P_1$/EDG1 (GPCR for S1P) with overexpressed PDGFβ receptor using either anti-Myc or anti-PDGFβ receptor antibodies. This therefore suggested the formation of a functional signalling complex between these two receptors in HEK293 cells (Alderton et al., 2001). The termination of S1P and LPA signalling to ERK-1/2 has also been the subject of research and the LPP enzymes have been implicated with a role in this process.

1.13. Potential for 'ecto'-LPP activity

It has been shown that the LPP enzymes catalyse the dephosphorylation of several potent phosphorylated lipid mediators such as PA, LPA and S1P. However, the precise biological role of LPP activity remains the topic of much research. There is a large body of evidence proposing that LPPs may be involved in the metabolism of PA that is produced following activation of PLDs or DGKs (for reviews, see Brindley & Waggoner, 1996, Kanoh et al., 1999, Waggoner et al., 1999). However,

all LPPs are predicted to be six transmembrane domain enzymes, localised to the plasma membrane and are predicted to possess an extracellular facing catalytic site (Zhang et al., 2000, Waggoner et al., 1999). This therefore, raises the possibility of an 'ecto'-LPP activity which functions to regulate signalling by exogenous PA, LPA and S1P (for review, see Waggoner et al., 1999). Indeed, the first suggestion of a possible 'ecto'-LPP action was made by Perry and colleagues who suggested that 'ecto'-LPP dephosphorylates exogenous PA to provide a 'timed release diacylglycerol' signal in neutrophil superoxide generation (Perry et al., 1993). Following this, Xie and Low reported a similar 'ecto'-LPP activity in PAM212 keratinocytes which was found to hydrolyse LPA in addition to PA (Xie & Low, 1994). English and colleagues then reported the partial purification of the previously reported 'ecto'-LPP activity from neutrophils and showed it to hydrolyse several molecular species of PA and also LPA (English et al., 1997).

The cloning of several LPP isoforms has facilitated investigations into the physiological roles of the LPP isoforms and reports have now detailed 'ecto' activities for several LPP isoforms when overexpressed (Jasinska et al., 1999, Ishikawa et al., 2000, Roberts & Morris, 2000, Xu et al., 2000, Hooks et al., 2001, Alderton et al., 2001). In support of an 'ecto'-LPP model, recent studies have detailed the attenuation of signalling events mediated by the extracellular lipid phosphates LPA and S1P, following overexpression of LPP isoforms in rat-2 fibroblasts and HEK293 cells (Jasinska et al., 1999, Xu et al., 2000, Hooks et al., 2001, Alderton et al., 2001) (see Chapter 5).

1.14. LPPs as a point of 'cross-talk' between glycerolipid and sphingolipid signalling

A complication in the investigation of LPP activity and its physiological role is created through the presence of apparent 'cross-talk' between the glycerolipid and sphingolipid signalling pathways. As detailed earlier, the regulation of LPP activity by products of sphingolipid metabolism has been demonstrated in several cell types (Wu et al., 1993, Mullmann et al., 1991, Lavie et al., 1990, Gomez-Munoz et al.,

1994). The study by Lavie and colleagues also demonstrated the inhibition of PAP-1 activity by sphingoid bases and so represents a further example of 'cross-talk' between the glycerolipid and sphingolipid signalling pathways (Lavie et al., 1990). A further dimension to these findings is that in addition to inhibiting LPP activity, sphingosine was found to activate DGK in GPASM (Tolan et al., 1997) and also stimulate PLD activity (Zhang et al., 1990). The net effect of these actions would be potentially to increase the levels of PA within cells while decreasing the levels of DG. Thus, sphingosine is seen to modulate the levels of two important glycerolipid signalling molecules through the regulation of specific enzyme activities such as the LPPs. In addition to this, the precursor of sphingosine, ceramide has been demonstrated to inhibit PLD while stimulating LPP activity (Gomez-Munoz et al., 1994). These effects are in contrast to the effects of sphingosine and show that the levels of two sphingolipid signalling molecules may have profound effects on the levels of the glycerolipid signalling molecules PA and DG in cells. The interaction between glycerolipid and sphingolipid signalling pathways further highlights the need to uncover the complex mechanisms of metabolism of the many lipid mediators produced within cells. In relation to this, the diverse signalling roles, in addition to the glycerolipid and sphingolipid origins of the many LPP substrates and LPP-catalysed products, place the LPPs in a unique position in the regulation of the levels of potent glycerolipid and sphingolipid signalling molecules.

1.15. Aims

LPP research is now at an exciting point where many isoforms have been identified and studied. However, the need for such molecular diversity is not yet apparent. Isoform specific regulation, subcellular localisation, substrate preference, kinetics and their precise physiological roles remain to be established. In relation to this, the discovery of the physiological substrates of the individual LPP isoforms may yield important clues in the discovery of the individual roles of each LPP isoform in normal and pathological states. Furthermore, the 'ecto'-LPP mechanism of action versus an alternative intracellular function is an issue that remains to be resolved and

represents one of the major questions in the field of LPP research. In light of these issues, the aims of the present study were as follows:

(i) To establish a bacterial expression system for the expression and purification of individual LPP isoforms to allow the extensive characterisation of the enzyme family.

(ii) To use HEK293 cells, separately stably transfected with each LPP isoform to further characterise and investigate the substrate preference and kinetics of LPPs in a model system.

(iii) To assess the role of individual LPP isoforms in the signalling events mediated by their lipid substrates.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2 General Materials and Methods

2.1. Materials

2.1.1. General reagents

All biochemical reagents were of the highest quality available commercially and were purchased from Sigma Chemical Company (U.K.) or BDH (U.K.), unless otherwise stated.

Anachem (U.K.)

30% (w/v) acrylamide/bis-acrylamide (29:1).

Amersham Bioscience (U.K)

Hybond™ECL™ Nitrocellulose membrane, DNA Polymerase mix (dNTPs), GFX™ PCR and gel purification kit.

BIO-RAD (U.K.)

BIO-RAD protein assay reagent.

Calbiochem-Novabiochem (U.K.)

Diacylglycerol Kinase (recombinant, *E.coli*).

Clontech Laboratories Inc. (U.S.A.)

Human Glyceraldehyde-3-Phosphate Dehydrogenase (G3PDH) Control Amplimer Set.

DIFCO laboratories (U.K.)

Bacto-Yeast extract, Bacto-Tryptone, Bacto-Agar.

H. A. West (U.K)

Kodak LX24 developer and Kodak Industrex fixer.

Invitrogen (U.K.)

pTrcHis B and pcDNA3.1 expression vectors, Competent *E. coli* (TOP-10), DNase I Amplification Grade, Oligo dt(12-18) Primers, Superscript II Reverse Transcriptase, Taq Polymerase, 100bp Ladder, All general Cell Culture materials.

Lipid Products (U.K)

Phosphatidylserine and 1,2-dioleoyl-sn-glycerol.

New England Biolabs Inc.

pMAL™ Protein Fusion and Purification System.

Pierce-Warriner (U.K.)

Horse-radish peroxidase-linked INDIA™HisProbe-HRP.

Qiagen (U.K.)

Ni-NTA Superflow resin, QIA Shredder, RNeasy Total RNA Isolation Kit.

Web Scientific (U.K.)

Non-Interfering Protein Assay™ kit.

Whatman International (U.K.)

K6 Silica (G60) thin layer chromatography plates.

2.1.2. Antibodies

BD Transduction Laboratories (U.K.)

Anti-phospho-p42/p44 MAPK, and Anti-total (p42) MAPK antibodies.

BPU of Strathclyde University

Anti-LPP1/LPP1a antibody raised to amino acids 259-268 and 260-269 near the C-terminus of both LPP1 and LPP1a respectively (peptide E-R-K-E-E-D-S-H-T-T) in New Zealand White rabbits.

Anti-LPP Catalytic domain antibody raised to amino acids 216-227, 217-228, 213-224 and 244-255 of LPP1, LPP1a, LPP2 and LPP3 respectively. The amino acids are located in the conserved catalytic region of all LPP isoforms (peptide S-R-V-S-D-Y-K-H-H-W-S-D) in New Zealand White rabbits.

New England Biolabs Inc.

Anti-Maltose Binding Protein antibody (provided with pMAL™ Protein Fusion and Purification System).

Scottish Antibody Production Unit (U.K.)

Horse-radish peroxidase-linked anti-rabbit / anti-mouse IgG.

2.1.3. Radioisotopes

Amersham Bioscience (U.K.)

Adenosine 5'-[γ-^{32}P] triphosphate (specific activity 2Ci/mmol, 74 GBq/mmol), [^{3}H]-cAMP (37mCi/mmol, 1850kBq/mmol).

2.2. Protein assay

The protein content of samples was calculated using the BIO-RAD micro protein assay system, based on the method of Bradford (1976). 10µl of sample was mixed with 200µl BIO-RAD reagent and 800µl of H_2O. The OD_{595} was then measured in a spectrophotometer and the protein content estimated through comparison with a standard curve constructed using known concentrations of Bovine Serum Albumin (BSA).

2.3. Non-interfering protein assay

The protein content of samples containing detergents (which interfere with the BIO-RAD method) were measured using the Non-Interfering Protein Assay™ (Web Scientific, U.K.). This involved the precipitation of protein to remove interfering agents from the protein solution. Precipitation of protein was achieved by addition of 0.5ml UPPA™-1 to each protein sample and incubation at room temperature for 2 minutes. Following this, 0.5ml UPPA™-2 was added to the solutions and mixed thoroughly. Samples were then centrifuged (10000 x **g**, 5 minutes) to sediment the precipitated protein. Precipitated protein was then redissolved in 100µl of copper solution plus 400µl H_2O and assayed colorimetrically on the basis of the specific binding of copper ions to the peptide backbone of proteins. Unbound copper was measured with a colour producing agent and is inversely proportional to the amount of protein in the sample. The OD_{480} was measured in a spectrophotometer and the protein content estimated through comparison with a standard curve constructed using known concentrations of Bovine Serum Albumin (BSA).

2.4. SDS-PAGE and Western Blot analysis

2.4.1. Sample preparation

All bacterial samples prepared following the procedures detailed in sections 2.7.1.-2.7.8. were resuspended in 100µl of electrophoresis sample buffer (62.5mM Tris/HCl (pH 6.7), 0.6M mercaptoethanol, 12.5% glycerol (v/v), 1.25% SDS (w/v) and 0.02% (w/v) bromophenol blue). Samples were prepared from Human Embryonic Kidney cells, clone 293 (HEK293) cells or airway smooth muscle cells (ASMC's) following cell stimulation (Section 2.8.6.) by aspiration of the culture medium followed by resuspension in 200µl of sample buffer. The samples were boiled for approximately five minutes in order to denature the proteins and also disrupt disulphide bonds prior to electrophoresis.

2.4.2. Preparation of acrylamide gels

Proteins were resolved by SDS-PAGE (Laemmli, 1970) using a BIO-RAD mini-Protean-II™ unit. The separating gel contained a final concentration of 10% acrylamide [components of separating gel: 10% acrylamide, 0.375M Trizma Base (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.05% (v/v) TEMED, and H_2O] and the stacking gel contained a final concentration of 6% acrylamide [components of stacking gel: 6% acrylamide, 0.125M Trizma Base (pH6.7), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.1% (v/v) TEMED, and H_2O].

2.4.3. Polyacrylamide gel electrophoresis

The electrophoresis equipment and the prepared acrylamide gels were assembled and the upper and lower buffer chambers were filled with electrophoresis buffer (0.21M glycine, 3.5mM SDS and 25mM Tris). The prepared samples were equalised for protein and the required volume of each was applied to the stacking gel using a Hamilton syringe. Pre-stained SDS-PAGE molecular weight markers (5µl) were also applied to one well of the stacking gel and resolved in parallel. The gels were run at 150 volts/1mA. Proteins were visualised using either Coomasie Blue staining (2.4.4.) or Western blotting following transfer to nitrocellulose (2.4.6.) and detection using specific probes (2.4.7.) or antibodies (2.4.8.-2.4.9.)

2.4.4. Coomasie Blue staining of proteins

SDS-PAGE gels were soaked for 3 hours in 0.25% (w/v) Coomasie Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid. Destaining was performed overnight in 50% (v/v) methanol, 10% (v/v) acetic acid.

2.4.5. Molecular weight determination of unknown proteins from SDS-PAGE

The molecular weights of the unknown proteins were estimated by comparing their mobility with pre-stained SDS-PAGE molecular weight markers. The proteins used as molecular weight markers were α2-macroglobulin (205kDa), β-galactosidase (130kDa), fructose-6-phosphate kinase (90kDa), pyruvate kinase (64kDa), fumarose (53kDa), lactic dehydrogenase (38kDa) and triosephosphate isomerase (33.5kDa). The molecular weights given are not native molecular weights but are the apparent molecular weights when run on SDS-PAGE.

2.4.6. Transfer to nitrocellulose

Following gel electrophoresis, proteins were transferred to nitrocellulose using a BIO-RAD Mini Trans-Blot^{TM} electrophoretic Transfer cell. Transfer was achieved at 100 volts / 0.6mA for at least 60 minutes using transfer buffer which contained 0.21M glycine and 25mM Tris in 20% (v/v) methanol.

2.4.7. Western Blotting Using of INDIA^{TM} HisProbe-HRP

After transfer of proteins (2.4.6.), the nitrocellulose was incubated in blocking buffer [Phosphate buffered saline (PBS) / 0.2% Igepal- CA630 (PBSI) supplemented with 5% (w/v) non-fat milk powder] overnight at 4°C to prevent non-specific binding. The nitrocellulose was subsequently washed (4 x 10 minutes) with PBSI before addition of INDIA^{TM} HisProbe-HRP (HisProbe) in PBSI (1:5000 v/v dilution with PBSI) for 1 hour at room temperature. The nitrocellulose was washed (4 x 10 minutes) with PBSI before detection of the hexa-histidine or deca-histidine tagged, recombinant protein by enhanced chemiluminescence (ECL). This was achieved by incubating the nitrocellulose in a mixture of equal volumes of reagents 1 (2.5mM Luminol, 1.1mM *p*-coumaric acid, 0.1M Trizma base, pH 8.5) and 2 (0.02% hydrogen peroxide, 0.1M Trizma base, pH 8.5) for 1-2 minutes. Excess ECL reagent was drained from the nitrocellulose which was placed between two sheets of

transparent film before exposure to X-ray film (1-10 minutes) to visualise immunoreactive bands.

2.4.8. Western Blotting using the anti-LPP1/LPP1a, anti-catalytic domain and anti-Maltose binding protein antibodies

Following transfer of proteins (2.4.5.), the nitrocellulose was incubated in PBSI solution supplemented with 3% Bovine Serum Albumin (BSA) (w/v) for 2 hours at 37°C, to prevent non-specific antibody binding. The nitrocellulose was subsequently washed (4 x 10 minutes) with PBSI. The nitrocellulose was incubated overnight at 4°C in a solution of PBSI containing 3% BSA (w/v) and the appropriate concentration of primary antibody. The anti-LPP1/LPP1a antibody and anti-LPP catalytic domain antisera were used at a 1:200 (v/v) dilution and the anti-Maltose Binding Protein (MBP) antibody used at a working dilution of 1:10,000 (v/v). The nitrocellulose was washed (4 x 10 minutes) in PBSI before incubation for 2 hours in PBS with 3% BSA (w/v) containing horse-radish peroxidase (HRP)-linked anti-rabbit secondary antibody (1:2000 dilution). The nitrocellulose was washed for a further 4 x 10 minutes with PBSI and briefly dried in air. Immunoreactive bands were then detected using ECL as described before (2.4.7.)

2.4.9. Western Blotting using the Anti-phospho ERK-1/2 and anti-ERK-1 antibodies

When using the anti-phospho ERK-1/2 and anti-ERK-1 antibodies, the nitrocellulose was incubated in blocker [Tris buffered saline (TBS) / 0.1% Tween 20 (TBST) solution supplemented with 5% (w/v) non-fat milk powder] for 2 hours at room temperature, to prevent non-specific antibody binding. The nitrocellulose was subsequently washed (2 x 2 minutes) with TBST and incubated overnight at 4°C in a solution of TBST containing 3% BSA (w/v) plus the appropriate concentration of primary antibody (1:1000 (v/v) dilution). The primary antibody was removed and the nitrocellulose washed (3 x 10 minutes) in TBST before incubation for 1 hour in TBST with 5% (w/v) non-fat milk powder containing either HRP-linked anti-rabbit

IgG (1:2000 v/v) or anti-mouse IgG (1:2000 v/v) for nitrocellulose previously incubated with anti-phospho ERK-1/2 or anti-ERK-1 antibodies respectively. The nitrocellulose was washed (3 x 10 minutes) with TBST and briefly dried in air. Immunoreactive bands were detected using ECL reagents as described before (2.4.7.).

2.4.10. Reprobing of nitrocellulose membranes

In order to reprobe nitrocellulose membranes with several antibodies, the nitrocellulose membranes were stripped as follows. The nitrocellulose membrane was incubated with gentle agitation, for 60 minutes at 70°C in a solution containing 100mM β-mercaptoethanol, 2% SDS (w/v) and 62.5mM Trizma-hydrochloride, pH6.7. The membranes were then washed thoroughly in the appropriate buffer (PBSI or TBST) before addition of the required primary antibody. The Western blotting procedure was then conducted as described in sections 2.4.7. – 2.4.9.

2.5. Preparation of [^{32}P]–lipids

2.5.1. Preparation of various species of [^{32}P]–PA

[^{32}P]PA was synthesised enzymatically from various species of diradylglycerol (DRG) and [^{32}P]-γ-ATP (50mCi/mmol, 1.85GBq/mmol) using *E. coli* diacylglycerol kinase (DGK) (Walsh & Bell, 1986). The species of PA made were dioleoyl$_{(18:1,18:1)}$, dipalmitoyl$_{(16:0,16:0)}$, dioctanoyl$_{(8:0,8:0)}$, and stearoyl-arachidonyl$_{(18:0,20:4)}$.

1μmole of the appropriate DRG was dispersed in a mixed micelle containing 0.3% (v/v) Triton X-100 and 0.15mM phosphatidylserine (PS) (final concentrations). 50μl of a 15.4mg/ml solution of dithiolthreitol (DTT) was then added along with 1μmole of Mg^{2+}-ATP, 50μCi/μmole (1.85MBq/mmol) [^{32}P]-γ-ATP and 100μl of a buffer containing 250mM imidazole, 250mM NaCl, 62.5mM $MgCl_2$ and 5mM EGTA at pH 6. The incubation was started by the addition of 100μl DGK and maintained for 2

hours at 30°C. The reaction was stopped by the addition of 4.032ml $CHCl_3$:CH_3OH:10mM HCl (15:30:2, v/v) before incubation at room temperature for 15 minutes to allow the lipids to extract. Organic and aqueous phases were separated by the addition of 1.32ml of $CHCl_3$ and 1.06ml H_2O before mixing and centrifugation at 350 x g for 2 min. The final ratio of $CHCl_3$:MeOH:aqueous was 1:1:0.9 (Bligh & Dyer, 1959). The lower organic phase which contained the ^{32}P-labelled PA was removed and the solvent evaporated under a stream of nitrogen gas. The remaining lipids were re-dissolved in a mixture of $CHCl_3$:CH_3OH (19:1, v/v) and the [^{32}P]PA resolved by thin layer chromatography (TLC) on 10x20cm silica G60 plates using a solvent system containing $CHCl_3$:CH_3OH:CH_3COOH (26:6:3, v/v) (silica G60 plates). The position of unlabelled standards resolved in parallel was visualised by staining the plate with iodine vapour.

The [^{32}P]PA was visualised by autoradiography before excision and transfer of the corresponding area of silica to a centrifuge tube. [^{32}P]PA therein, was succesively extracted with (i) 3ml of $CHCl_3$:CH_3OH:CH_3COOH:H_2O (25:15:4:2, v/v) for 15 mins at 30°C, (ii) as (i) but using 2ml, (iii) CH_3OH (2ml, 15 mins, 30°C and (iv) CH_3OH:CH_3COOH:H_2O (95:1:5, v/v), 2ml, 30°C for 15 mins (Skipski & Barclay, 1969). The organic and aqueous phases were then separated by the addition of 2.8ml of $CHCl_3$ and 4.2ml H_2O and the lower organic phase containing the [^{32}P]PA was retained as a working stock. The yield of [^{32}P]PA, calculated from the specific activity of the [^{32}P]-γ-ATP used, ranged from 15-35%.

2.5.2. Preparation of [32P]-oleoylLPA

[^{32}P]-LPA was synthesized by mild alkali hydrolysis of [^{32}P]-dioleoylPA as described by Gomez-Munoz et al., (1994). [^{32}P]-dioleoylPA was dissolved in 3ml $CHCl_3$:CH_3OH (1:1, v/v). To this was added 0.5ml of 0.35M NaOH in 96% CH_3OH and the mixture incubated for 10 mins at room temperature before the solution was adjusted to pH 7 by addition of 0.9ml of 0.2M HCl. Seaparation of the organic and aqueous phases was achieved by the addition of 0.5ml $CHCl_3$, the solution gently mixed and subsequently centrifuged at 1000 x g for 10 mins. The lower phase was

removed and retained. The residual upper phase was washed with 1ml $CHCl_3$ by mixing and centrifugation. The resulting $CHCl_3$ was pooled with the original lower phase and dried under a stream of N_2 gas. The remaining lipids were dissolved in $CHCl_3$:MeOH (19:1, v/v) and the [^{32}P]-oleoyl LPA isolated by TLC on silica G60 plates, developed with $CHCl_3$: CH_3OH:H_2O: CH_3COOH (50:25:3:7, v/v). Unlabelled standards were resolved in parallel and visualised by staining the plate with iodine vapour. The position of the [^{32}P]-LPA was detected by autoradiography and the silica from the corresponding area of the plate excised and the [^{32}P]-LPA therein, extracted as described above for [^{32}P]-PA species (2.5.1.).

2.5.3. Preparation of [^{32}P]-C_8-Ceramide-1-Phosphate (C1P)

[^{32}P]-C_8-C1P was synthesized enzymatically from [^{32}P]-γ-ATP and C_8-ceramide using *E. coli* DGK as described for PA above but with the following changes. The [^{32}P]-C1P was resolved on silica gel G60 K6 plates using a solvent system consisting of $CHCl_3$:CH_3OH:CH_3COOH:H_2O (25:10:1:2, v/v). All other steps were as described for the synthesis of [^{32}P]-PA species (2.5.1.).

2.5.4. Preparation of [^{32}P]-Sphingosine 1-Phosphate (S1P)

[^{32}P]-S1P was prepared by deacylation of [^{32}P]-C1P (see 2.5.3.) as described by Desai et al., (1992). [^{32}P]-C1P was treated with 1ml of 6M HCl/Butan-1-ol (1:1, v/v) in a securely sealed vessel for one hour at 100°C. After cooling, The organic and aqueous phases were separated by the addition of 555µl of $CHCl_3$ and 55µl of CH_3OH. The solvents of the lower phase were dried under nitrogen and the remaining [^{32}P]-S1P isolated by TLC as described for the [^{32}P]-C1P preparation (2.5.3.). All other steps were as described for the synthesis of [^{32}P]-PA species (2.5.1.).

2.6. Assesing Lipid phosphate phosphatase (LPP) activity

2.6.1. LPP assay

LPP activity was determined by measuring the release of $[^{32}P]P_i$ from $[^{32}P]$lipid (PA, LPA, C1P, S1P) (Lin & Carman, 1989). For the purposes of the methods, PA will be used as an example. $[^{32}P]$PA (50000 cpm/assay, approximately 6500cpm/nmol) was presented as a mixed micelle using PA and Triton X-100 at a fixed ratio of PA:Triton X-100 (1:10). The initial concentration of TX-100 used to form PA/TX-100 micelles was 0.01% (0.15mM). All assays were performed at 30°C and incubations were for 15 mins unless otherwise stated.

Incubations were initiated by the addition of 25µl of the appropriate sample (e.g. a bacterial lysate (Section 2.7.) or a HEK293 cell membrane preparation (2.8.3.) to an incubation mixture containing 150mM Tris maleate (pH7), 30mM mercaptoethanol, 8mM magnesium chloride ($MgCl_2$) and 0.8mg/ml bovine serum albumin, in a final volume of 0.1ml. The reaction was terminated by the addition of 500µl of a mixture containing $CHCl_3$:CH_3OH:10mM HCl (15:30:2, v/v). The organic and aqueous phases were then separated by the addition of 150µl of $CHCl_3$ and 150µl of 0.1M HCl. The upper aqueous phase was removed to a 5ml scintillation vial and the radioactivity quantified in a Wallac 1209 Rackbeta scintillation counter. Specific LPP activity was calculated as a function of the total concentration of PA in the assay.

In substrate saturation experiments, $[^{32}P]$-lipid substrates were presented as described above but with increasing concentrations of unlabelled $PA_{(18:1/18:1)}$ substrate while maintaining the lipid to TX-100 ratio at 1:10 (constant mole fraction of 0.091).

2.6.2. LPP assay against $[^{32}P]$-S1P

LPP activity was measured as described above with the following additional steps. Incubations were terminated as described above. The organic and aqueous phases

were separated by the addition of 150µl of $CHCl_3$ and 150µl of 0.1M HCl. 400µl of the upper phase was removed to another vial and S1P was subsequently extracted. The upper phase mixture was adjusted to 0.88M KCl by the addition of 400µl of 1.76M KCl and the [^{32}P]-S1P subsequently extracted by the addition of 800µl of water saturated butan-1-ol. The butan-1-ol phase was washed twice using 800µl 1.76M KCl and the remaining radioactivity in the washed, upper, butan-1-ol phase removed for quantification (Brindley, D., personal communication).

2.7. Purification of recombinant Lipid phosphate phosphatase fusion proteins

All procedures were performed at 4°C unless otherwise stated.

2.7.1. *Escherichia coli* transformation

LPP1 and LPP1a were separately cloned into the bacterial expression vectors pTrcHis B (pTrcHis6) (Figures 3.1 and 3.2) at the BamH1 and Kpn 1 sites and pTrcHis10 (Figure 3.19) at the BamH1 and HindIII sites (R. Tate – Strathclyde University) to generate pTrcHis6-LPP1, pTrcHis6-LPP1a, pTrcHis10-LPP1 and pTrcHis10-LPP1a plasmid constructs. pTrcHis6 enables expression of an N-terminal, hexa-histidine tagged fusion protein and pTrcHis10 an N-terminal deca-histidine tagged fusion protein following exposure to isopropyl-β-D-galactoside (IPTG) which activates an inducible promoter region that precedes the multiple cloning site into which the gene of interest has been inserted. In addition, LPP1a was cloned into the bacterial expression vector pMal-c2x at the BamH1 and HindIII sites (R. Tate – Strathclyde University) to generate a pMal-c2x-LPP1a plasmid construct. pMal-c2x enables expression of an N-terminal maltose binding protein (MBP) tagged fusion protein following exposure to IPTG as described above for the pTrcHis vectors. *Escherichia coli* (TOP-10, competent cells) were therefore, separately transformed with the bacterial expression vectors pTrcHis6, pTrcHis10 and pMal-c2x or the plasmid constructs pTrcHis6-LPP1, pTrcHis6-LPP1a, pTrcHis10-LPP1, pTrcHis10-LPP1a or pMal-c2x-LPP1a.

100ng of vector or construct DNA was added to competent *E. coli* before incubation on ice for 30 minutes, followed by heat shock at 42°C for 30 seconds and further incubation on ice for 2 minutes. 200µl of SOB medium [(20g Bacto-tryptone, 5g Bacto-yeast extract, 0.5g NaCl in 1l distilled water (H_2O), pH7, autoclaved 20mins at 15 pounds/square inch. 5ml of an autoclaved (20mins at 15 pounds/square inch) 2M $MgCl_2$ solution is also added immediately before use)] was added to the *E. coli* cultures which were incubated with shaking, at 37°C for 1 hour. The resulting cultures were plated on selective agar plates (see section 2.7.2.) and incubated overnight at 37°C. A single colony was picked from the resulting colonies and cultured in bulk for experimentation. Plates could be kept at 4°C for up to 3 weeks for repeated picking of single, viable colonies.

2.7.2. Preparation of agar plates

Agar plates were prepared by the addition of 1.5g of Bacto-agar to 100ml Luria-Bertani (LB) medium [(10g Bacto-tryptone, 5g Bacto-yeast extract, 10g NaCl in 1l distilled water (H_2O), pH7, autoclaved 20mins at 15 pounds/square inch)]. The solution was autoclaved and allowed to cool before the addition of 100µl of a 50mg/ml (50µg/ml final concentration) ampicillin solution. The mixture was poured into 9cm diameter petri dishes and allowed to solidify at room temperature.

2.7.3. Preparation of overnight cultures

5ml of LB medium was mixed with 5µl of a 50mg/ml stock solution of ampicillin in a 25ml vial. The LB (ampicillin) medium was inoculated with a single colony of the transformed bacteria from agar plates, sealed and placed in an orbital shaking incubator for approximately 16 hours at 37°C.

2.7.4. Preparation of Glycerol stocks

Glycerol stocks of the transformed *E. coli* cultures were prepared by mixing 800µl of sterile glycerol with 200µl of the appropriate culture. These were frozen and maintained at -80°C for use in subsequent plating and bulk culturing.

2.7.5. Induction of protein expression from the plasmid constructs

50mls of LB medium (plus 50µl of a 50mg/ml ampicillin stock) was inoculated with 2.5mls of the previously prepared overnight culture and incubated in a sterile 250ml flask, with shaking at 37°C. The culture was maintained until the linear phase of growth was achieved. This was estimated by measuring OD_{600} of a 1ml sample. The bacteria were assumed to be in the linear phase of growth when OD_{600} was 0.5-0.7 (generally after 1.75 hours). Once this point was reached, 1ml of the culture was removed and centrifuged at 15000 x g, 20°C, 3 mins before removal of the supernatant and freezing of the subsequent pellet at -20°C. This acts as a pre-induction sample. IPTG was added (final concentrations of 1mM for pTrcHis vectors and 0.3mM for pMal-c2x vector) to the remaining culture to induce His 6, His 10 or MBP-LPP fusion protein expression. Certain experiments were conducted with varying concentrations of IPTG (1mM and 0.2mM) or cultures were given 4 hours pre-induction growth followed by one hour of induction with 1mM IPTG. An induction timecourse was determined by sampling every hour and a bacterial pellet isolated as detailed above. Routinely, the culture was induced for 3 hours before isolation of the bacterial pellet by centrifugation at 7800 x g, 4°C, for 15mins. The pellet was frozen at -70°C for use in further experimentation.

2.7.6. Preparation of soluble and particulate fractions from the bacterial cultures

The bacterial pellet harvested from a 50ml culture was resuspended in 12ml of lysis buffer (50mM NaH_2PO_4/Na_2HPO_4 / 500mM NaCl pH 7) and freeze/thawed twice

before sonication at 200 – 300W for 6 x 10 second pulses (10 second pauses). The lysate was centrifuged at 5000 x **g**, 4°C for 15 minutes to pellet the cell debris. The resulting supernatant was centifuged at 100000 x **g**, 4°C for 60 minutes to prepare soluble and particulate (membrane) fractions. The particulate fraction was re-suspended in 6ml lysis buffer. The soluble and particulate fractions were stored at -20°C or subjected to further analysis e.g. SDS-PAGE and Western blotting or activity assays. Only the particulate fraction was used in solubilisation and purification experiments.

2.7.7. Solubilisation of the particulate fraction

Solubilisation of the particulate fraction from the induced *E-coli* cultures was attempted using several detergents at a final concentration of 1% (w/v or v/v) as well as various concentrations (10μg/ml, 50μg/ml, or 100μg/ml) of Phosphonate-1 which is a non-hydrolysable analogue of dioctanoyl PA ($C_{8:0}$,$C_{8:0}$). Table 2.1 details the properties of each of the detergents used.

Table 2.1: Properties of biological detergents used for extraction of the LPP isoforms from *E-coli* particulate fractions.

BIOLOGICAL DETERGENT	**CMC (Mm)**	**AGGREGATION No.**	**MOLECULAR WEIGHT**
Anionic			
Sodium dodecyl Sulphate (SDS)	8.27	62	288.4
Zwitterionic			
CHAPS	6-10	4-14	614.9
Non-Ionic			
n-Dodecyl β-D-maltoside	0.1-0.6	98	510.6
n-Octyl β-D-glucopyranoside	20-25	84	292.4
Triton X-100	0.24	140	625

Solubilisation was achieved through incubation with mixing of the particulate fraction (6mg/ml) at 4°C a solution containing lysis buffer (**2.2.5.**) and 1% (v/v) of each detergent tested or phosphonate-1. The resulting bacterial extract was then

centrifuged at 100000 x **g**, 4°C for 30 minutes to isolate a second soluble and particulate fraction. The second soluble fraction (SN2) was then used for subsequent purification experiments.

2.7.8. Immobilised Metal Affinity Chromatography (IMAC)

IMAC relies on the formation of weak bonds between basic groups on proteins (mainly histidine residues) and a metal ion [mainly divalent ions of the transition metals e.g. Nickel (Ni), Cobalt (Co) and Copper (Cu)] immobilised on a column. The column used in the purification procedure employed here was Ni^{2+} coupled via nitrilo triacetic acid to sepharose beads. This resin has been shown to have a high affinity for His 6-fusion proteins (Hancock, K. 2001). A 2ml slurry (1ml bed volume) of sepharose beads charged with nickel (Ni) was added to a 5ml plastic vial. The slurry was equilibrated (using 10 bed volumes) with buffer A [50mM NaH_2PO_4, 10% glycerol (v/v), 1% TX-100, 10mM β-Mercaptoethanol – pH7]. The detergent solubilised sample was added to the slurry and incubated at 4°C for 2 hours with mixing. The slurry was transferred to a disposable column cartridge and allowed to settle to form a column. Bound proteins were eluted with successive application of 5 x 2ml imidazole (10mM, 100mM, 500mM) pH 7 in buffer A which were collected as 0.5ml fractions. All procedures were conducted at 4°C and samples analysed by Western blotting to detect the protein of interest.

2.8. Cell Culture

All cell culture was performed in a Class II laminar flow cabinet.

2.8.1. HEK293 cell stable transfection and maintenance

Guinea pig LPP1 and LPP1a in addition to human LPP2, and LPP3 (generous gift from A.J.Morris which were subcloned by A. McKie) were separately cloned into the mammalian expression vector pcDNA3.1 Zeo(-) (Tate et al., 1999). HEK293 cells (maintained in Minimal essential medium (MEM)/Glutamax/10% foetal calf

serum/1% non-essential amino acids/50U/ml penicillin/50µg/ml streptomycin: complete medium) were transfected using Lipofectamine Plus (Life Technologies) with either pcDNA3.1, pcDNA3.1-LPP1, pcDNA3.1-LPP1a, pcDNA3.1-LPP2, or pcDNA3.1-LPP3. After 24 hours, the cells were incubated and subsequently maintained in selection medium (complete medium supplemented with Zeocin @ 200µg/ml) until Zeocin-resistant colonies became evident. These were separately isolated and grown in the continued presence of Zeocin. Surviving HEK293 clones were tested for LPP over-expression by LPP activity measurements and clones possessing high copy numbers were propagated and maintained for future experiments. HEK293 clones into which pcDNA3.1 alone had been successfully transfected were identified by their Zeocin resistance alone.

2.8.2. Maintenance of stably transfected HEK293 cells

HEK293 cell clones were sub-cultured by trypsinisation. This involved removal of the culture medium, followed by rinsing with 10ml of MEM and incubation for 3-5mins, in 2ml of sterile trypsin solution [0.1% (w/v) Trypsin, 2.69mM EDTA, 10mM glucose, 145mM NaCl, 5.4mM KCl, 8.1mM Na_2HPO_4 and 1.5mM KH_2PO_4 (pH 7.4) (Phosphate-buffered saline, PBS)] to detach the cells. The trypsinised cell suspension was diluted in 8ml of complete medium and transferred to a sterile 11ml centifuge tube. Cell suspensions were centrifuged at 65 x **g** for 2 min and the resultant pellet re-suspended in complete medium before being seeded into 75cm^2 culture flasks or 12 well plates as required. Cells were maintained in complete medium at 37°C in humidified air/CO_2 (95:5, v/v) and the medium was replaced every 48-72 hours. In addition, sustained overexpression of the LPP isoforms in the stably transfected HEK293 cell clones was monitored periodically by activity measurements.

2.8.3. Preparation of HEK293 cell membranes

HEK293 cells were grown to confluence in 75cm^2 flasks. The medium was aspirated and the cells were rinsed twice with 5ml of ice-cold PBS. Cells were then harvested into 2ml of ice-cold homogenisation buffer (50mM Tris maleate (pH7), 1mM EDTA, 150mM NaCl and 10mM mercaptoethanol) using a cell scraper. Cell extracts were repeatedly passed through a 0.24mm guage syringe and membranes obtained by centrifugation (15000 x **g**, 10 minutes). The resultant pellet was resuspended in homogenisation buffer by repeatedly passing through a 0.24mm guage syringe and samples stored at -20°C until required.

2.8.4. Guinea-pig airway smooth muscle (GPASM) primary culture

GPASM primary cell culture was performed using a method based on that of Panettieri et al., (1989). This involved dissection of the smooth muscle strip from a guinea-pig trachea, followed by digestion of the smooth muscle tissue in a solution containing 1ml Dulbecco's Modified Eagle Medium (DMEM) (supplemented with 2mM glutamine and 50U/ml penicillin/streptomycin) containing collagenase (Type II, 1mg/ml), elastase (Type IV, 0.2mg/ml) and soya bean trypsin inhibitor (50µg/ml) at 37°C for 2 - 2.5 hours. The cell suspension was diluted with DMEM containing 10% European foetal calf serum (EFCS) and 10% donor horse serum (DHS) before being transferred to a 75cm^2 culture flask for incubation at 37°C in humidified air/CO_2 (95:5, v/v). Surviving cells were termed passage 1 (P1). Medium was replaced after 24 hours with fresh DMEM containing 10% EFCS and 10% DHS and then every 48 – 72 hours until the GPASM cells were deemed > 80% confluent (Pyne & Pyne, 1993). The cells were then subjected to secondary culture.

2.8.5. Secondary culture of GPASM cells

Secondary culture of the GPASM cells was conducted as described in section 2.8.2. for the HEK293 stably transfected cell clones except that the culture medium used was DMEM containing 10% EFCS and 10% DHS. Experiments were routinely

conducted on GPASM cells passaged twice (P3), i.e. approximately 15-21 days after primary culture (Pyne & Pyne, 1993).

2.8.6. Transient transfection of Wild Type HEK293 and GPASM cells

Wild Type (WT) HEK293 and GPASM cells were transfected with various vector constructs using the following procedure. MEM was used for WT HEK293 cell transfection and DMEM for GPASM cell transfection. Cells were grown to approximately 90% confluence in 12 well plates before the medium on the cells was replaced with 0.9ml of MEM/DMEM with 2% serum. For each well to be transfected, 1μg of the relevant cDNA construct was then mixed with 1.5μl of LipofectAMINE^{TM} 2000 (LF2000) Reagent and 97.5μl of MEM/DMEM in a sterile eppendorf and incubated at room temperature for 20 minutes. 100μl of the DNA-LF2000 reagent complexes was then added directly to each well and the cells incubated for 24 hours at 37°C in humidified air/CO_2 (95:5, v/v). After 24 hours, cells were rendered quiescent (24 hours in serum free MEM/DMEM) prior to stimulation.

2.8.7. Cell stimulation

Transiently transfected HEK293 cells and GPASM cells were rendered quiescent (24 hours in serum free growth medium) prior to stimulation. Cells were stimulated for 10 minutes with S1P (5μM) or PMA (1μM). Following stimulation, lysates were taken (2.4.1.) for Western blotting with antibodies towards the phosphorylated/activated forms of ERK-1/2 and ERK-1 (2.4.9.). In experiments using PTX, cells were pre-incubated with/without PTX (0.1μg/ml) for 18 hours before stimulation with S1P or PMA.

2.8.8. Cell counting

HEK293 cells stably transfected with blank vector, LPP1, LPP1a, LPP2 and LPP3 were seeded onto 12 well plates at a density of approximately 33000 cells/well. Cell counting was conducted daily for a period of 8 days using a hemacytometer as recommended by the manufacturers (Clonetics Corporation ©) in order to monitor basal growth rates. Briefly, a cell suspension was made and subsequently diluted until between 100 and 400 cells could be counted in the hemacytometer. The number of cells per ml of cell suspension was calculated using the equation: Cells/ml = (n) x 10^4, n = average cell count. The number of cells counted per ml of cell suspension was then multiplied according to the total volume of cell suspension produced following dilution.

2.9. Molecular analysis

2.9.1. RNA isolation from stably transfected HEK293 cells

All procedures were conducted with RNase free plasticware and protocols were followed as per the manufacturers instructions (Qiagen). Briefly, a 75cm^2 flask of cells was washed twice in 5ml of sterile PBS and lysed by adding 600µl of RNeasy lysis buffer (containing 14.5M β-mercaptoethanol) – (RLT buffer). The cells were then scraped and homogenised by passing the lysate through a 25 gauge (g) needle at least six times.

The lysates were loaded onto the provided QIAshredder columns before centrifugation at 10000 x g for 2 minutes to homogenize the sample. Total RNA was extracted by addition to a RNeasy mini column followed by sequential washing and elution into a 2ml collection tube as per the manufacturers instructions (RNeasy protocol – Qiagen). The purified RNA was incubated at 37°C for 15 minutes with 4 units of DNase1 in order to degrade any possible contaminating genomic DNA. The

RNA was re-extracted and purified a second time before elution with RNase free H_2O and storage at -20°C until required.

2.9.2. Quantification and confirmation of purity of total RNA

In order to determine the concentration and purity of the isolated RNA, the optical density at 260nm (OD_{260}) and 280nm (OD_{280}) was measured in a spectrophotometer (Genequant II^{TM}). RNA was used for subsequent experimentation if a yield of greater than 25µg RNA/ml and a ratio of OD_{260}:OD_{280} (which gives an estimate of RNA purity) between 1.5 and 2 was obtained.

2.9.3. Reverse Transcription

Synthesis of first strand cDNA from the isolated RNA was achieved using 2µg of total RNA and Superscript II reverse transcriptase (200 Units). The reaction contained 500ng of oligo (dT_{12-18}) primers, 200µM dNTP, first strand buffer [50mM Tris-HCl (pH 8.3), 75mM KCl, 3mM $MgCl_2$] and 1mM DTT in a final volume of 20µl. The reaction was incubated for 1.5 hours at 42°C before inactivation by incubation at 70°C for 15 minutes. Template RNA was degraded by the addition of 2 Units of *E-coli* RNase H and incubation at 37°C for 20 minutes. A separate reaction containing no Superscript II reverse transcriptase was conducted in parallel as a control. 2µg of the synthesised cDNA was then used as a template for subsequent Polymerase Chain Reactions (PCRs).

2.9.4. Semi-quantitative Polymerase Chain Reaction

The polymerase chain reaction (PCR) allows the *in vitro* amplification of specific cDNA sequences through the use of a pair of gene-specific forward and reverse oligonucleotide primers. The primers were designed to unique, gene-specific nucleotide sequences using information published in the GenBank database. Primer

sequences were checked by BLAST® analysis to exclude the possibility of homology with other non-specific genes. The primers used to amplify human $S1P_1$/EDG1 are detailed below. The primers used to amplify human Glyceraldehyde 3-Phosphate Dehydrogenase (G3PDH) were obtained from Clontech and are also detailed below.

Primers: $S1P_1$/EDG1 sense, 5' – TCC GCA AGA ACA TTT CCA AGG – 3'
antisense, 5' – GCT GCG GCT GAA TTC CAT G – 3'

G3PDH sense, 5' – TGA AGG TCG GAG TCA ACG GAT TTG GT – 3'
antisense, 5' - CAT GTG GGC CAT GAG GTC CAC CAC – 3'

The PCR amplification was performed in a total reaction volume of 100µl containing: PCR reaction buffer (10mM Tris-HCl, 50mM KCl), 1.5mM $MgCl_2$, 0.2mM dNTP mix, 1µM of each sense and antisense primer, 200ng cDNA, and 2.5 units of Taq DNA polymerase. Amplification of human G3PDH was conducted in parallel to confirm equal addition of cDNA in the reaction mixtures. The PCR reaction was programmed in a Phoenix thermal cycler as follows: 5 minutes initial denaturation at 94°C, followed by 15-30 cycles of amplification. One cycle of amplification involved denaturation for 1 minute at 94°C, annealing for 1 minute at 49°C and extension for 2 minutes at 72°C. A final extension was then performed for 10 minutes at 72°C, and samples were then stored at 4°C until further experimentation. RT-PCR conditions, which yielded linear amplification of EDG1 (25 cycles) were then employed to investigate the levels of EDG1 transcript.

2.9.5. Agarose gel electrophoresis

Products obtained following PCR were analysed by agarose gel electrophoresis on a 1% agarose gel made in Tris-Borate-EDTA (TBE): 45mM Tris-Borate and 1mM EDTA plus 0.5µg/ml ethidium bromide. Gels were cast in a Mini-Q apparatus (Bioscience Services) and immersed in TBE buffer. 15µl of each PCR product was mixed with 3µl of gel loading dye (50% v/v glycerol, 0.05% v/v bromophenol blue,

0.05% w/v xylene cyanol) and all 18µl applied to the gel alongside 10µl of 100 base pair (bp) ladder (15 blunt end fragments ranging from 100 to 1500bp in multiples of 100bp with an additional fragment of 2072bp). The gel was run at 70 volts for 1 hour and amplified DNA bands visualised using an Ultra Violet transilluminator and photographed using a Kodak DC120 digital camera and Kodak Digital Science 1D image analysis software (Eastman Kodak Company). Quantification was then achieved through densitometry measurements using Scion Image© - Version 3b.

2.9.6. Sequencing of DNA products

In order to verify amplification of $S1P_1$/EDG1, amplified DNA bands were cut from the agarose gel and purified using the GFX™ PCR gel band purification kit (Amersham Biosciences). A PE-Applied Biosystems Division Model 373A automated DNA sequencer and a BigDye Dye terminator cycle sequencing kit was then used to sequence the purified amplicons. The amplicons were sequenced in both directions using the gene-specific forward and reverse primers that had been used in the PCR reaction and all sequencing was performed in the Molecular Biology facility at the University of Strathclyde (by R.J. Tate).

2.10. Statistical analysis

Unless otherwise stated, data is expressed as mean ± standard error of the mean (SEM) for experiments repeated at least three times with each assay conducted in triplicate. Statistical analysis was by students t-test with $p<0.05$ deemed to be significant. Data denoted by * was significantly different from control values.

CHAPTER 3

PURIFICATION OF LPP1 AND LPP1A FUSION PROTEINS USING A BACTERIAL EXPRESSION SYSTEM

3. Purification of LPP1 and LPP1a fusion proteins using a bacterial expression system

3.1. Introduction

The first purification of mammalian LPP was reported by Kanoh et al. (1992) from porcine thymus. They suggested it to be an 83 kDa protein that co-purified with LPP activity and was enriched 2300-fold. Subsequently, Fleming and Yeaman (1995a) purified two distinct PAP activities from rat liver, PAP-1 and PAP-2 (LPP). A further study by Fleming & Yeaman detailed the presence of two LPP activities in rat liver, the first containing an 83 kDa protein identified through silver staining and the second containing three proteins with molecular masses of 83 kDa, 53 kDa and 34 kDa (Fleming & Yeaman, 1995b).

Waggoner and colleagues also reported the purification of a 51-53kDa LPP activity which was a glycoprotein from rat liver (Waggoner et al., 1995). However, two different ionic forms of LPP were suggested. The cationic form had an apparent molecular weight of 51 kDa and the anionic form 53 kDa. The conclusion of this study was that the anionic enzyme was a sialated form of the enzyme and that LPP was an extensively N-glycosylated protein (Waggoner et al., 1995). The 51-53 kDa protein was detected immunologically in several other tissues including heart, kidney, skeletal muscle, testes and brain. Subsequently, Kanoh and co-workers demonstrated that the LPP activity, isolated previously from porcine thymus, was a 35 kDa protein which co-purified with the original 83 kDa protein (Kai et al., 1996). Furthermore, Siess & Hoftstetter (1996) using immunological techniques, demonstrated that rat liver LPP was associated with a 31kDa protein. These apparent discrepancies in the molecular weights of purified LPP enzymes may suggest the formation of multimers or their post-translational modification within cells.

A role for LPP enzymes in signal transduction has been implicated for several years. However, the precise biological functions of these enzymes remains unknown. As described previously, LPP activity has been purified from pig thymus (Kanoh et al.,

1992, Kai et al., 1996) and also from rat liver (Fleming & Yeaman, 1995, Waggoner et al., 1995). However, these studies have utilised total membrane fractions to purify LPP activity from native tissues and so may contain several different LPP enzymes. The cloning of individual LPP isoforms has enabled the investigation of each LPP when over-expressed. An additional approach, adopted here, was to use molecular biological techniques to prepare purified LPP1 and LPP1a using a bacterial expression system.

Bacterial expression systems allow the production of large quantities of recombinant proteins within the chosen bacterial host such as *E. coli* and have become invaluable in the purification of proteins. There are a variety of bacterial expression vectors available, which are designed to produce recombinant protein with epitope tags at either the N- or C-terminus to facilitate their purification. The pTrcHisB vector was employed as it is designed, (1), for efficient protein expression in *E. coli* and (2), to add a hexa-histidine tag to the N-terminus of the fusion protein which aids detection and purification through specific probes and Ni^{2+}-affinity resins respectively. However, as will be described, the N-terminal, hexa-histidine tag was not sufficient to enable purification. Therefore, the pTrcHisB vector construct was modified to encode a deca-histidine tag at the N-terminus of the recombinant LPP1 and LPP1a. In addition, an alternative strategy of using a Maltose-binding protein tagged fusion protein was also investigated. The successful purification of the individual LPP1 and LPP1a would represent the first of its kind and would allow the detailed investigation of individual LPP isoforms with respect to their kinetic properties and their regulation by processes such as protein association or phosphorylation.

3.2. Results

3.2.1. Expression of hexa-histidine tagged-LPP1 and LPP1a in *E. coli*

E. coli (TOP-10) were separately transformed with the bacterial expression vector pTrcHis or a construct of pTrcHis plus the LPP1 open reading frame (ORF) (pTrcHis6–LPP1) or pTrcHIS plus the LPP1a ORF (pTrcHis6–LPP1a). These constructs were designed to generate LPP1 and LPP1a fusion proteins with hexa-histidine tags at their N-terminal (His 6-LPP1 and His 6-LPP1a respectively). Figures 3.1 and 3.2 show the vector constructs used for LPP1 and LPP1a respectively. Cultures were prepared as described in 2.7.4 and the purification monitored by Western blot analysis (2.4.7.-2.4.9.) and *in vitro* LPP activity measurements (2.6.). Figures 3.3, 3.4 and 3.5 demonstrate the specificity of the anti-LPP1/LPP1a antibody, the anti-LPP catalytic domain antibody and the HisProbe for the His 6-LPP1 and His 6-LPP1a fusion proteins. As shown in Figure 3.3, no IPTG-induced proteins were detected in any of the samples probed with the pre-immune serum or in the pre induction sample probed with the 1st bleed antibody. However, clear bands were detected at the predicted molecular weight of the LPP fusion proteins (35.95kDa: His 6-LPP1 and 35.91kDa: His 6-LPP1a) in the induced samples probed with the 1st bleed anti-LPP1/LPP1a antibody. Furthermore, Figure 3.4 shows that again, the anti-LPP1/LPP1a but also the anti-LPP catalytic domain antibody detected specific products at the correct molecular weight in the pTrcHis 6-LPP1 and pTrcHis 6-LPP1a cultures induced for 3 hours with IPTG which were not detected in pre-induced samples. In addition, Figure 3.5 demonstrates the ability of the HisProbe to detect similar reactivity in pTrcHis 6-LPP1 and pTrcHis 6-LPP1a cultures induced for 3 hours with IPTG but not in pre-induced samples. This demonstrates the ability of three distinct probes to detect the induced His 6-LPP1 and His 6-LPP1a fusion proteins. Furthermore, the results show that the induced products not only contain the histidine tag, but also contain the specific regions of the LPPs to which the anti-LPP1/LPP1a antibody and the anti-LPP catalytic domain antibody were raised.

Bacterial expression of both His 6-LPP1 and His 6-LPP1a was initially confirmed by Coomassie-blue staining of SDS-PAGE gels (2.4.4.). However, the IPTG-induced fusion proteins could not be measurably detected using this method (data not shown). This suggested relatively low levels of expression of the LPP fusion proteins, estimated to be around 1% of total *E. coli* protein. Bacterial expression systems are known to produce recombinant foreign proteins to levels representing up to 10% of total protein. However, 1% of total protein is generally considered to be sufficient, although not ideal, for purification protocols (Scopes, 1994). Therefore, Western blot analysis was conducted on lysates derived from IPTG-induced pTrcHis6-LPP1 and pTrcHis6-LPP1a transformants using INDIA^{TM}Hisprobe-HRP (Hisprobe). The HisProbe is a Nickel activated derivative of horseradish peroxidase which has been designed to detect histidine-rich proteins, including hexa-histidine tagged fusion proteins. In both the pTrcHis6-LPP1 and pTrcHis6-LPP1a transformants, two products (35 and 38kDa) were detected (Figures 3.5a and 3.5b). The induction time course of the two products was very similar, being detected and expressed maximally within one hour (Figures 3.5a and 3.5b). The maximal level of expression was maintained up to five hours post-induction (results not shown). The appearance of two products following induction was unexpected and may represent modification of the induced fusion protein by the *E. coli*. host. LPP activity measurements were also conducted on lysates derived from IPTG-induced transformants. Significantly higher activity was present in the pTrcHis 6-LPP tranformants compared to the blank vector transformant (Figure 3.6). The pTrcHis 6-LPP1 transformant was found to exhibit an 11.08+/-2 fold increase in activity compared to an 7.66+/-2.08 fold increase in the pTrcHis 6-LPP1a transformant.

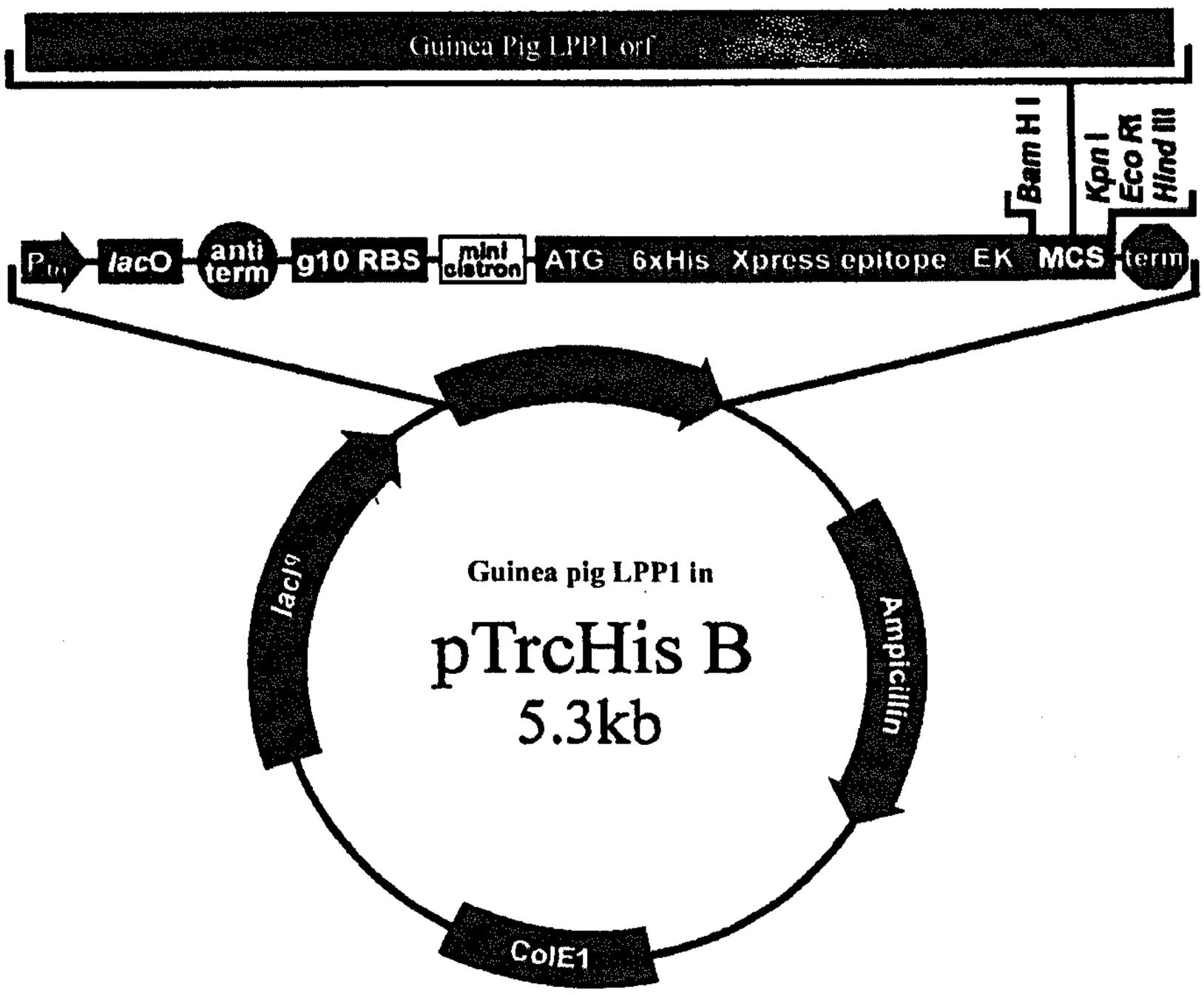


Figure 3.1: pTrcHis B vector construct plus gpLPP1 insert

The open reading frame of guinea pig LPP1 (GenBank Accession No. AF088283) was inserted into the pTrcHis B vector at the Bam H1 and Kpn 1 sites within the multiple cloning site (R.J.Tate, Molecular Biology Facility, University of Strathclyde). The pTrcHis B-LPP1 construct was designed to express N-terminal hexa-histidine tagged LPP1 fusion protein upon induction of transformed *E. coli* with IPTG.

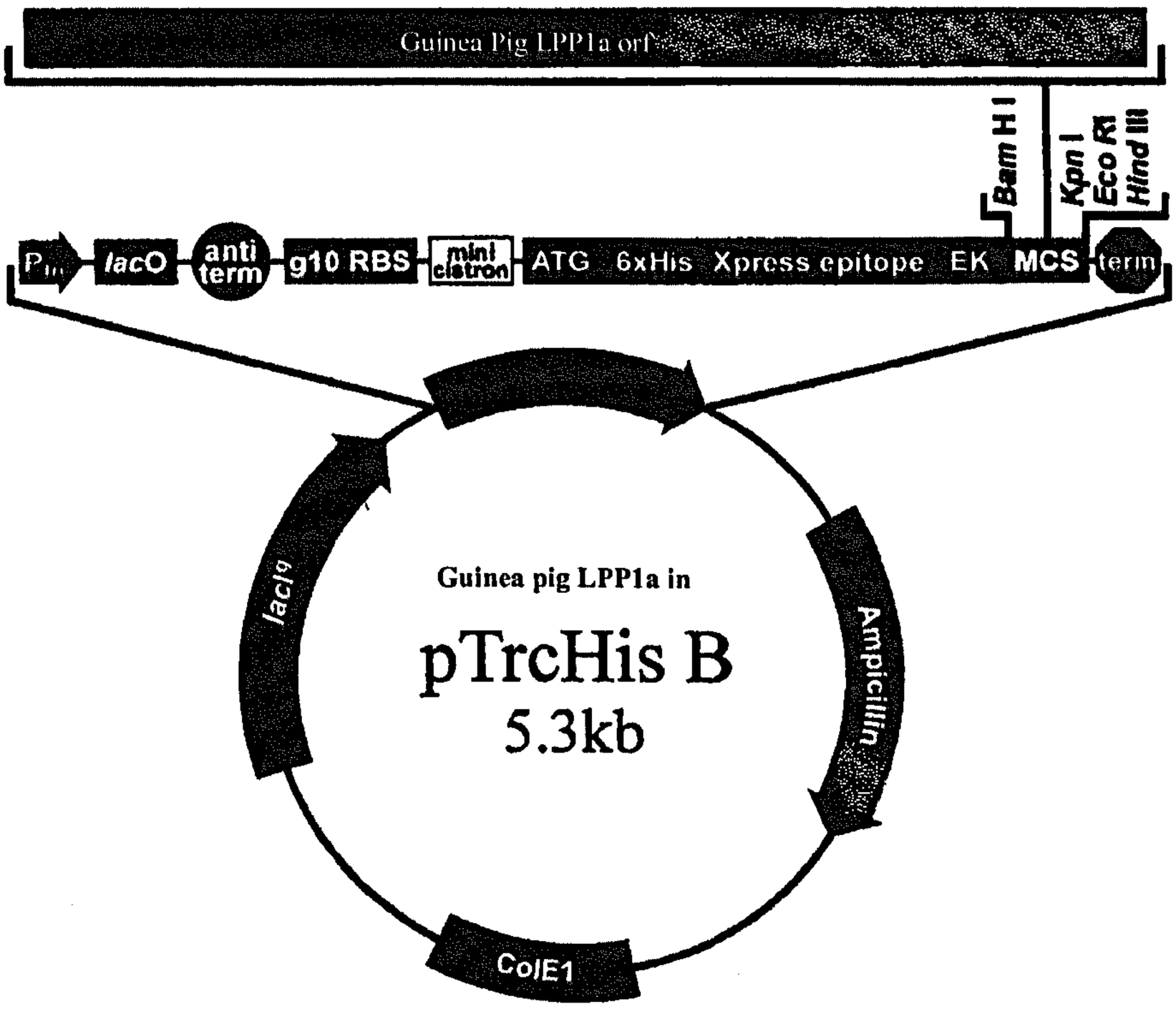


Figure 3.2: pTrcHis B vector construct plus gpLPP1a insert

The open reading frame of guinea pig LPP1a (GenBank Accession No. AF088284) was inserted into the pTrcHis B vector at the Bam H1 and Kpn 1 sites within the multiple cloning site (R.J.Tate, Molecular Biology Facility, University of Strathclyde). The pTrcHis B-LPP1a construct was designed to express N-terminal hexa-histidine tagged LPP1a fusion protein upon induction of transformed *E. coli* with IPTG.

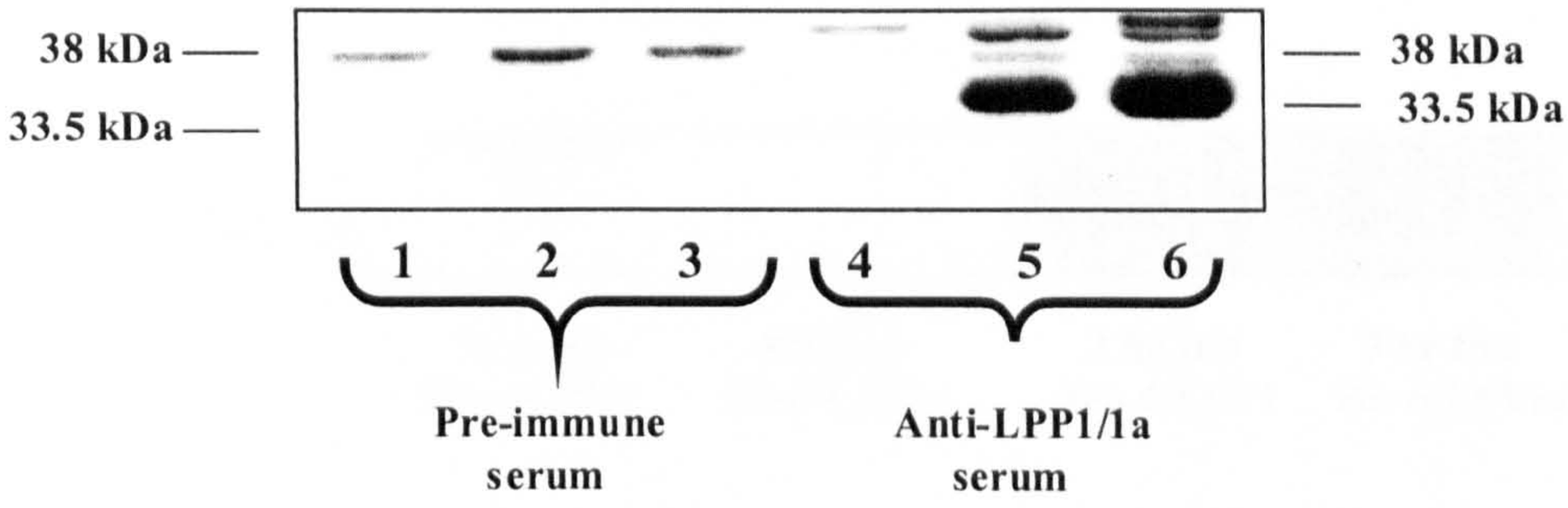


Figure 3.3: Western blot analysis of pre-induced and induced bacterial lysates.

The expression of His 6-LPP1 and His 6-LPP1a was induced by addition of IPTG (1mM) to cultures of *E-coli* that had been separately transformed with pTrcHis 6-LPP1 or pTrcHis 6-LPP1a as described in 2.7.4. Samples of pre-induced and induced cultures were collected and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. His 6-LPP1 and His 6-LPP1a were detected by Western blot analysis using an anti-LPP1/LPP1a polyclonal antibody (1:200 dilution) as described in 2.4.8. (lanes 4-6). Immunoreactivity was compared with that obtained using pre-immune serum (lanes 1-3). Positions of molecular weight markers are indicated. This is a representative result of an experiment performed at least three times.

Lanes 1 and 4 – pTrcHis 6-LPP1, pre-induced
Lanes 2 and 5 – pTrcHis 6-LPP1, induced
Lanes 3 and 6 – pTrcHis 6-LPP1a, induced

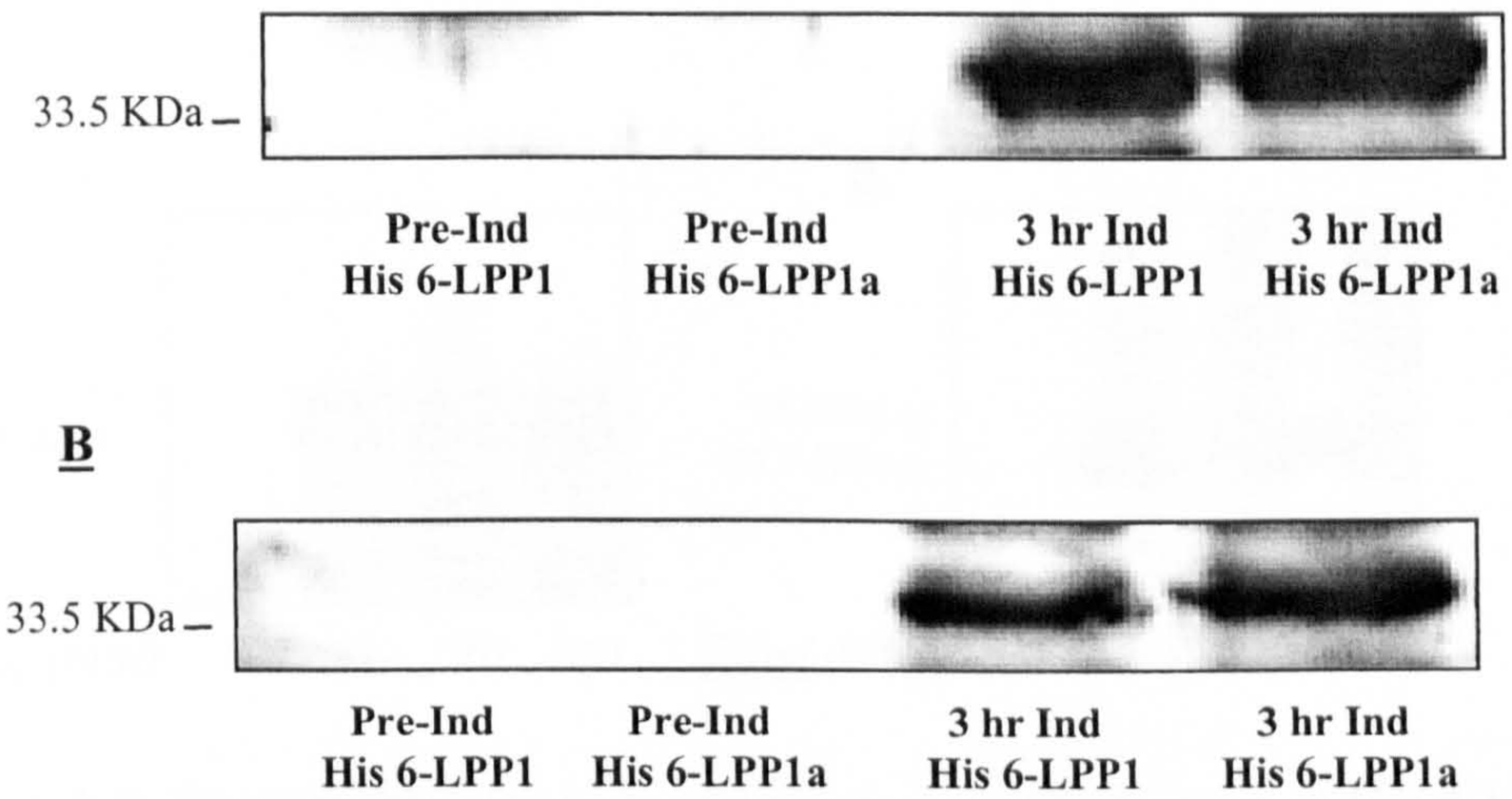


Figure 3.4: Western blot analysis of pre-induced and induced bacterial lysates.

The expression of His 6-LPP1 and His 6-LPP1a was induced by addition of IPTG (1mM) to cultures of *E-coli* that had been separately transformed with pTrcHis 6-LPP1 or pTrcHis 6-LPP1a as described in 2.7.4. Samples of pre-induced and induced cultures were collected and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. His 6-LPP1 and His 6-LPP1a were detected by Western blot analysis using **A** - anti-LPP1/LPP1a polyclonal antibody (1:200 dilution), **B** - anti-LPP catalytic domain antibody (1:200 dilution) as described in 2.4.7.-2.4.8. Positions of molecular weight markers are indicated. This is a representative result of an experiment performed at least three times.

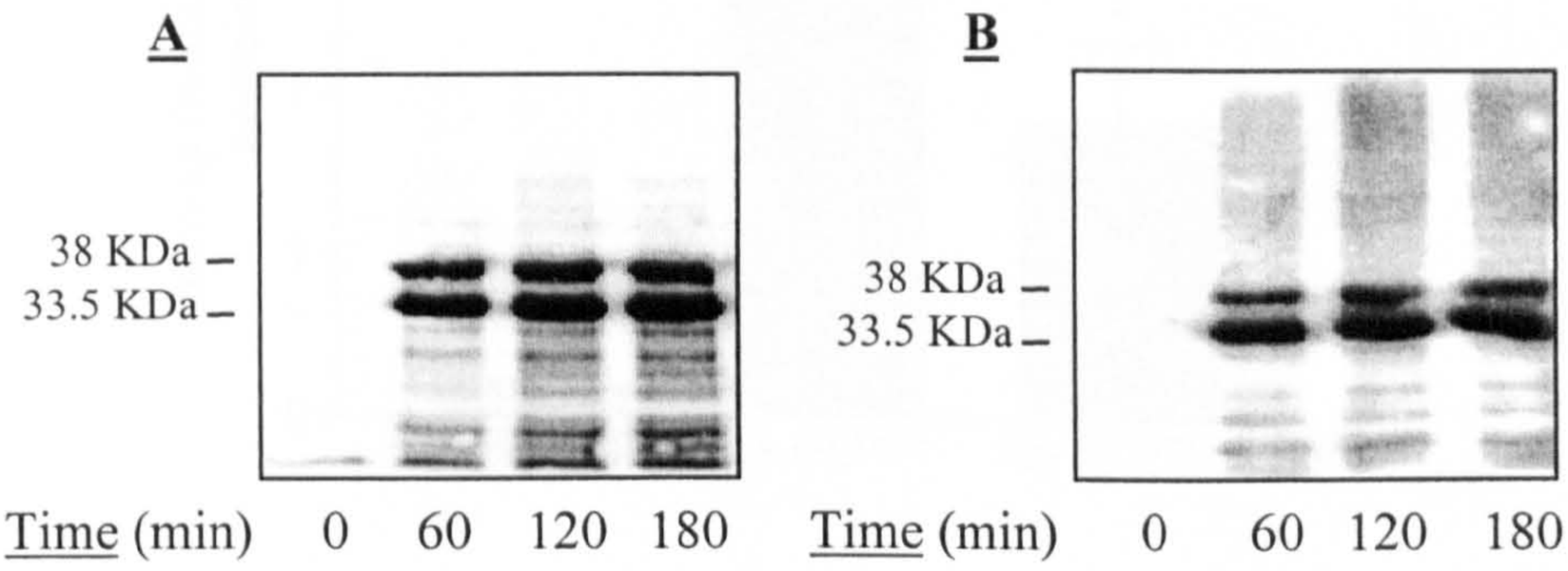


Figure 3.5: Western blot analysis of timecourse of induction of His 6-LPP1 and His 6-LPP1a expression.

The expression of His 6-LPP1 (A) and His 6-LPP1a (B) was induced using 1mM IPTG in *E-coli* that had been separately transformed with pTrcHis 6-LPP1 or pTrcHis 6-LPP1a and a time course performed as described in 2.7.5. Samples were collected at each time point and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. Proteins were detected by Western blot analysis using the INDIA^{TM}HisProbe-HRP (Pierce) as described in 2.4.7. Positions of molecular weight markers are indicated. This is a representative result of an experiment performed at least three times.

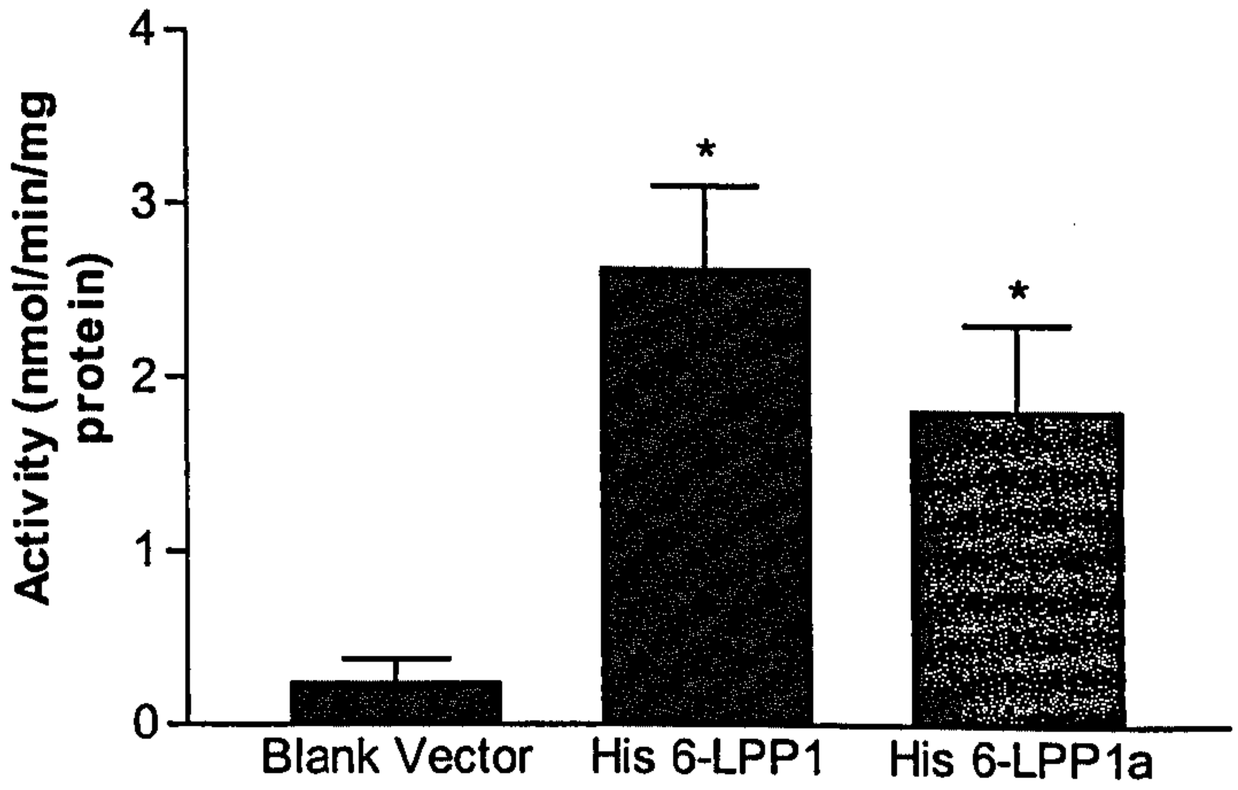


Figure 3.6: LPP activity measurements of lysates derived from IPTG-induced *E.coli* cultures after transformation with pTrcHIS alone, pTrcHIS - LPP1 or pTrcHIS - LPP1a.

LPP activity was assayed against dioleoyl PA (500μM) as described in 2.6.1. using total lysates from each induced *E-coli* culture at protein concentrations between 7 and 10μg/assay. Results are expressed in nmoles Pi/min/mg protein (mean +/- SEM, n =3). * $p < 0.05$: Students t-test versus blank vector lysate.

The expression of foreign proteins in cells can change cell physiology, protein folding, or susceptibility to proteolysis (Sambrook et al., 1989). Therefore, experiments were conducted to optimise the induction of the His 6-LPP1 and His 6-LPP1a fusion proteins by altering the kinetics of the synthesis of the recombinant LPP fusion proteins. This involved induction with either 0.2mM IPTG or 1mM IPTG for 3 hours following growth of the *E-coli* to mid-log phase, or by growing transformants for four hours before induction with 1mM IPTG for one hour. The results of these experiments are shown in Figures 3.7 to 3.9 with bacterial lysates for each of the three transformants, induction and growth conditions being probed with the HisProbe, the anti-LPP1/LPP1a antibody and the anti-LPP catalytic domain antibody, respectively. The results detailed in Figures 3.7 to 3.9 demonstrate that induction with 1mM IPTG for 3 hours was the most efficient method of induction for the expression of the recombinant His 6-LPP fusion proteins. In addition, altering of the kinetics of expression was found to have no effect on the LPP activity measured (results not shown).

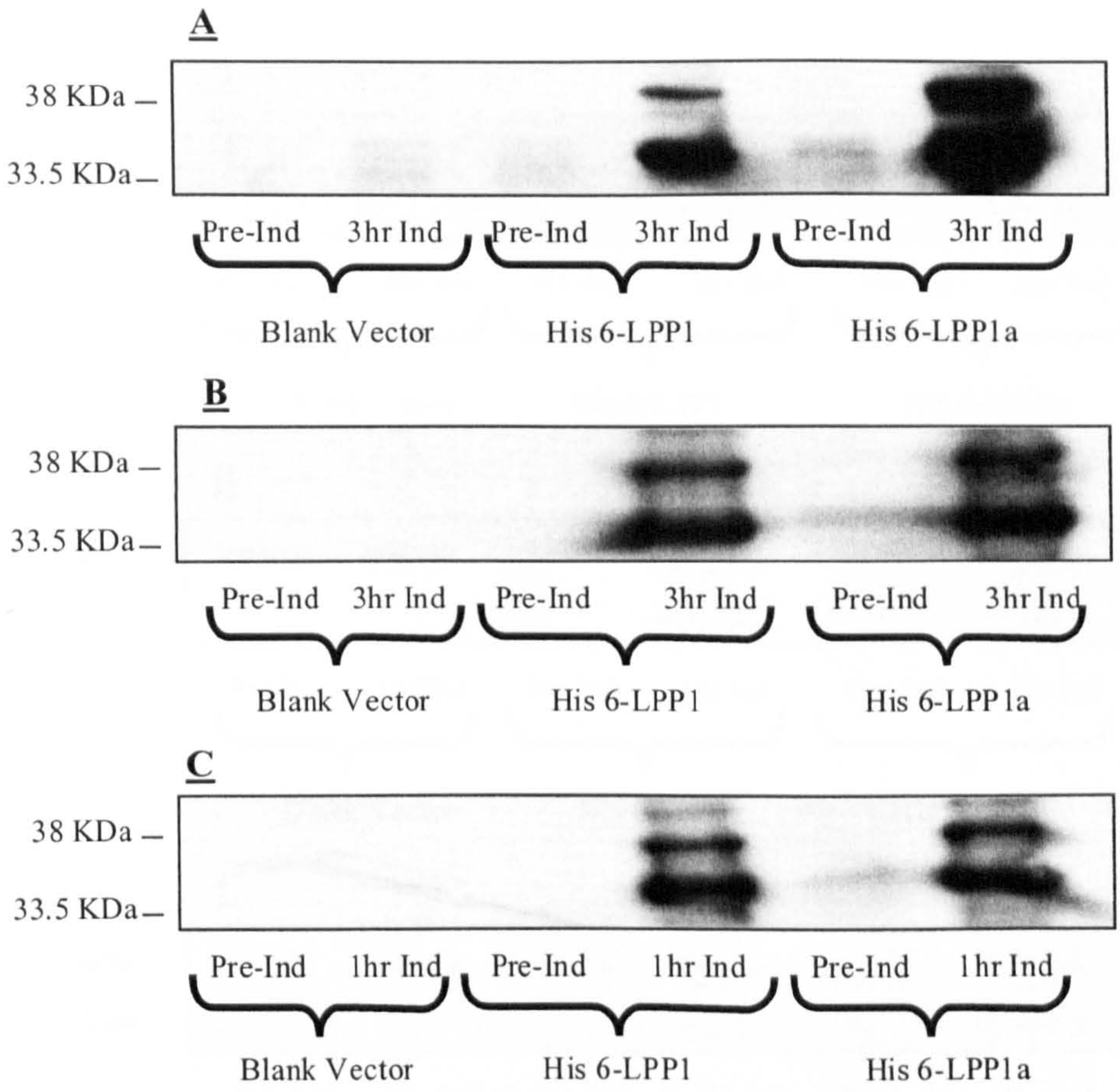


Figure 3.7: Western blot analysis of His 6-LPP1 and His 6-LPP1a expression: comparison of induction protocols (INDIA^{TM}HisProbe-HRP).

The expression of His 6-LPP1 and His 6-LPP1a was induced in pTrcHis-LPP1 and pTrcHis-LPP1a transformants using three different induction protocols. A vector transformed culture was incubated in parallel. Samples were collected and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. His 6-LPP1 and His 6-LPP1a were detected by Western blot analysis using INDIA^{TM}HisProbe-HRP (Pierce) as described in 2.4.7. These are representative results from an experiment performed at least three times. Positions of molecular weight markers are indicated. **A** – 1mM IPTG, **B** – 0.2mM IPTG, **C** – 4 hours growth followed by 1 hour induction (1mM IPTG).

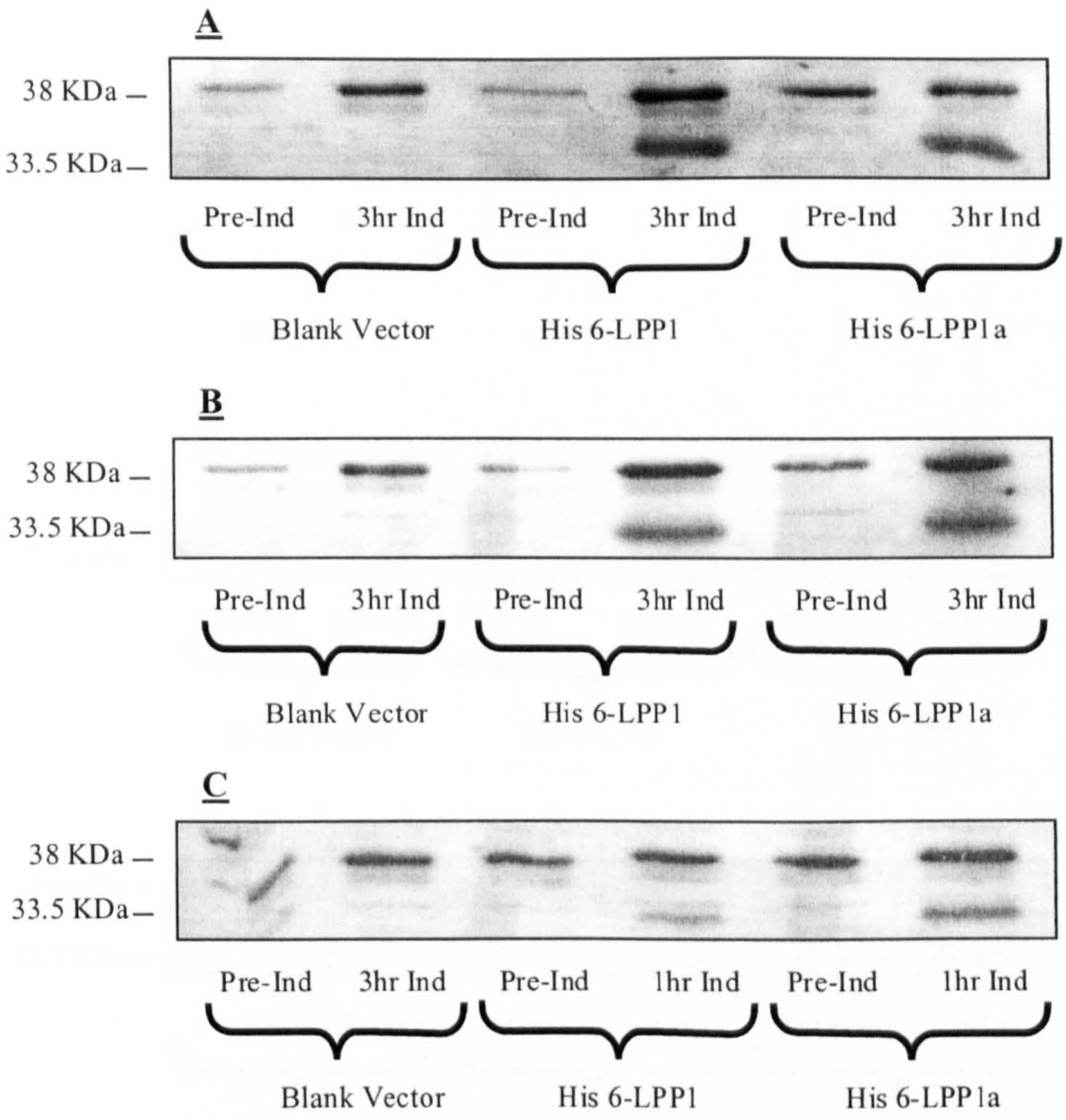


Figure 3.8: Western blot analysis of His 6-LPP1 and His 6-LPP1a expression: comparison of induction protocols (anti-LPP1/LPP1a antibody).

The expression of His 6-LPP1 and His 6-LPP1a was induced in pTrcHis-LPP1 and pTrcHis-LPP1a transformants using three different induction protocols. A vector transformed culture was incubated in parallel. Samples were collected and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. His 6-LPP1 and His 6-LPP1a were detected by Western blot analysis using anti-LPP/LPP1a antibody as described in 2.4.8. These are representative results from an experiment performed at least three times. Positions of molecular weight markers are indicated. **A** – 1mM IPTG, **B** – 0.2mM IPTG, **C** – 4 hours growth followed by 1 hour induction (1mM IPTG).

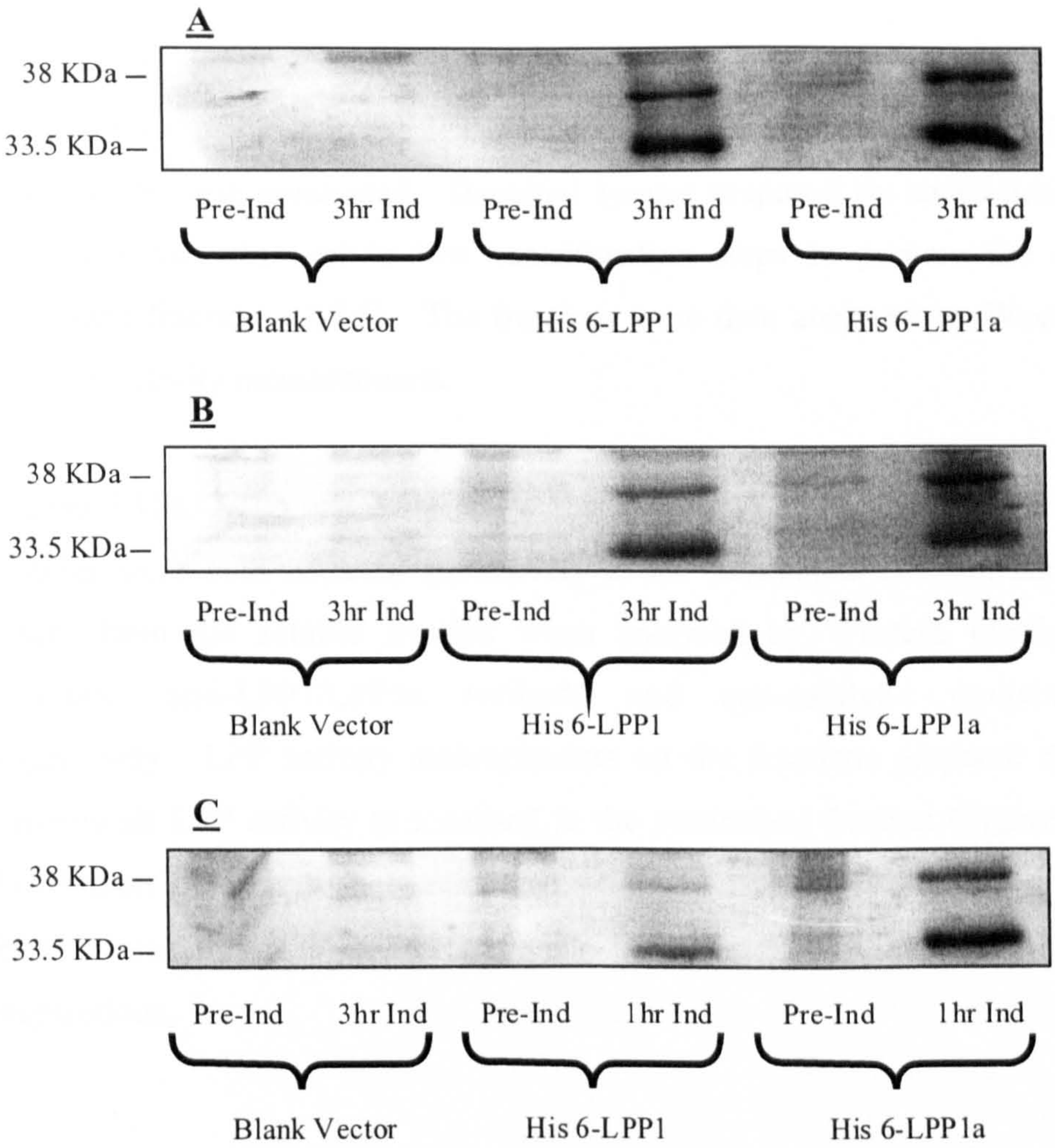


Figure 3.9: Western blot analysis of His 6-LPP1 and His 6-LPP1a expression: comparison of induction protocols (anti-LPP catalytic domain antibody).

The expression of His 6-LPP1 and His 6-LPP1a was induced in pTrcHis-LPP1 and pTrcHis-LPP1a transformants using three different induction. A vector transformed culture was incubated in parallel. Samples were collected and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. His 6-LPP1 and His 6-LPP1a were detected by Western blot analysis using an antibody raised to the Catalytic site of the LPP isoforms as described in 2.4.8. These are representative results from an experiment performed at least three times. Positions of molecular weight markers are indicated. **A** – 1mM IPTG, **B** – 0.2mM IPTG, **C** – 4 hours growth followed by 1 hour induction.

3.2.2. Subcellular localisation of recombinant His 6-LPP1 and His 6-LPP1a

To characterise the particulate and cytoplasmic distribution of His 6-LPP1 and His 6-LPP1a fusion proteins in the IPTG-induced transformants, subcellular fractionation experiments were conducted. Bacterial lysates prepared by sonication and freeze thawing were subjected to two centrifugation steps to prepare the soluble and membrane fractions (2.7.6). The fractions were then analysed by Western blotting and LPP activity measurements.

Figures 3.10a, 3.10b and 3.10c show that the His 6-LPP1 and His 6-LPP1a fusion proteins were both detected exclusively in the particulate fraction and apparently absent from the soluble fraction when analysed by Western blotting with the HisProbe, anti-LPP1/LPP1a antibody and anti-catalytic domain antibody, respectively. LPP activity measurements on the fractions prepared demonstrated virtually all LPP activity to localised in the particulate fraction (Figure 3.14). The distribution of activity between the particulate fraction compared to the soluble fraction was 99%:1%, respectively for both the His 6-LPP1 and His 6-LPP1a preparations.

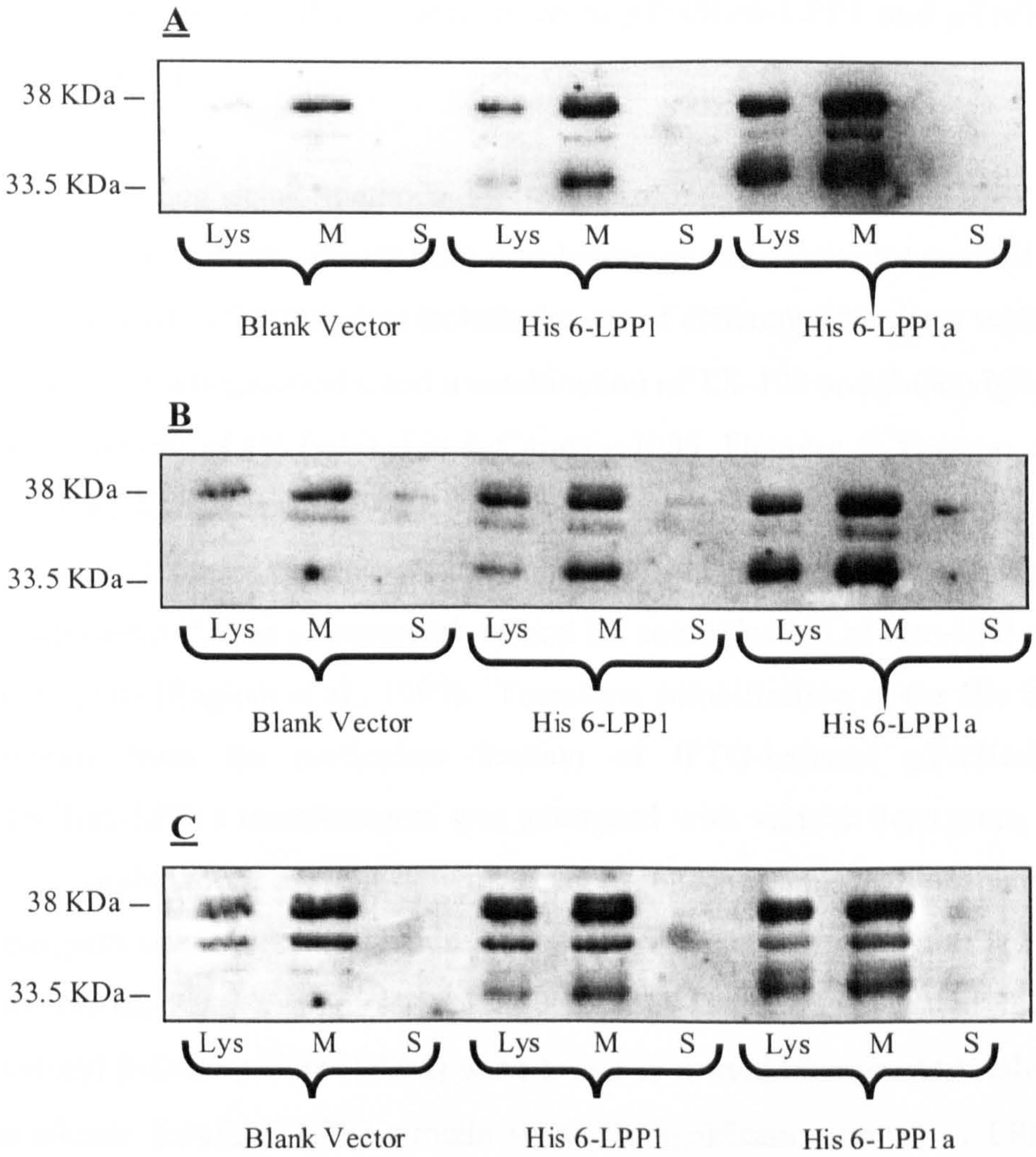


Figure 3.10: Subcellular Localisation of His 6-LPP fusion proteins when expressed in pTrcHis 6-LPP1 and pTrcHis 6-LPP1a transformed *E-coli*.

The expression of His 6-LPP1 and His 6-LPP1a was induced in *E-coli* transformants as described in (2.7.5.). Soluble and particulate fractions were prepared as described in 2.7.6. Samples were collected and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. Proteins were detected by Western blot analysis using **A** INDIA^{TM}HisProbe-HRP (Pierce), **B** anti-LPP1/LPP1a antibody and **C** anti-catalytic domain antibody as described in 2.4.7.and 2.4.8. Positions of molecular weight markers are indicated. This is a representative result of an experiment performed at least three times. **Lys**= Lysate, **M**= Membrane fraction, **S**= Soluble fraction.

3.2.3. Solubilisation of LPP activity from pTrcHis6-LPP1 and pTrcHis6-LPP1a transformants

Several studies detail methods for solubilisation of either total LPP activity or specific LPP isoform activity from membranes of various tissues and cell types. The methods used in these studies include the use of different detergents such as TX-100, CHAPS, β-Octylglucoside, and a combination of TX-100 and β-Octylglucoside all at concentrations of 1% (w/v) (Lin & Carman, 1989, Fleming & Yeaman, 1995, Kai et al., 1996, Kanoh et al., 1992). As an alternative to these detergents, English and colleagues reported the use of phosphonate-1, which is a non-hydrolysable analogue of dioctanoyl PA, as a successful method for solubilisation of *ecto*-LPP activity from neutrophils (English et al., 1997). Therefore, solubilisation of the His 6-LPP fusion proteins from the particulate fraction of IPTG-induced pTrcHis6-LPP1 and pTrcHis6-LPP1a transformants was attempted with various detergents, either alone or in combination, as well as with several concentrations of Phosphonate-1. The detergents used were chosen due to their differing properties as detailed in Table 2.1 (see section 2.7.7.). Figures 3.11 to 3.14, and Table 3.2 show that TX-100 and n-Dodecyl β-D-maltoside (DDM) were found to provide the greatest solubilisation of membrane localised LPP1 protein where a significant amount of LPP was being solubilised. Similar results were obtained for LPP1a (data not shown). Activity measurements confirmed measurable LPP activity in the TX-100 solubilised fractions with around 12+/-2% of LPP1 and 14+/-5% of LPP1a activity from the particulate fraction routinely being solubilised (Fig. 3.13). However, n-Dodecyl β-D-maltoside (DDM) was found to completely inhibit the LPP activity which is in contrast to the results of Roberts et al. (1998) who showed DDM to have no effect on LPP activity (results not shown).

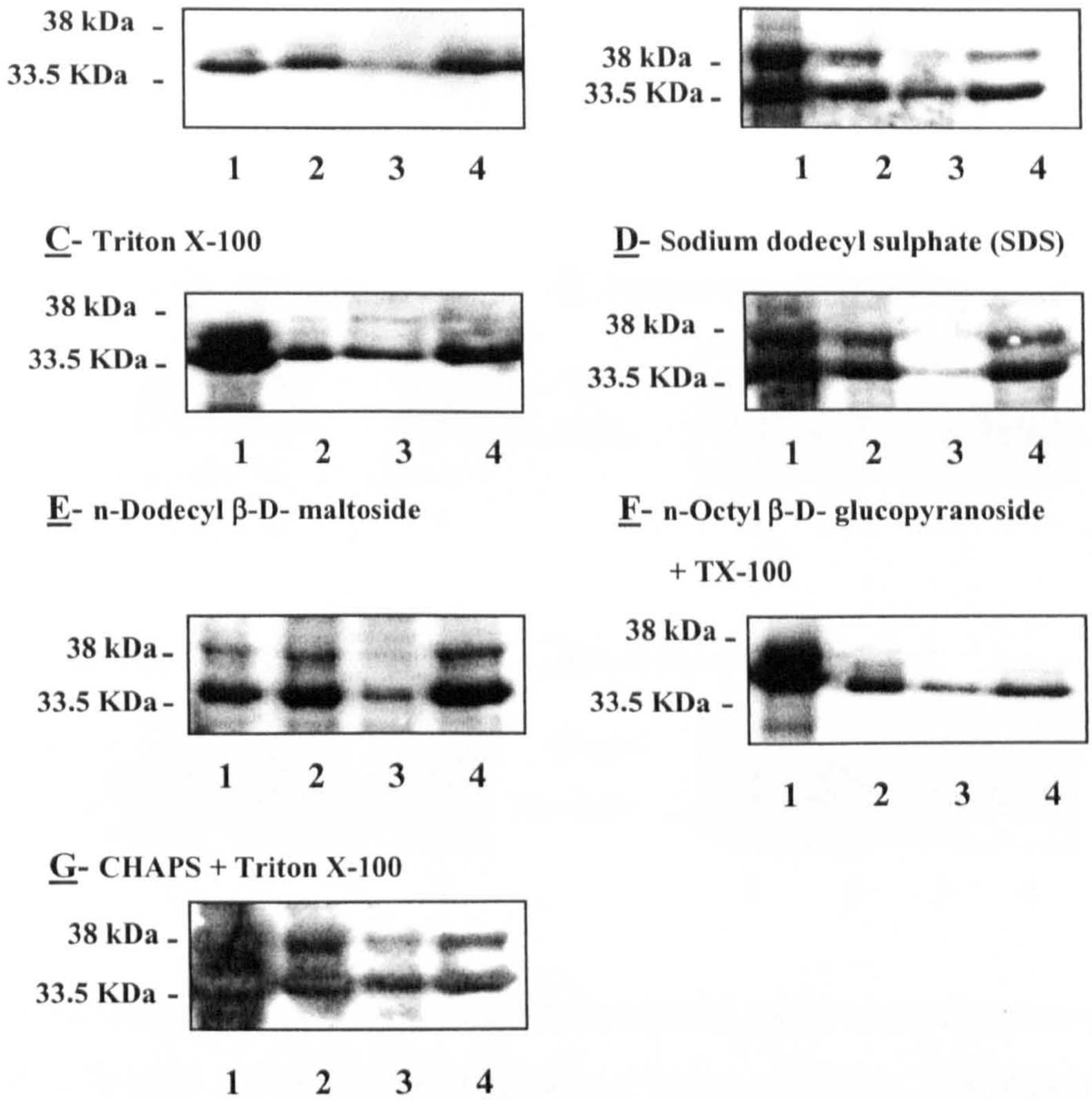


Figure 3.11: Solubilisation of His 6-LPP1 from the particulate fraction of IPTG-induced pTrcHis 6-LPP1 transformants using various detergents.

pTrcHis 6-LPP1 transformants, induced with 1mM IPTG for 3hrs, were lysed and a membrane fraction prepared (2.7.6). The membrane fraction was incubated for 2 hours at 4°C, in a solution containing Lysis buffer plus the appropriate detergent or combination of detergents. All detergents were added at 1% (w/v) or (v/v). Samples were collected and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. Solubilisation was assessed by Western blot analysis using INDIA^{TM}HisProbe-HRP of **1**-lysates, **2**-membranes, **3**-detergent solubilised membranes, and **4**-detergent insoluble membrane fraction (2.4.7). Similar results were obtained for LPP1a (data not shown). This is a representative result from an experiment performed at least three times. Positions of molecular weight markers are indicated.

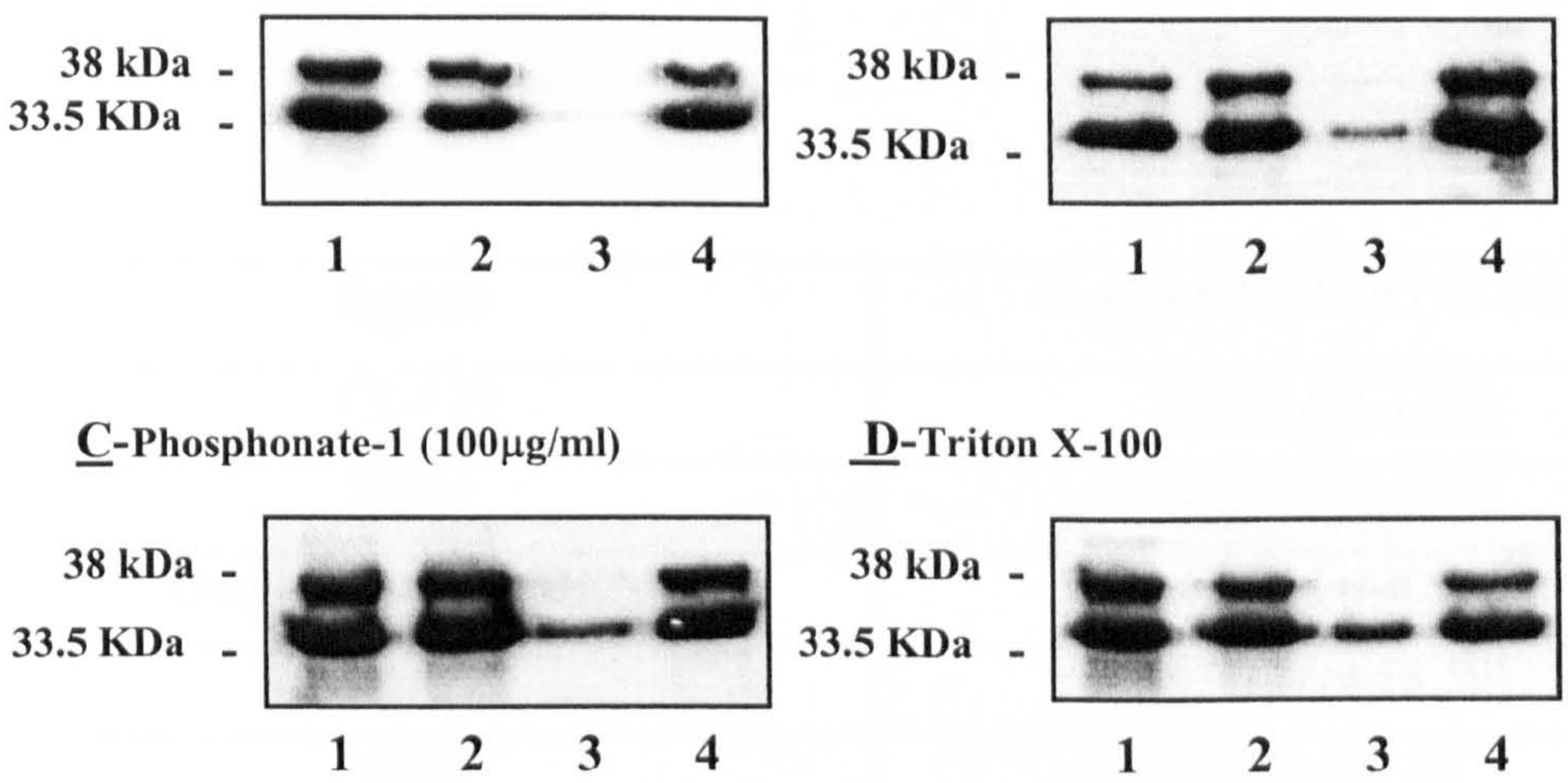


Figure 3.12: Solubilisation of His 6-LPP1 from the particulate fraction of IPTG-induced pTrcHis 6-LPP1 transformants using various concentrations of Phosphonate-1.

pTrcHis 6-LPP1 transformants, induced with 1mM IPTG for 3hrs, were lysed and a membrane fraction prepared (2.7.6). The membrane fraction was incubated for 2 hours at 4°C, in a solution containing Lysis buffer plus 10, 50, and 100μg/ml of Phosphonate-1 or TX-100 at 1% (v/v). Samples were collected and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. Solubilisation was assessed by Western blot analysis using INDIA^{TM}HisProbe-HRP of **1**-lysates, **2**-membranes, **3**-phosphonate-1 solubilised membranes, and **4**-phosphonate-1 insoluble membrane fraction (2.4.7). Similar results were obtained for LPP1a (data not shown). This is a representative result from an experiment performed at least three times. Positions of molecular weight markers are indicated.

Agent	% solubilisation (+/- SEM)
TX-100	**79.32 (+/-1.09)**
DDM	46.07 (+/-1.99)
Octyl-glucopyranoside	45.86 (+/-6.37)
CHAPS	53.07 (+/-13.59)
SDS	22 (+/-4.78)
Octyl-glucopyranoside + TX-100	48.69 (+/-1.08)
CHAPS + TX-100	70.79 (+/-3.77)
Phosphonate-1 (10μg/ml)	5.63 (+/-1.98)
Phosphonate-1 (50μg/ml)	32.07 (+/-1.77)
Phosphonate-1 (100μg/ml)	46.14 (+/-1.04)

Table 3.1: Percentage solubilisation of His 6-LPP1 fusion protein from the particulate fraction using various detergents and several concentrations of Phosphonate-1.

Values shown are percentage of the membrane fraction solubilised as assessed by densitometry using Scion Image© software version 3b.

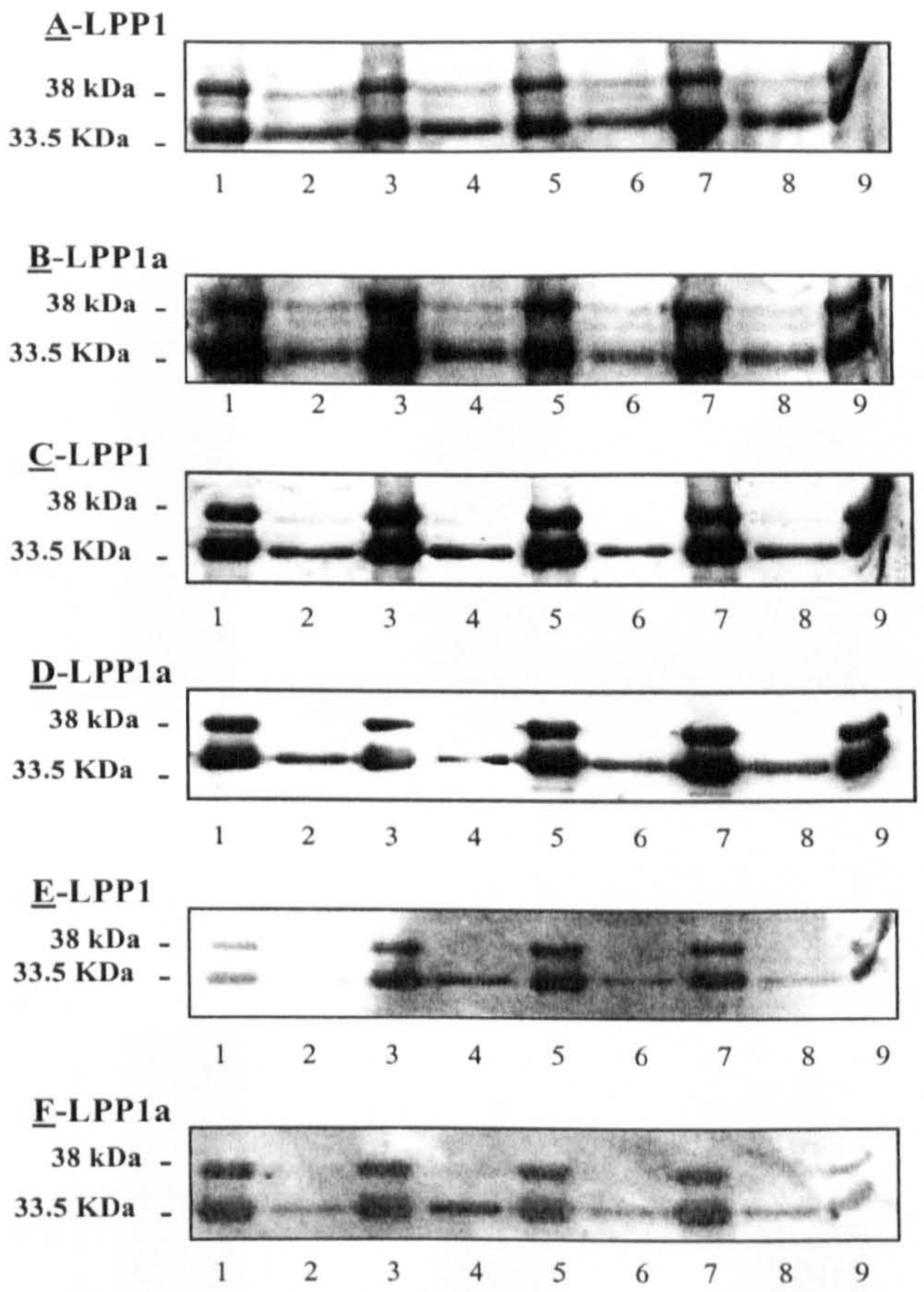
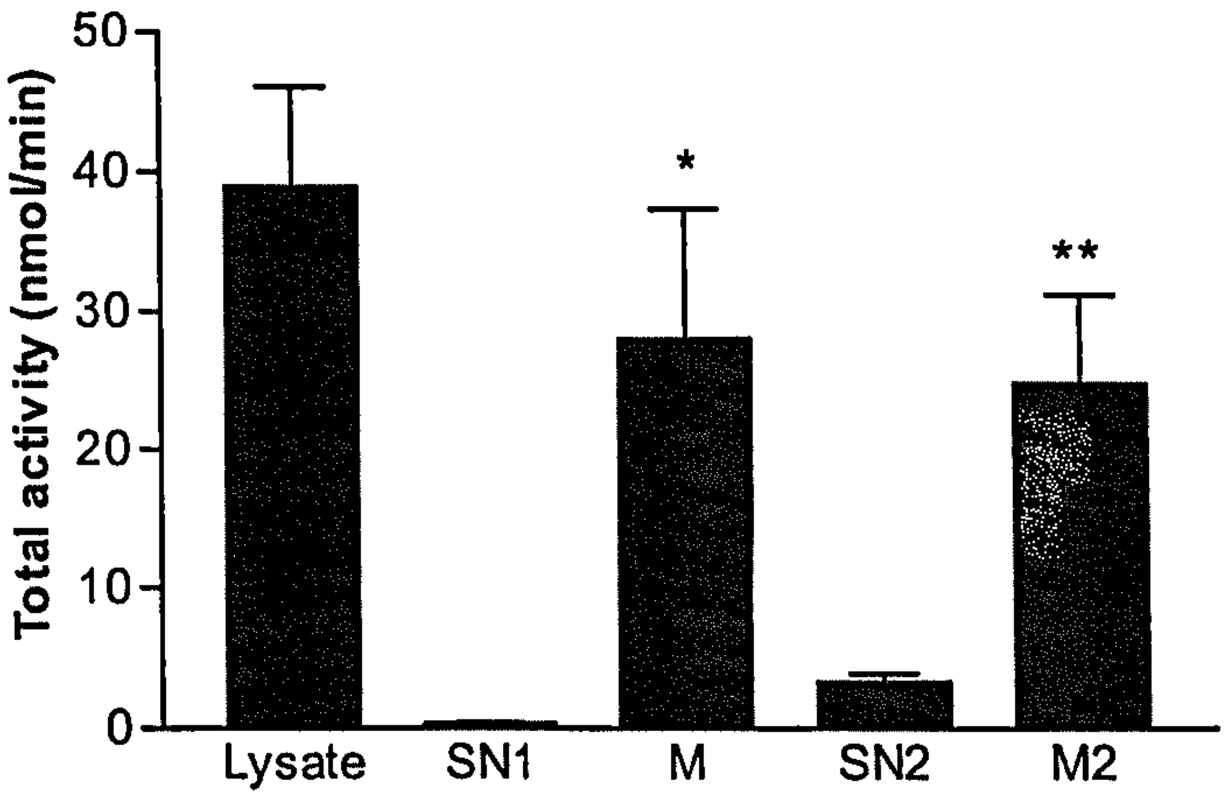


Figure 3.13: Timecourse of solubilisation of His 6-LPP1 and His 6-LPP1a from the particulate fraction of IPTG-induced pTrcHis 6-LPP1 and pTrcHis 6-LPP1a transformants using TX-100.

pTrcHis 6-LPP1 and pTrcHis 6-LPP1a transformants, induced with 1mM IPTG for 3hrs, were lysed and a membrane fraction prepared (2.7.6). The membrane fraction was incubated in a solution containing Lysis buffer plus 1% TX-100 for 1 hour, 2 hours, 3 hours or overnight (O/N) at 4°C. Samples were collected and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. Solubilisation was assessed by Western blot analysis using **A/B**-INDIA^{TM}HisProbe-HRP – Pierce, **C/D**-anti-LPP1/LPP1a antibody and **E/F**- anti-LPP catalytic domain (2.4.7.-2.4.8.). This is a representative result from an experiment performed at least three times. Positions of molecular weight markers are indicated. **1**-Lysate, **2**-1 hour TX-100 solubilised, **3**-1 hour TX-100 insoluble, **4**-2 hour TX-100 solubilised, **5**-2 hour TX-100 insoluble, **6**-3 hour TX-100 solubilised, **7**-3 hour TX-100 insoluble, **8**-O/N TX-100 solubilised, **9**-O/N TX-100 insoluble.

A



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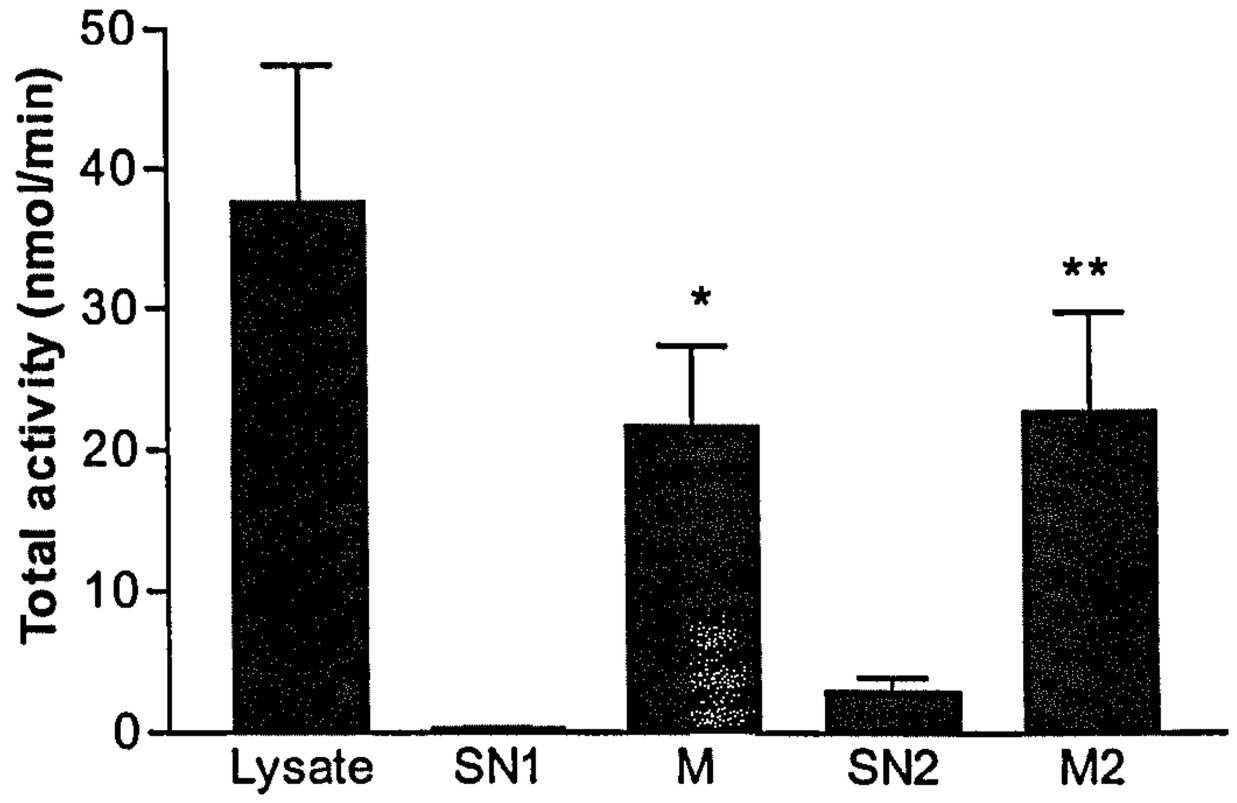


Figure 3.14: LPP activity measurements from fractions prepared from IPTG-induced pTrcHis 6-LPP1 and pTrcHis 6-LPP1a transformants following subcellular fractionation and solubilisation of the particulate fraction with 1% TX-100 for 2 hours.

LPP activity was assayed as described in 2.6.1. using L = total lysates, SN1 = Soluble fraction, M = membrane fraction, SN2 = TX-100 solubilised fraction, M2 = TX-100 insoluble fraction, from IPTG-induced **A** pTrcHIS/LPP1 and **B** pTrcHIS/LPP1a induced transformants. Results are expressed in nmoles Pi/min/mg protein (mean +/- SEM, n =3). * $p<0.05$: Students T-test compared to SN1; ** $p<0.05$: Students T-test compared to SN2. This is a representative result of an experiment performed at least three times.

3.2.4. Initial purification of His 6-LPP1 and His 6-LPP1a by immobilised metal affinity chromatography

The TX-100 solubilised extract of membranes derived from IPTG-induced pTrcHis 6-LPP1 and pTrcHis 6-LPP1a transformants was added to a Nickel (Ni^{2+}) affinity matrix. As described in section 2.7.8., the hexa-histidine tag is expected to form bonds with the Ni^{2+} matrix and figures 3.15 and 3.16 demonstrate this to have occurred as evidenced by the absence of reactive bands around the predicted molecular weight of the LPP fusion proteins in the flow through fractions from the affinity column.

The bound fusion proteins were then eluted with increasing concentrations of Imidazole (10mM, 100mM and 500mM). Imidazole is of very similar chemical structure to histidine and acts to displace bound histidine residues from the Ni^{2+} matrix through competition. A sample from each elution fraction was analysed by SDS-PAGE (section 2.4.) and the presence of His 6-LPP1 (Figure 3.15) and His 6-LPP1a (Figure 3.16) established through Western blotting initially using the HisProbe. Blots were successively stripped (section 2.4.6.) and reprobed with the anti-LPP1/LPP1a antibody and the anti-LPP catalytic domain antibody. The results in figures 3.15 and 3.16 demonstrate elution of the His 6-LPP1 and His 6-LPP1a fusion proteins in fractions 2 to 5 of the 10mM elution step. The 10mM elution is designed to be a wash step to remove weakly associated, non-specifically bound proteins and so suggests a weak association of the recombinant fusion proteins. Additionally, figures 3.15 and 3.16 also demonstrate a smearing of the recombinant fusion proteins from the column in all of the subsequent 100mM and 500mM Imidazole elutions rather than an expected optimal, well-defined peak of elution. The apparent weak association of a portion of the His 6 LPP1 and LPP1a fusion proteins and the subsequent smearing observed suggests that the fusion proteins are binding to the Ni^{2+} affinity matrix with varying degrees of strength. This demonstrates that the hexa-histidine tag is not very effective for affinity purification of these particular fusion proteins. In addition to Western blot analysis, LPP activity

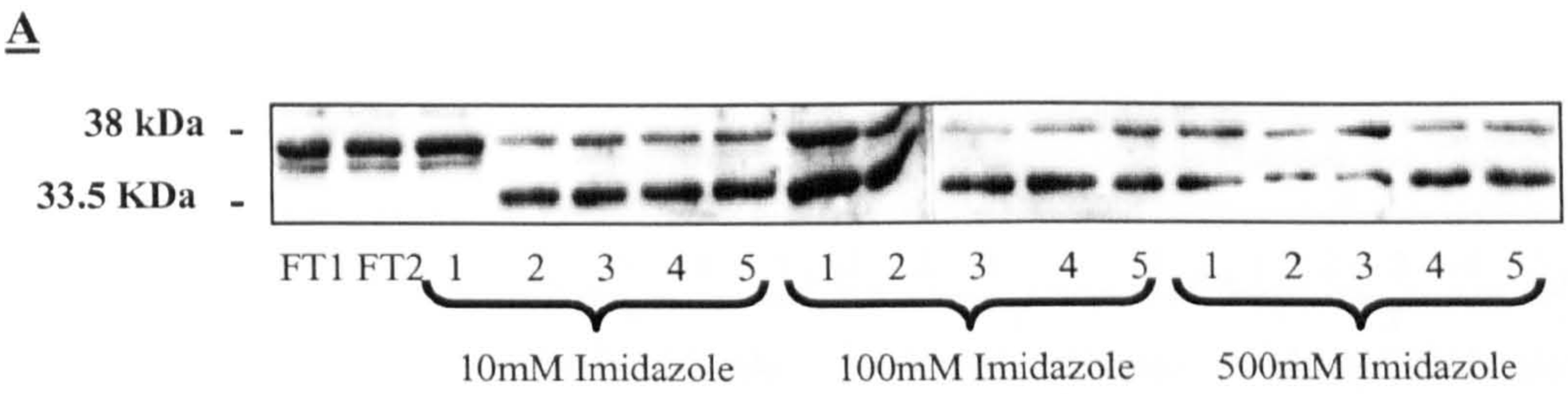


Figure 3.15: Western blot analysis of detergent solubilised His 6-LPP1 following immobilised metal affinity chromatography (IMAC)

Detergent solubilised His 6-LPP1 was subjected to IMAC on a Ni^{2+} affinity resin. The flow through was collected as the sample was applied and the column eluted with 10, 100, and 500mM imidazole and five 2ml fractions of each collected. Fractions were numbered 1 to 5 and equalised for protein prior to SDS-PAGE analysis (2.4.-2.4.3.). Western blot analysis was then conducted using **A**-INDIA^{TM}HisProbe-HRP – Pierce, **B**-anti-LPP1/LPP1a antibody and **C**- anti-LPP catalytic domain (2.4.7.-2.4.8.). This is a representative result of an experiment performed at least three times. Positions of molecular weight markers are indicated.

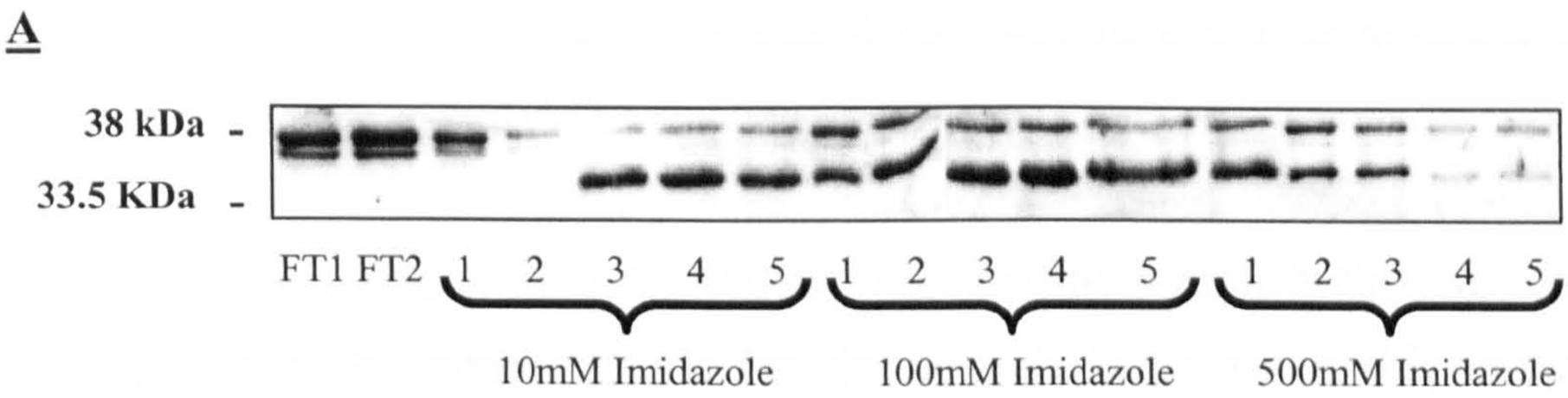


Figure 3.16: Western blot analysis of detergent solubilised His 6-LPP1a following immobilised metal affinity chromatography (IMAC)

Detergent solubilised His 6-LPP1a was subjected to IMAC on a Ni^{2+} affinity resin. The flow through was collected as the sample was applied and the column was eluted with 10, 100, and 500mM imidazole and five, 2ml fractions of each collected. Fractions were numbered 1 to 5 and equalised for protein prior to SDS-PAGE analysis (2.4.-2.4.3.). Western blot analysis was then conducted using **A**-INDIA™HisProbe-HRP – Pierce, **B**-anti-LPP1/LPP1a antibody and **C**- anti-LPP catalytic domain (2.4.7.-2.4.8.). This is a representative result of an experiment performed at least three times. Positions of molecular weight markers are indicated.

measurements were conducted on the eluted fractions. However, no measurable LPP activity could be detected in any fraction.

In summary, the results from the IMAC show that the hexa-histidine tag achieved binding to the Ni^{2+}-affinity matrix but that this binding was variable in strength leading to smearing from the column. The hexa-histidine tag therefore, did not provide an efficient means of capturing the recombinant LPP isoforms via IMAC. Therefore, an alternative strategy was adopted whereby decahistidine-tagged fusion proteins, pTrcHis 10-LPP1 and pTrcHis 10-LPP1a, were generated.

3.2.5. Expression of His 10-LPP1 and His 10-LPP1a in *E. coli*

Modification of the pTrcHis B vector was made to encode the addition of ten histidine residues to the N-terminus of the LPP enzymes, instead of six. A restriction site flanked deca-histidine amplicon was generated by PCR. This was then digested along with the pTrcHis6 B vector and the two fragments ligated together to produce a deca-histidine pTrcHis B (pTrcHis10) vector (Figure 3.17). LPP1 and LPP1a inserts were then cloned into pTrcHis10 vector to produce pTrcHis10-LPP1 and pTrcHis10-LPP1a (Tate, R., unpublished data). *E. coli* (TOP-10) were subsequently transformed separately with pTrcHis10, pTrcHis10-LPP1 and pTrcHis10-LPP1a. Cultures were prepared as described previously (2.7.2.-2.7.5.) and expression of His 10-LPP1 and His 10-LPP1a fusion proteins and their subsequent purification monitored by Western blot analysis and LPP activity measurements.

Expression of both His 10-LPP1 and His 10-LPP1a in the *E. coli* cultures was again, initially confirmed by Coomasie-blue staining of SDS-PAGE gels. However, as with the His 6-LPP experiments, induced fusion proteins could not be measurably detected on Coomassie-blue stained gels (data not shown) and represented around an estimated 1% of total *E.coli* protein. Therefore, Western blot analysis using the anti-LPP1/LPP1a antibody was conducted on lysates derived from IPTG-induced pTrcHis10-LPP1 and pTrcHis10-LPP1a transformants (figure 3.18). As with the His 6-LPP fusion proteins, it was found that incubation of the pTrcHis10-LPP1 and

pTrcHis10-LPP1a transformants with 1mM IPTG for 3 hours was the most efficient method of induction of expression for His10-LPP1 and His10-LPP1a (Figure 3.18 and results not shown). Thus, a protein around the expected molecular weight of the His 10-LPP1 and His 10-LPP1a fusion proteins (36.5kDa – LPP1 and 36.46kDa – LPP1a) was detected in bacterial lysates induced with 1mM IPTG but not in pre-induction samples probed with the anti-LPP1/LPP1a antibody. Furthermore, LPP activity measurements conducted using lysates derived from IPTG induced pTrcHis10-LPP1 and pTrcHis10-LPP1a transformants showed significantly higher activity compared to the pTrcHis10, blank vector transformant (Figure 3.19). The His 10-LPP1 transformants were found to exhibit a 8.2+/-1.2 fold increase in activity and the His 10-LPP1a transformants a 4.2+/-0.8 fold in activity, compared to blank vector transformants.

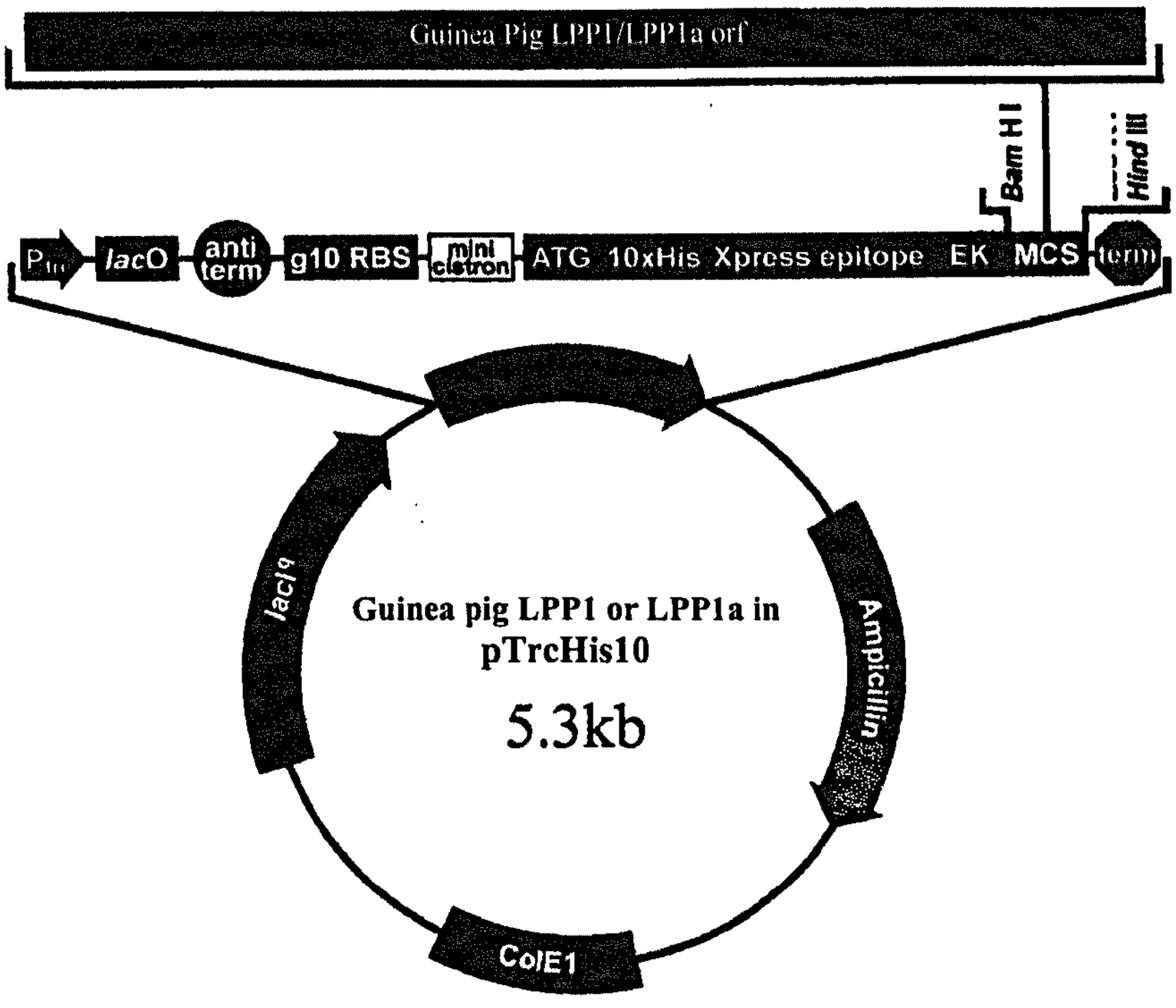


Figure 3.17: pTrcHis10 vector construct plus gpLPP1 or gpLPP1a insert

The open reading frame of guinea pig LPP1 or LPP1a (GenBank Accession No. AF088283 and AF088284 respectively) was inserted into the pTrcHis10 vector at the Bam H1 and Hind III sites within the multiple cloning site (R.J.Tate, Molecular Biology Facility, University of Strathclyde). The pTrcHis10-LPP1 and pTrcHis10-LPP1a constructs were designed to express N-terminal hexa-histidine tagged LPP1 and LPP1a fusion proteins upon induction of transformed *E. coli* with IPTG.

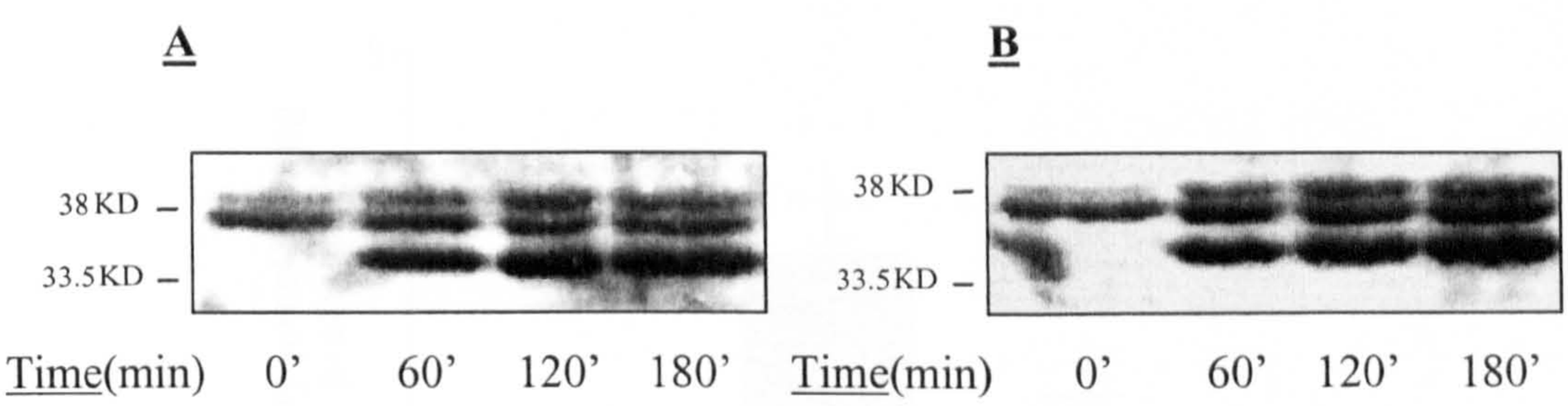


Figure 3.18: Western blot analysis of a timecourse of induction of His 10-LPP1 and His 10-LPP1a expression.

The expression of His 10-LPP1 (**A**) and His 10-LPP1a (**B**) was induced in *E-coli* cultures that had been separately transformed with pTrcHis10-LPP1 or pTrcHis10-LPP1a using 1mM IPTG and a time course performed as described in 2.7.5. Samples were collected at each time point and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. His 10-LPP1 and His 10-LPP1a were detected by Western blot analysis using the anti-LPP1/LPP1a antibody as described in 2.4.8. Positions of molecular weight markers are indicated. This is a representative result of an experiment performed at least three times.

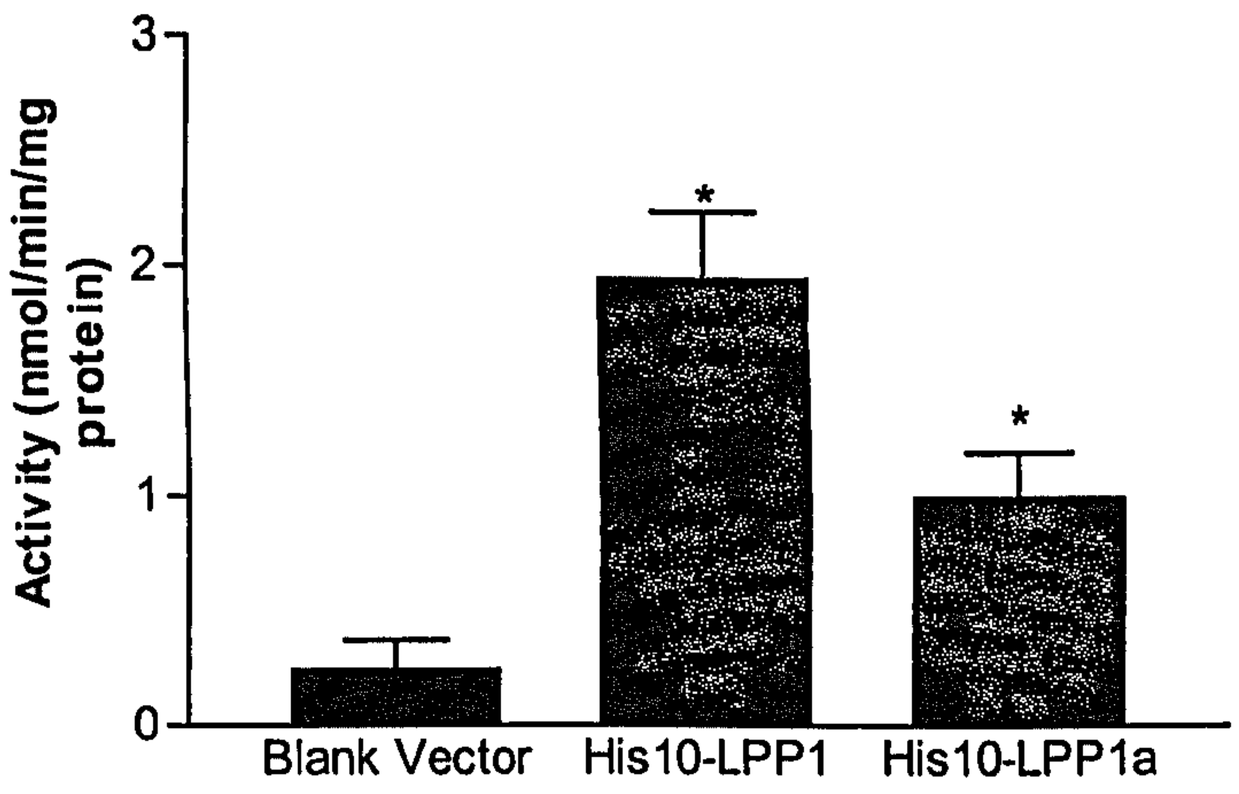


Figure 3.19: LPP activity measurements of lysates derived from IPTG-induced *E.coli* cultures after transformation with pTrcHis10 alone, pTrcHis10-LPP1 or pTrcHis10-LPP1a.

LPP activity was assayed against dioleoyl PA (500μM) as described in 2.6.1. using total lysates from each induced *E-coli* culture at protein concentrations between 7 and 10μg/assay. Results are expressed in nmoles Pi/min/mg protein (mean +/- SEM, n =3). * $p < 0.05$: Students t-test versus blank vector lysate.

3.2.6. Subcellular localisation of the recombinant His 10-LPP1 and His 10-LPP1a

Lysates derived from IPTG-induced pTrcHis10-LPP1 and pTrcHis10-LPP1a transformants were subjected to subcellular fractionation (2.7.6.) to establish the distribution of the expressed His 10-LPP1 and His 10-LPP1a fusion proteins between the soluble and membrane fractions. Samples were collected and analysed by Western blotting and LPP activity measurements.

Figures 3.20 A, 3.20 B and 3.20 C show that His 10-LPP1 and His 10-LPP1a fusion proteins were both detected exclusively in the particulate fraction when analysed by Western blotting using the HisProbe, the anti-LPP1/LPP1a antibody and the anti-catalytic domain antibody respectively. Measurement of LPP activity indicated that as described for the His 6-LPP fusion proteins, virtually all LPP activity was associated with the particulate fraction with the distribution between the particulate and soluble fractions routinely 99%:1%, respectively for the His 10-LPP1 and His 10-LPP1a transformants respectively (Figure 3.21).

These results are similar to those obtained with the His 6-LPP1 and His 6-LPP1a fusion proteins and indicate that the extension of the N-terminal poly-histidine tag has no effect on the subcellular distribution of the expressed fusion proteins.

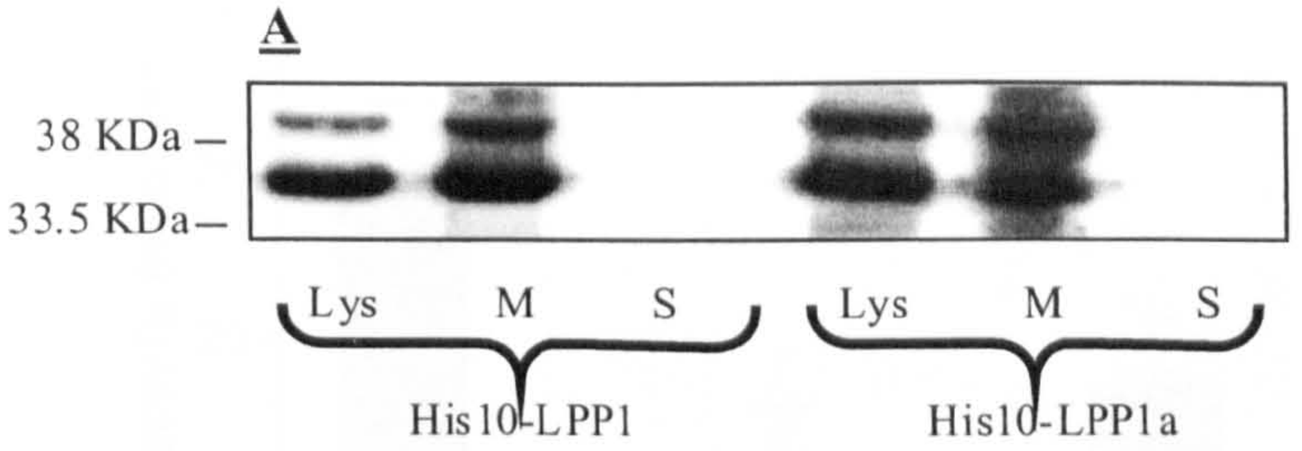
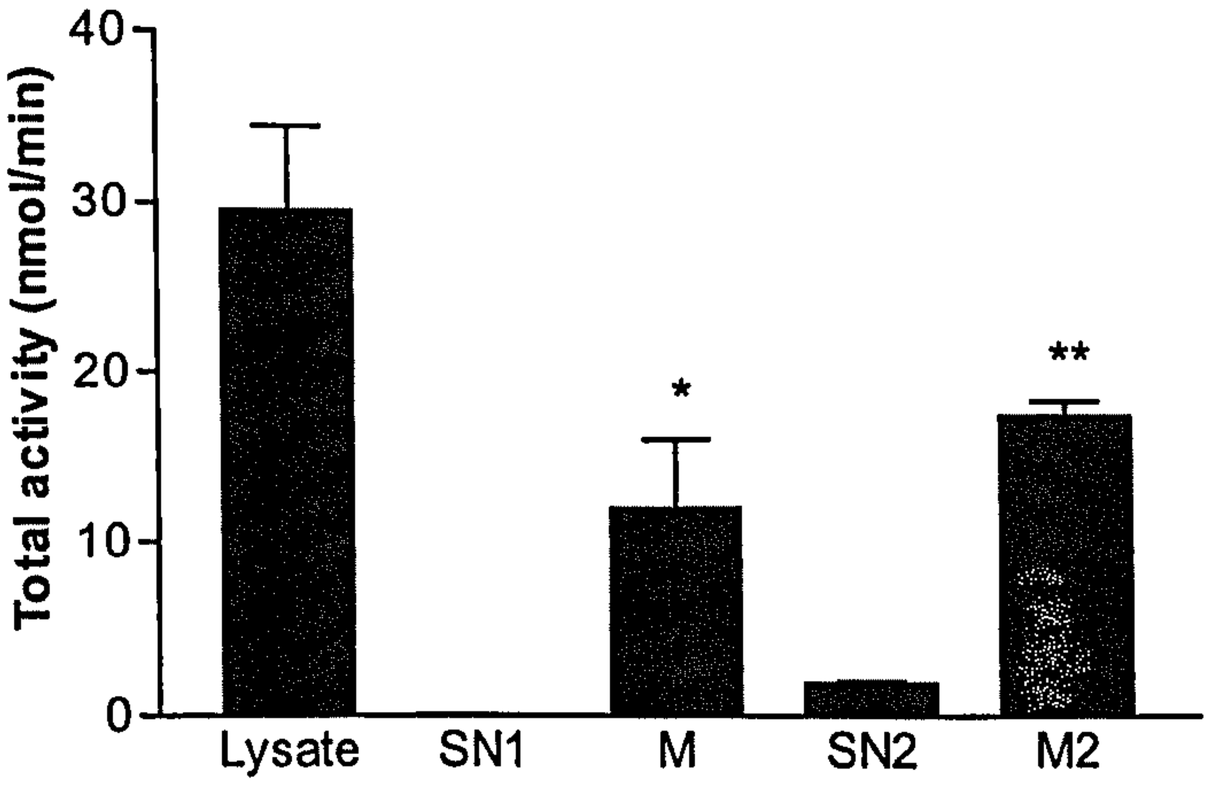


Figure 3.20: Subcellular Localisation of the His 10-LPP1 and His 10-LPP1a fusion proteins when expressed in pTrcHis10-LPP1 and pTrcHis10-LPP1a transformed, *E-coli*.

The expression of His 10-LPP1 and His 10-LPP1a was induced in *E-coli* transformants as described in 2.7.5. Soluble and particulate fractions were prepared as described in 2.7.6. Samples were equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. His 10-LPP1 and His 10-LPP1a were detected by Western blot analysis using **A** INDIA^{TM}HisProbe-HRP, **B** anti-LPP1/LPP1a antibody and **C** anti-LPP catalytic domain antibody as described in 2.4.7.-2.4.8. Positions of molecular weight markers are indicated. This is a representative result of an experiment performed at least three times. **Lys**= Lysate, **M**= Membrane fraction, **S**= Soluble fraction.

A



B

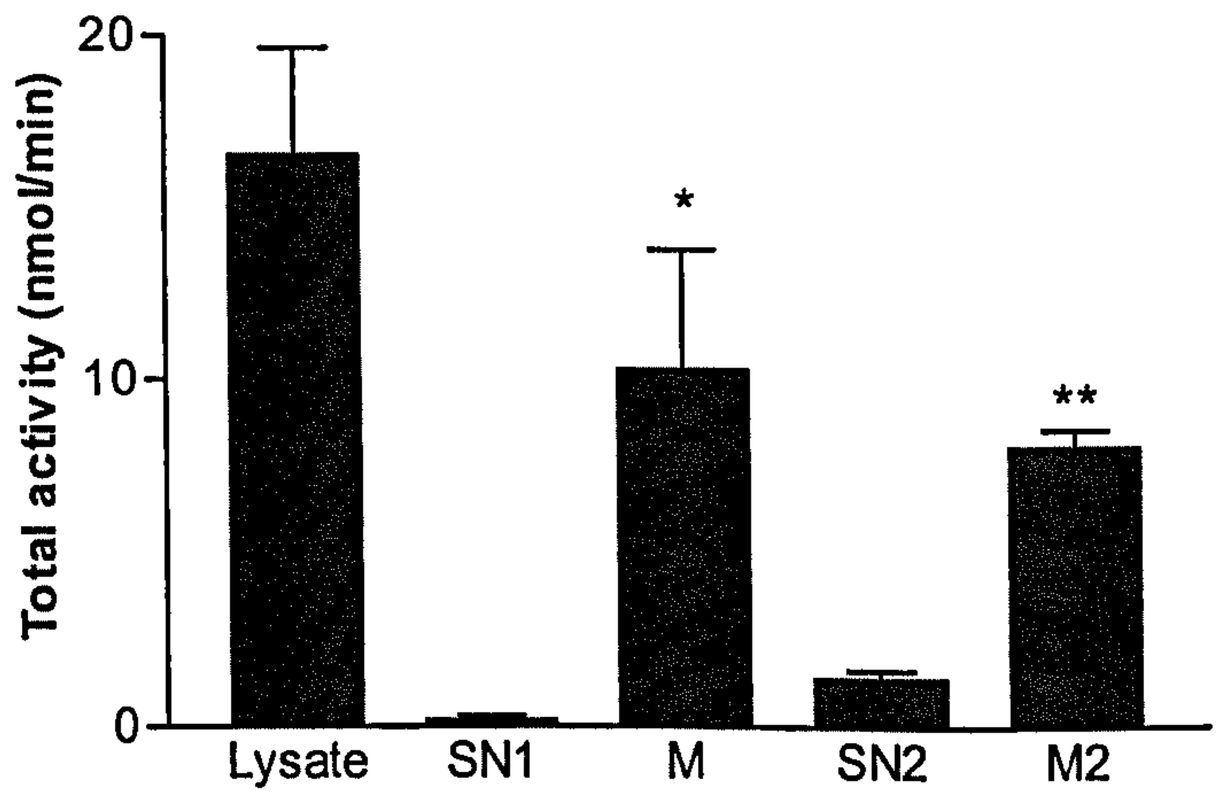


Figure 3.21: LPP activity measurements from fractions prepared from IPTG-induced pTrcHis10-LPP1 and pTrcHis10-LPP1a transformants following subcellular fractionation and solubilisation of the particulate fraction with 1% TX-100 for 2 hours.

LPP activity was assayed as described in 2.6. using Lysates, SN1 = Soluble fraction, M = membrane fraction, SN2 = TX-100 solubilised fraction, M2 = TX-100 insoluble fraction, from IPTG-induced **A** pTrcHis10-LPP1 and **B** pTrcHis10-LPP1a transformants. Results are expressed in nmoles Pi/min/mg protein (mean +/- SEM, n =3). * $p < 0.05$: Students t-test versus SN1, ** $p < 0.05$: Students t-test versus SN2. This is a representative result of an experiment performed at least three times.

3.2.7. Solubilisation of His 10-LPP1 and His 10-LPP1a from *E. coli* transformants

It was assumed that the His 10-LPP fusion proteins would display similar solubilisation properties to the His 6-LPP fusion proteins, since they differ only by 4 N-terminal histidine residues. Therefore, solubilisation of His 10-LPP1 and His 10-LPP1a from the particulate fraction of IPTG-induced pTrcHis10-LPP1 and pTrcHis10-LPP1a transformants was attempted using 1% TX-100 for 2 hours. This routinely gave a solubilisation of 46.07+/-2.29% of His 10-LPP1 and 44.1+/-5.25% of His 10-LPP1a membrane LPP protein as determined through densitometric analysis of Western blotting (results not shown). LPP activity measurements showed that TX-100 solubilisation recovered 16+/-1% of His 10-LPP1 activity and 14+/-2% of His 10-LPP1a activity from the membrane fractions (Figure 3.21).

3.2.8. Initial purification of His 10-LPP1 and His 10-LPP1a by Immobilised Metal Affinity Chromatography

As with the hexa-histidine LPP recombinant enzymes, the solubilised extract of membranes derived from IPTG-induced pTrcHis10-LPP1 and pTrcHis10-LPP1a transformants was added to a nickel affinity matrix and the recombinant fusion proteins eluted with increasing concentrations of imidazole (10mM, 100mM, 500mM). A sample from each fraction was analysed by SDS-PAGE (2.4.-2.4.3.) and Western blotting using the HisProbe, the anti-LPP1/LPP1a antibody and the anti-LPP catalytic domain antibody (2.4.7.-2.4.8.). Blots were successively stripped and reprobed with each of the antibodies after the HisProbe and figures 3.22 and 3.23 show the results obtained for His 10-LPP1 and His 10-LPP1a respectively. The results show that, in contrast to the results obtained with the His 6 fusion proteins, there was no reactivity in the five 10mM imidazole elutions as well as the flow through for both His 10-LPP1 and His 10-LPP1a. Furthermore, the results show decreased reactivity in the five 500mM imidazole elution fractions and a concentration of the His 10-LPP fusion proteins in the five 100mM imidazole elution

fractions compared to the results obtained using the His 6-LPP fusion proteins. From these results, it was anticipated that LPP activity would be detected in the 100mM elution fractions. However, this was not the case and no measurable LPP activity could be detected.

The results described above demonstrate that the deca-histidine tag provided a significant improvement in the effectiveness of the IMAC as a means of initial capture and purification of the LPP1 and LPP1a fusion proteins compared to the hexa-histidine tag. However, even though the LPP fusion proteins were being concentrated on the Ni^{2+}-affinity column, there was still no measurable activity. In a further attempt to achieve a purification protocol for the LPP1 and LPP1a isoforms, it was decided to conduct a preliminary investigation of an alternative vector system, the pMal-c2x vector system which is designed to express cloned genes in the soluble fraction of *E. coli*, with a Maltose binding protein tag as opposed to a histidine tag.

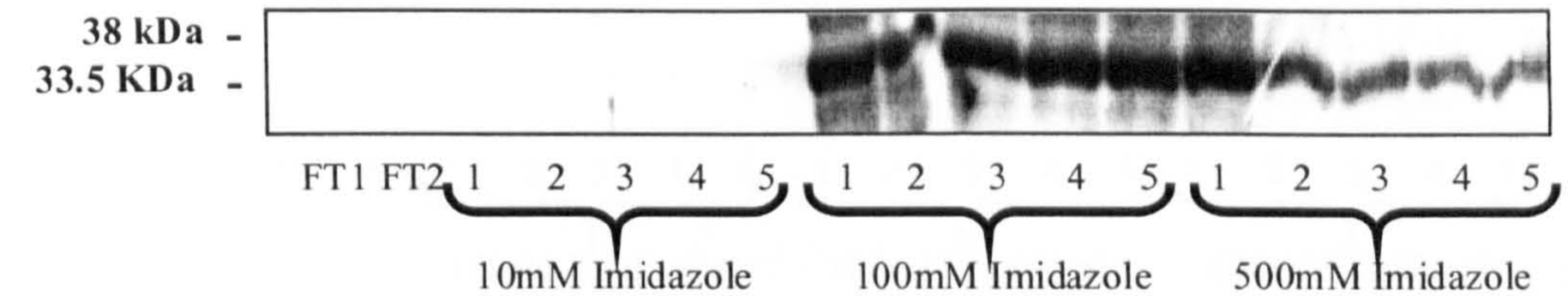
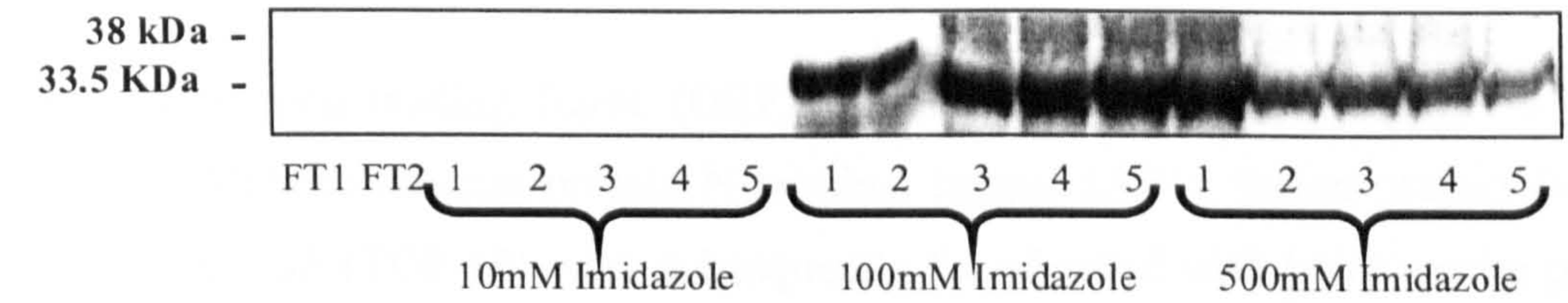


Figure 3.22: Western blot analysis of detergent solubilised His 10-LPP1 following immobilised metal affinity chromatography (IMAC)

Detergent solubilised His 10-LPP1 was subjected to IMAC on a Ni^{2+} affinity resin (2.7.8.). The flow through was collected and the column eluted with 10, 100 and 500mM imidazole and five 2ml fractions of each collected. Fractions were equalised for protein prior to SDS-PAGE analysis (2.4.-2.4.3.). Western blot analysis using **A** INDIA^{TM}HisProbe-HRP, **B** anti-LPP1/LPP1a antibody and **C**- anti-LPP catalytic domain antibody (2.4.7.-2.4.8). This is a representative result of an experiment performed at least three times. Positions of molecular weight markers are indicated.

A



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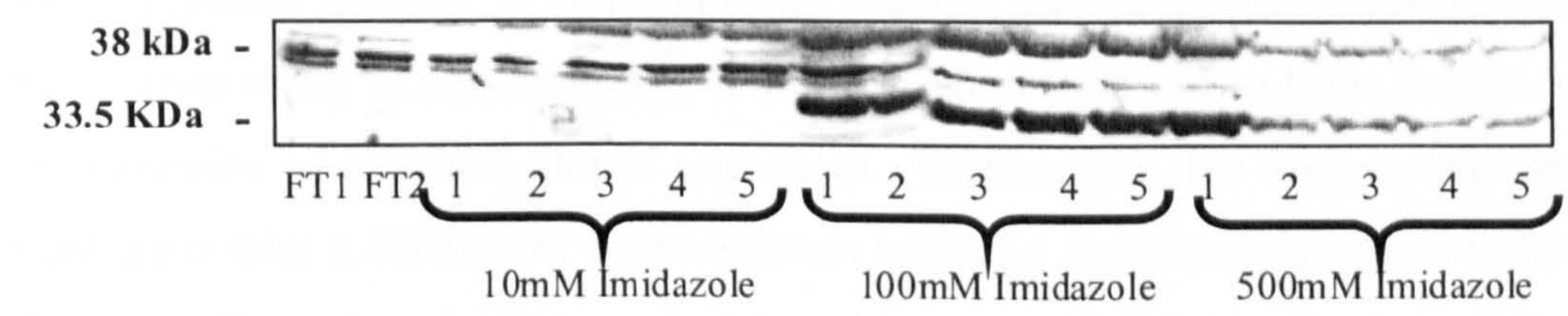

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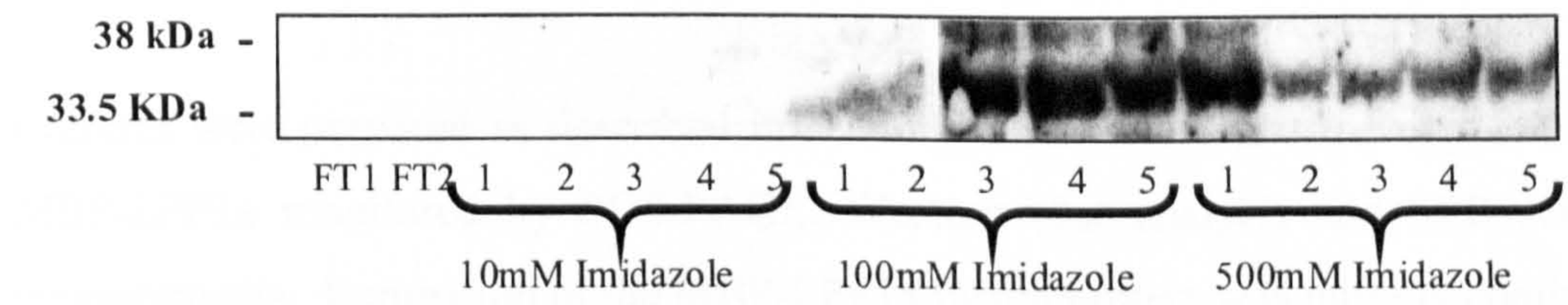


Figure 3.23: Western blot analysis of detergent solubilised His 10-LPP1a following immobilised metal affinity chromatography (IMAC)

Detergent solubilised His 10-LPP1a was subjected to IMAC on a Ni^{2+} affinity resin (2.7.8.). The flow through was collected and the column eluted with 10, 100 and 500mM imidazole and five 2ml fractions of each collected. Fractions were equalised for protein prior to SDS-PAGE analysis (2.4.-2.4.3.). Western blot analysis using **A** INDIA^{TM}HisProbe-HRP, **B** anti-LPP1/LPP1a antibody and **C**- anti-LPP catalytic domain antibody (2.4.7.-2.4.8). This is a representative result of an experiment performed at least three times. Positions of molecular weight markers are indicated.

3.2.9. Expression of LPP1a in *E. coli* with a Maltose binding protein tag

The LPP1a open reading frame (ORF) was inserted into the pMal-c2x vector to encode a Maltose binding protein N-terminal tagged LPP1a fusion protein (MBP-LPP1a). *E. coli* (TOP-10) were subsequently transformed with blank vector pMal-c2x and pMal-c2x-LPP1a. Figure 3.24 shows the pMal-c2x vector construct. The pMal-c2x vector includes the *malE* gene of *E. coli* which encodes the Maltose binding protein (MBP). In addition to the maltose binding protein allowing affinity purification using an amylose resin, it is an *E. coli* protein and so leads to high levels of expression of attached, cloned sequences. Furthermore, the vector possesses the *malE* gene with a deletion of the membrane targeting signal sequence and so has the ability to direct the expression of a cloned gene to the soluble fraction of *E. coli*. Thus, it was anticipated that the pMal-c2x-LPP1a construct may lead to the production of a cytosolic fusion protein with a molecular weight of 75.48kDa.

Cultures were prepared as described in sections 2.7.2.-2.7.5. and the expression of MBP-LPP1a monitored by SDS-PAGE, Western blot analysis and LPP activity measurements. Expression of the MBP-LPP1a fusion protein was initially confirmed through Coomasie blue staining of SDS-PAGE gels (2.4). Figure 3.25 shows that in contrast to the hexa- and deca-histidine tagged fusion proteins, Coomassie blue staining of SDS-PAGE gels detected a significant amount of induced MBP-LPP1a fusion protein of the correct molecular weight. This result was confirmed through Western blotting using the anti-Maltose binding protein antibody, anti-LPP1/LPP1a antibody and the anti-LPP catalytic domain antibody (data not shown). The results show that expression of MBP-LPP1a fusion protein was detected by 1 hour induction and increased to a maximal level by 3 hours induction. This level of induction was then maintained, even five hours post IPTG induction. LPP activity measurements were also conducted on lysates derived from IPTG-induced pMal-c2x and pMal-c2x-LPP1a transformants. LPP activity in the pMal-c2x-LPP1a transformants was significantly greater (1.77 +/-0.15) fold than in the blank vector transformants (Figure 3.26). The observed increase in LPP activity was significantly less than that

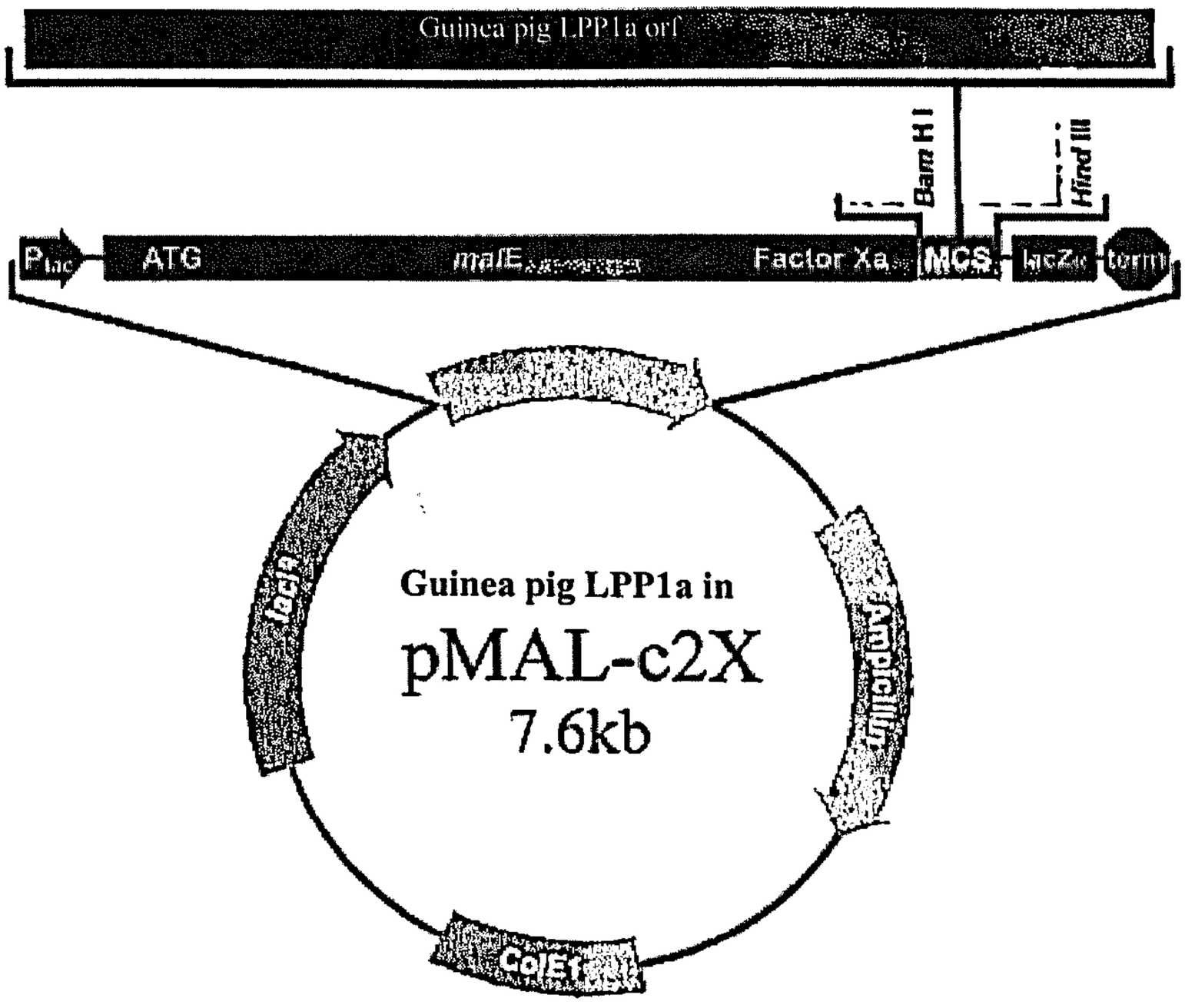


Figure 3.24: pMal-c2x vector construct plus gpLPP1a insert

The open reading frame of guinea pig LPP1a (GenBank Accession No. AF088284) was inserted into the pMal-c2x vector via the Bam H1 and Hind III sites within the multiple cloning site (R.J.Tate, Molecular Biology Facility, University of Strathclyde).. The pMal-c2x-LPP1a construct was designed to express N-terminal maltose binding protein tagged LPP1a upon induction of transformed *E. coli* with IPTG.

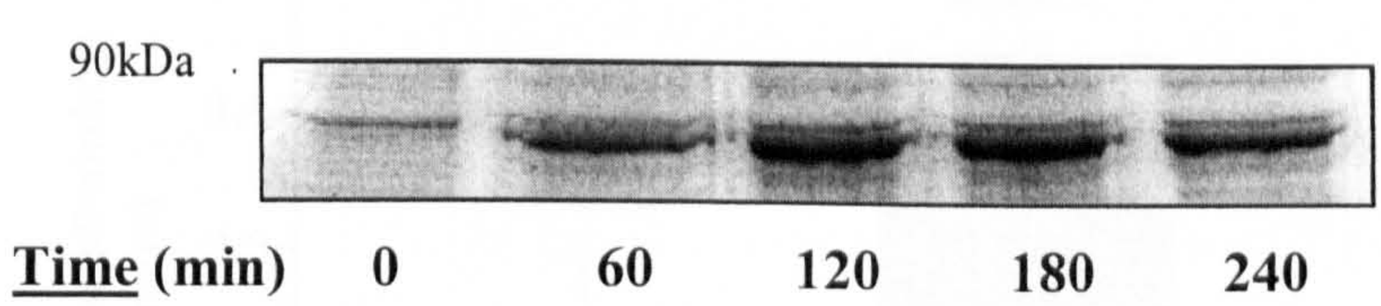


Figure 3.25: SDS-PAGE analysis of a timecourse of induction of MBP-LPP1a expression.

The expression of MBP-LPP1a was induced using 0.3mM IPTG in *E-coli* that had been transformed with pMal-c2x-LPP1a and a time course performed as described in 2.7.5. Samples were collected at each time point and equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. Proteins were detected by Coomassie blue staining as described in 2.4.4. Positions of molecular weight markers are indicated. This is a representative result of an experiment performed at least three times.

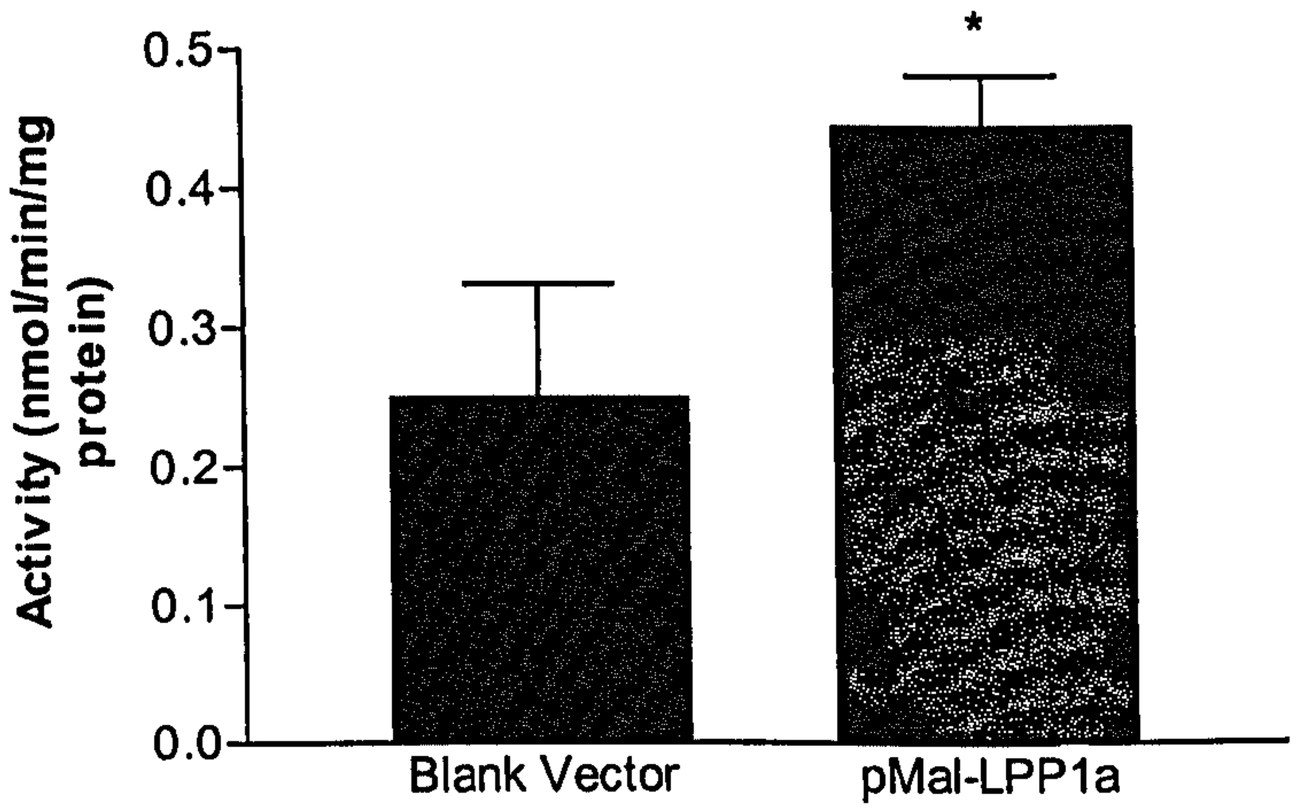


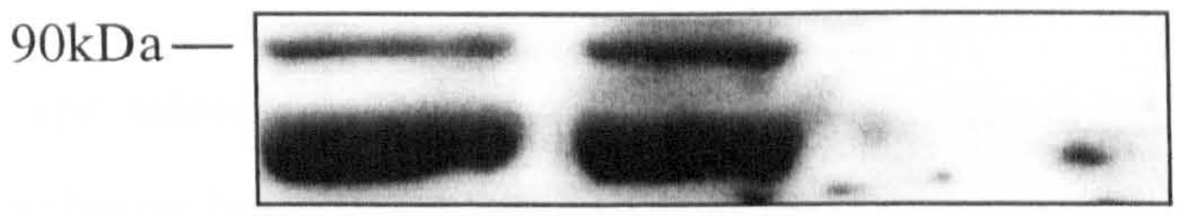
Figure 3.26: LPP activity measurements of lysates derived from IPTG-induced *E.coli* cultures after transformation with pMal-c2x alone or pMal-c2x-LPP1a.

LPP activity was assayed against dioleoyl PA (500μM) as described in 2.6.1. using total lysates from each induced *E-coli* culture at protein concentrations between 7 and 10μg/assay. Results are expressed in nmoles Pi/min/mg protein (mean +/- SEM, n =3). * $p < 0.05$: Students t-test versus blank vector lysate.

obtained with either the His6-LPP1/LPP1a, or His10-LPP1/LPP1a expression systems.

Lysates from the pMal-c2x-LPP1a transformants were then subjected to subcellular fractionation in order to establish the distribution of the MBP-LPP1a fusion protein between the soluble and membrane fractions and so confirm whether the pMal-c2x vector had led to expression of the fusion proteins in the soluble fraction. Figures 3.27A, 3.27B and 3.27C show that the MBP-LPP1a fusion protein was detected exclusively in the membrane fraction when analysed by Western bloting using the anti-MBP antibody, anti-LPP1/LPP1a antibody and the anti-LPP catalytic domain antibody respectively. This suggested that the pMal-c2x vector had not achieved expression of the MBP-LPP1a in the soluble fraction. However, it is possible that due to the high levels of expression which were obtained with the pMal-c2x vector system, the protein was expressed in inclusion bodies which are aggregates of denatured fusion protein expressed in the soluble fraction but which are collected following centrifugation. The purification protocol utilising the pMal-c2x vector system was therefore, found to lead to greater amounts of protein compared to the pTrcHis vectors but the fusion proteins were less active suggesting expression in the form of insoluble inclusion bodies.

A



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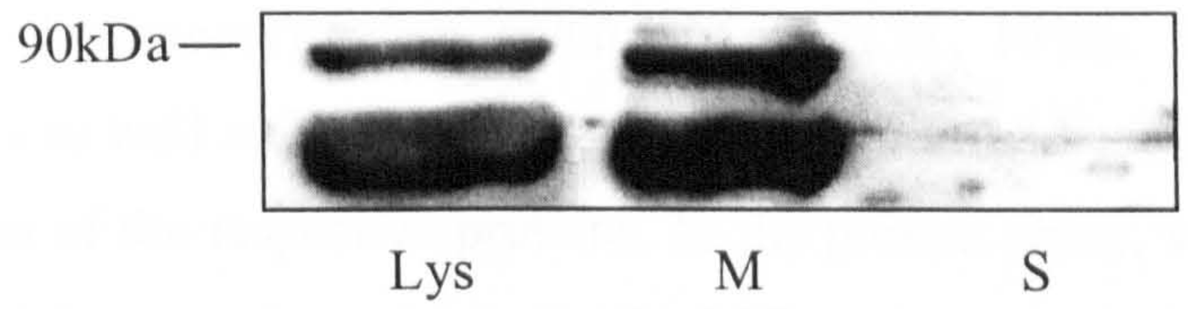

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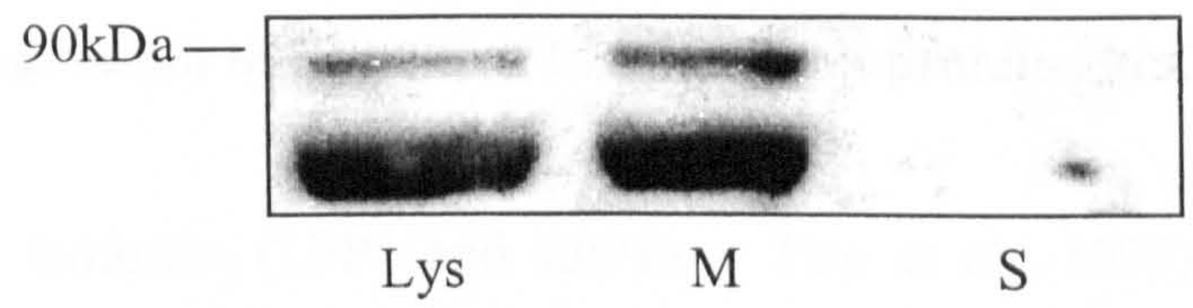


Figure 3.27: Subcellular localisation of MBP-LPP1a fusion proteins when expressed in pMal-c2x-LPP1a transformed, *E-coli*.

The expression of MBP-LPP1a was induced in *E-coli* transformants as described in 2.7.5. Soluble and particulate fractions were prepared as described in 2.7.6. Samples were equalised for protein prior to SDS-PAGE analysis as described in 2.4.-2.4.3. Proteins were detected by Western blot analysis using **A** Anti-Maltose binding protein antibody, **B** anti-LPP1/LPP1a antibody and **C** anti-LPP catalytic domain antibody as described in 2.4.7.-2.4.8. Positions of molecular weight markers are indicated. This is a representative result of an experiment performed at least three times.

Lys= Lysate

M= Membrane fraction

S= Soluble fraction.

3.3. Discussion

Bacterial expression systems have been successfully used to express and purify several membrane bound and integral membrane proteins such as the H^+/phosphate co transporter, Pho84p, from *Saccharomyces cerevisiae* (Fristedt et al., 1999), a rat neurotensin receptor (Tucker & Grisshammer, 1996), and the lactose transport protein of *Streptococcus thermophilus* (Knol et al., 1996). Furthermore, Knol and co-workers as well as Fristedt and colleagues utilised a hexa-histidine tag to achieve purification of the respective proteins. In the present study, we have established the first bacterial expression systems for the LPPs.

3.3.1. Expression of LPP1 and LPP1a fusion proteins in *Escherichia coli*

Two LPP isoforms (LPP1 and LPP1a – Tate et al., 1999) were cloned into the bacterial expression vector pTrcHis B and a modified form of pTrcHis B, namely pTrcHis 10, and His6- and His10-LPP1 and LPP1a fusion proteins expressed in *E. coli*. Additionally, LPP1a was cloned into the bacterial expression vector pMal-c2x before expression as a maltose binding protein (MBP) tagged fusion protein in *E. coli*. Notably, all of the LPP fusion proteins were absent from transformed *E. coli* grown in the absence of IPTG and from *E.coli* transformed with the vector alone. Furthermore, all fusion proteins were detected with an anti-LPP1/LPP1a antibody and an anti-LPP catalytic domain antibody. In addition to Western blot analysis, LPP activity measurements conducted on the lysates of IPTG-induced LPP1 and LPP1a transformants displayed significantly increased LPP activity compared with the blank vector transformants. The increases in activity observed compared to the blank vector transformants were substantially less in the pMal-c2x vector system compared to the pTrcHis vector system. An explanation for this is that the large MBP epitope tag, which is larger than the cloned LPP1a itself, may have caused misfolding of the cloned LPP1a or could be occluding the active site of LPP1a when expressed.

Growth conditions were manipulated in order to achieve the most efficient method of induction of the fusion proteins. However, it was found that altering the time of induction, or the concentration of IPTG used to induce expression had a minimal effect on the magnitude of expression, or the subcellular localisation of the fusion proteins. Of the fusion proteins expressed, only the MBP-LPP1a fusion protein was detected by Coomasie blue staining of SDS-PAGE gels (Figure 3.25). The inability to detect His6- or His10-fusion proteins by Coomasie blue staining suggests that lower levels of expression were achieved using these bacterial expression systems. In general, the low levels of expression of the fusion proteins may be because these particular eukaryotic proteins are toxic to the prokaryotic *E. coli* host.

A surprising finding during the study was that Western blot analysis revealed the induction of two histidine tagged products in the pTrcHis vector system. Whilst it is conceivable that only one of the two histidine tagged products is a LPP isoform, this is unlikely, as it would require an alteration of the vector during induction. Alternatively, both products could be LPP isoforms with the larger of the two products (approximately 38kDa and 39kDa for the His6-LPP1/1a and His10-LPP1/1a, respectively) having undergone some form of modification from the '*native*' forms (approximately 36kDa and 36.5kDa for the His6-LPP1/1a and His10-LPP1/1a, respectively). For example, even though it is generally believed that bacteria lack the ability to post-translationally modify proteins, a recent report by Mironova et al., (2001) provided evidence for glycosylation in *E. coli*. In this study, glycation was demonstrated to occur to recombinant human interferon gamma when produced in *E. coli*. Despite this, the nature of the possible modification observed in the present study remains to be determined.

Subcellular fractionation of the transformed *E. coli* cultures revealed that all LPP fusion proteins were particulate. This was expected for the fusion proteins derived from the pTrcHis system whereas the pMal-c2x vector was designed to target the fusion proteins to the cytosolic fraction of the *E. coli* host. This suggested that the highly hydrophobic nature of the LPPs prevailed over the pMal-c2x vector in determining the subcellular localisation of the MBP-LPP1a fusion protein. However,

due to the large amounts of expression that was obtained with the pMal-c2x vector system, it is conceivable that the MBP-LPP1a fusion protein is expressed as insoluble inclusion bodies within the cytosolic compartment of the bacteria and that these protein aggregates were pelleted with the membranes.

3.3.2. Solubilisation of the LPP fusion proteins from the particulate fraction

In order to allow any significant purification of the recombinant LPP fusion proteins, it was necessary to solubilise the particulate fractions of the transformed cultures. As the LPPs are predicted to be integral membrane proteins and so have many hydrophobic regions on there surface, there were several factors which needed to be considered in order to retain the native structure of the proteins and avoid denaturation. For example, following solubilisation, a lipophilic component (e.g. detergent) needs to be retained throughout the subsequent purification to prevent aggregation of the fusion proteins. Furthermore, the lipophilic component needs to be compatible with the protein of interest. Selection of the correct detergent is critical to the success of any procedure of this kind since detergents can also cause a variety of problems. For example, the use of strong ionic detergents such as sodium dodecyl sulphate (SDS) will normally solubilise most proteins, but will most likely denature the proteins, sometimes irreversibly. Therefore, it is desirable to find a 'gentler' detergent, which may not necessarily achieve as much solubilisation of the protein of interest but retains the proteins' native state. In addition, purification techniques used following detergent extraction need to be compatible with the presence of the detergent (Scopes, 1994). Therefore, the solubilisation of LPP1/LPP1a fusion proteins was undertaken with several detergents of differing properties and with Phosphonate-1, a non-hydrolysable analogue of dioctanoyl PA which has been successfully used to solubilise LPP activity from neutrophils (English et al., 1997). The results show that each of the detergents had differing efficiencies at solubilising the LPP fusion proteins. This was similar to the findings of Fleming and Yeaman (1995b) who demonstrated the solubilisation of LPP from rat liver by detergents such as TX-100, CHAPS, deoxycholate and N-octyl-β,D-glucopyranoside. In the present study, 1% TX-100 was found to give the greatest

solubilisation of the LPP fusion proteins, achieving solubilisation of almost 80% of the His 6-LPP fusion proteins (Table 3.1), and around 45% of the His 10-LPP fusion proteins (as assessed by Western blotting and densitometry). This was in contrast to the findings of Fleming and Yeaman (1995b) who demonstrated optimal solubilisation of LPP from rat liver with N-octyl-β,D-glucopyranoside. However, TX-100 has been successfully used to preserve LPP activity from mammalian cells (Roberts et al., 1998) and also from porcine cells (Kanoh et al., 1992).

Nevertheless, in the present study, LPP activity measurements conducted on the solubilised fractions demonstrated that the majority of the LPP activity remained in the TX-100 insoluble fraction. The apparent lack of correlation of these activity measurements with the proportion of LPP1/1a fusion protein solubilised may be due to an underestimation of LPP activity due to the possible denaturation or aggregation of the LPP fusion proteins. Alternatively, an LPP co-factor present in the membrane fraction may have been lost during the solubilisation procedure. Interestingly, it was found that certain detergents, such as DDM, actually abolished LPP activity (results not shown). This was in contrast to results reported by Roberts et al (1998) who showed DDM to have no inhibitory effect on overexpressed LPP activity.

3.3.3. Partial purification of hexa- and deca-histidine tagged fusion proteins by immobilised metal affinity chromatography

As described above, Hexa-histidine tags have been used by several groups to purify membrane proteins. However, Grisshammer and colleagues, (1999) reported that the hexa-histidine tag was inefficient when used in affinity chromatography and that extension of the tag to include ten histidine residues achieved a significant improvement in the effectiveness of the technique. The hexa- and deca-histidine tags are attached to each fusion protein as an N-terminal extension (consisting of a chain of amino acids coding for several specific sequences such as restrictions sites in addition to the hexa- or deca-histidine tag) of approximately 4kDa. In the present study, the hexa-histidine tagged LPP fusion proteins were found to behave like a heterogeneous population of proteins which displayed variable binding efficiency to

the Ni^{2+}-affinity matrix (Figures 3.15 & 3.16). A possible reason for this may lie in the folding and conformation of the recombinant His6-LPP fusion proteins. These may exist in several different forms within the IPTG-induced pTrcHis6-LPP1 and pTrcHis6-LPP1a *E. coli* transformants. For example, His6-LPP fusion proteins may be in the form of the correctly folded protein, incorrectly folded LPP protein, or as large quantities of aggregated, denatured protein (known as inclusion bodies). Therefore, depending on the form the recombinant His6-LPP fusion proteins expressed, the hexa-histidine tag may be concealed to differing degrees, resulting in the observed, variable binding to the Ni^{2+}affinity matrix. The results obtained from IMAC for the hexa-histidine tagged (Figures 3.15 & 3.16) and the deca-histidine tagged (Figures 3.22 & 3.23) LPP fusion proteins show that extension of the hexa-histidine tag to a deca-histidine tag resulted in a significant improvement in the efficiency of the IMAC protocol, as was suggested by Grisshammer and colleagues, (1999). Therefore, the deca-histidine LPP fusion proteins were found to behave more like a homogeneous population of proteins resulting in a more 'well-defined' peak of elution from the IMAC column. Nevertheless, the apparent improved concentration of the His10-LPP fusion proteins did not improve the recovery of LPP activity. In light of these results, the pMal-c2x vector system was assessed as an alternative strategy. However, although the pMal-c2x system resulted in greater amounts of LPP fusion protein being expressed, the LPP activity detected was reduced.

3.3.4. Summary and future directions

Although bacterial expression of LPP1/LPP1a fusion proteins was established using three different strategies in the present study, purification of LPP1 and LPP1a was not possible within the time scale available. In all cases, activity was either low following expression, or lost following partial purification, most likely through denaturation of the LPP fusion proteins. Several possibilities for future directions to achieve successful purification of LPP1 and LPP1a fusion proteins are discussed below.

Cleavage of the epitope tags from the recombinant LPP fusion proteins would confirm whether the epitope tags are causing the incorrect folding of the LPP fusion proteins or are occluding the active site of the enzymes and, thereby, preventing proper presentation of the substrates. In addition, the epitope tags used in this study were all situated at the N-terminus of the LPPs. An alternative strategy would be to express fusion proteins with the epitope tag located at the C-terminus which may result in expression of more active proteins. However, with relation to the possible misfolding of the LPPs, several studies report the misfolding of recombinant proteins, especially integral or membrane bound proteins when expressed in *E. coli*. Equally, many protocols for the refolding of membrane proteins such as the use of liposomes have also been reported. For example, Knol and colleagues (1996 & 1998) report the detergent-assisted reconstitution of the lactose transport protein (lacS) of *Streptococcus thermophilus* into liposomes (proteoliposomes) made from *E. coli* lipids and egg phosphatidylcholine (PC). In a similar manner, Fristedt et al. (1999) and Berhe et al. (1995), successfully reconstituted the H^+/phosphate cotransporter (Pho84p) of *Saccharomyces cerevisiae* expressed as a histidine tagged fusion protein into proteoliposomes composed of PC, phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), and phosphatidylserine (PS). A detailed discussion of membrane protein reconstitution is provided in the review by Madden (1986). It should be noted that the LPP fusion proteins may require the use of different detergents and phospholipids from those detailed in published reports in order to reconstitute the proteins into proteoliposomes and this would need to be determined on a trial and error basis. Nonetheless, it may be possible to reconstitute the partially purified His 6 and His 10-LPP fusion proteins, from the present study, in this manner. Alternatively, the partially purified His 6- and His 10-LPP fusion proteins could be further purified via a technique such as Cation Exchange chromatography (CIEX) prior to reconstitution into proteoliposomes. CIEX is a natural choice for further purification of the His 6- or His 10-LPP fusion proteins as the pI's of the LPP1 and LPP1a fusion proteins are known. These are 7.26 and 6.96 for His 6-LPP1 and His 6-LPP1a, respectively, and 7.32 and 7.04 for His 10-LPP1 and His 10-LPP1a, respectively. Therefore, performing CIEX at pH 6 would be expected to achieve binding and subsequent further purification of the LPP fusion

proteins. Another possible method for purification of the histidine tagged LPP fusion proteins would be through denaturing conditions such as by the use of as guanidine hydrochloride (5-8M) or urea (6-8M) which would allow complete solubilisation of the LPP fusion proteins from the membrane fraction prior to purification and reconstitution. This approach was specifically not used in the present study in order to attempt to retain the native conformation and therefore, activity of the fusion proteins. However, since this did not prove to be possible, a denaturing purification protocol may be appropriate. The difficulty to be overcome would remain the reconstitution of an active protein by regaining the native conformation of the active site.

In contrast to the histidine tagged fusion proteins, the MBP-LPP1a is most likely expressed in the form of inclusion bodies. Therefore, before reconstitution into proteoliposomes, the inclusion bodies would have to be solubilised. Inclusion bodies can be solubilised as with the denaturing condition described above, through the use of various different conditions such as guanidine hydrochloride (5-8M), urea (6-8M) or acetonitrile/propanol (Sambrook et al., 1989).

The methods described above, if successful, would achieve the reconstitution of purified, or partially purified hexa and deca-histidine tagged LPP1 and LPP1a, or maltose binding protein tagged LPP1a. Furthermore, following the development of a successful purification protocol, this could potentially be applied unchanged to the other individual cloned LPP isoforms.

In addition to the methods described above, an alternative expression host cell could be considered. It is possible that post-translational modifications which can not occur in bacteria such as *E. coli* are required in order to achieve high activity recovery. Therefore, expression of the cloned LPP isoforms in mammalian cells using viral expression vectors may be an alternative strategy to achieve high expression levels in a mammalian system where all post-translational modifications can occur. Examples of these viral vectors include those derived from simian virus 40 (SV40), vaccinia virus, adenovirus, retroviruses and baculoviruses (Sambrook et

al., 1989). However, the use of these viral based systems requires some experience in the growth and plaque purification of the different viruses. Furthermore, following solubilisation of the LPP isoforms from the alternative expression system, loss of conformation may still occur and so reconstitution would still be required. In addition to the use of mammalian cell hosts, it is also possible to use yeast or insect cells as an alternative host system.

As detailed above, there is still no successful method for expression and purification of individual LPP isoforms. Therefore, in order to investigate the properties of the individual LPP isoforms further, the LPP isoforms were stably overexpressed in HEK293 cells. As will be described in chapter 4, the expression levels achieved were suitable for the investigation of the properties of the individual LPP isoforms.

CHAPTER 4

CHARACTERISATION OF LPP ISOFORMS WHEN STABLY EXPRESSED IN HEK293 CELLS

4. Characterisation of LPP isoforms when stably expressed in HEK293 cells

4.1. Introduction

The LPP's are multifunctional enzymes which were formerly known as the phosphatidic acid phosphatase Type 2 (PAP-2) enzyme family. The renaming of the PAP-2 enzymes to the LPPs was due to research demonstrating the ability of the then PAP-2 enzymes to dephosphorylate the lipid mediators PA, LPA, C1P and S1P (Brindley & Waggoner, 1998).

Preparations of partially purified LPP activity were originally used to investigate the substrate specificities of the enzyme (Brindley & Waggoner 1996, Kanoh et al., 1992, Fleming & Yeaman, 1995b). In these studies, total membrane fractions of native tissues were used to derive preparations that were substantially enriched in LPP activity. However, it is most probable that these preparations contained a heterogeneous, mixed population of LPP isoforms. Two LPP activities were isolated from rat liver by Fleming and Yeaman and were tested against different molecular species of PA, as well as phospholipids and other substrate analogues (Fleming & Yeaman, 1995b). The enzymes were active against all species of PA but were found to be least active against those containing intermediate-length saturated acyl groups. However, of the two activities isolated, one was twice as active against $PA_{(6:0/6:0)}$ compared with the other. In addition, phospholipids were found to have no effect on the activities of either enzyme. This contrasts with the findings of Jamal and colleagues (1991) who demonstrated inhibition of rat liver LPP by PC. Furthermore, DG, and to a lesser extent monoacylglycerol (MAG), were found to inhibit the LPP enzyme from rat liver suggesting that DG may act as a feedback inhibitor (Fleming & Yeaman, 1995b). Fleming & Yeaman also demonstrated LPA (which was also dephosphorylated by the LPP enriched preparation from rat liver) decreased LPP activity towards PA in an apparently competitive manner (Fleming & Yeaman, 1995b). In contrast however, LPA was reported to have no effect on the ability of the LPP activity partially purified from porcine thymus, to dephosphorylate PA (Kanoh et al., 1992). Interestingly, Waggoner and colleagues reported rat liver LPP

to hydrolyze LPA more effectively than PA (Waggoner et al., 1996). However, Fleming & Yeaman (1995b) showed that both LPP isoforms from rat liver were more effective against PA compared to LPA. These results therefore, suggested the presence of distinct LPP iso-enzymes that differ in their substrate preference.

Further work on partially purified LPP activity from rat liver membranes demonstrated that, in addition to PA and LPA, LPP dephosphorylated C1P and S1P (Waggoner et al., 1996). These lipids were reported to be mutually competitive, supporting the suggestion that the enzyme hydrolysed all four substrates (Waggoner et al., 1996). In addition, LPP activity from guinea pig airway smooth muscle (GPASM) cells was shown to dephosphorylate monounsaturated PA more readily than PA species with polyunsaturated fatty acyl chains (Tolan, 1997). The LPP activity from these cells was found to be inhibited by PC which was in agreement with the findings of Jamal and colleagues (1991). In addition, LPA and S1P, but not C1P were found to be competitive with respect to LPP activity against PA. Furthermore, DG, but not MAG, was found to inhibit the activity (Tolan, 1997). In contrast to the findings of Fleming and Yeaman (1995b), English and colleagues reported a preference for short chain PA species compared to PA species with longer fatty acid side chains for a neutrophil 'ecto'-LPP activity (English et al., 1997). However, LPP activity from GPASM exhibited no preference between $PA_{(8:0/8:0)}$ and $PA_{(18:1/18:1)}$ but showed significantly reduced activity against $PA_{(18:0/20:4)}$ and $PA_{(16:0/16:0)}$ (Tolan, 1997). The comparison between the results from the studies described above is difficult due to the use of different PA species such that Fleming and Yeaman did not test $PA_{(8:0/8:0)}$ as a substrate and English and colleagues did not test $PA_{(18:0/20:4)}$. However, they do suggest important differences between the activities investigated.

The substrate preferences of the cloned human LPP isoforms have also been investigated (Kai et al., 1997, Roberts et al., 1998, Hooks et al., 1998). Roberts and colleagues demonstrated a broad substrate profile for the LPPs with a rank order of preference LPA > PA > S-1-P > C-1-P for LPP1 (LPP1), PA > C-1-P > LPA > S-1-P for LPP2 (LPP2) and LPA ≈ PA > C-1-P > S-1-P for LPP3 (LPP3) (Roberts et al.,

1998). Interestingly, only the LPP1 isoform was found to result in high levels of cell surface activity when expressed in insect cells (Roberts et al., 1998). In contrast, Hooks and colleagues demonstrated an apparent preference for LPA over PA for the cloned human LPP1, LPP2 and LPP3 isoforms when over-expressed in HEK293 cells (Hooks et al., 1998). However, LPP activity against C1P or S1P was not measured in this study. Interestingly, Kai and colleagues reported that both cloned human LPP1 and LPP3, when over-expressed in HEK293 cells, dephosphorylated LPA, C1P and PA, whereas only LPP3 displayed activity towards S1P (Kai et al., 1997). The broad range of substrates for the LPPs has caused great debate over the possible physiological functions of the enzyme family. Furthermore, the issue of whether the LPPs function to degrade intracellular or extracellular phosphorylated lipids remains one of the most significant questions in the field. When addressing this question, it is invaluable to have as much information as possible on the kinetic properties of each of the LPP isoforms towards any potential physiological substrates.

In summary, all information on the substrate preference for different molecular species of PA has been generated in partially purified preparations which may contain a mixture of several LPP isoforms. Furthermore, investigations using the cloned LPP isoforms over-expressed in various systems, have revealed considerable variation in the reported substrate preferences with Roberts and colleagues (1998) reporting the only extensive kinetic investigation. Clearly, the LPPs represent a multi-substrate enzyme family, having several PA species, LPA, C1P and S1P as potential substrates. Therefore, even though simple kinetic studies provide important information on the ability of the enzymes to hydrolyse various substrates, it is important to investigate and consider the potential interactions that may occur between the various substrates and the enzymes in a physiological setting. For example, in a physiological setting, an enzyme may have to select between several substrates that are available simultaneously (Cornish-Bowden, 1995). To date there are no reports of a complex kinetic study using the individual cloned LPP isoforms such that all investigations using the cloned LPP isoforms have utilised classical, single substrate assay systems to investigate this multi-substrate enzyme family.

Therefore, the aims of this section were to (i) systematically investigate the LPP isoforms against various species of PA and also the other potential phosphorylated lipid substrates in a uniform assay system and (ii) to perform the first investigation of the substrate preference and kinetics of the LPP enzyme family in a multi-substrate assay of two alternative substrates. In order to extensively characterise the overexpressed LPP isoforms, several molecular species of PA with differing fatty acid chain length and degrees of saturation were used, as well as LPA and the potential substrates of sphingolipid origin, namely C1P and S1P. This therefore, represents the most detailed investigation of the substrate preference and kinetics of the LPP isoforms in a single system to date. Furthermore, this is the first study to investigate the cloned LPP isoforms in a multi-substrate assay system. Clarification of the substrate preference and kinetics of the LPP isoforms may provide an indication of the respective roles of the LPP isoforms and therefore, the reason for such molecular diversity within the family of enzymes.

4.2. Results

4.2.1. Expression of LPP1, LPP1a, LPP2 and LPP3 in HEK293 cells

gpLPP1, gpLPP1a, hLPP2, and hLPP3 were stably overexpressed in HEK293 cells as detailed previously (Alderton et al., 2001). In order to confirm overexpression of the LPP isoforms in the HEK293 cells, LPP activity was determined in a crude membrane fraction derived from control cells (Blank vector) and cells separately and stably transfected with each of the LPP isoforms using 500μM [^{32}P]-dioleoyl PA ($PA_{(18:1,18:1)}$) as a substrate. The HEK293 cells expressing LPP1, LPP1a, LPP2 and LPP3 exhibited 142 +/- 8, 252 +/- 36, 96 +/- 5 and 8 +/- 2 fold increases (mean+/- SEM, n=3) in LPP activity compared to control cells (4.8nmol/min/mg) respectively (Figure 4.1). Figure 4.1 also shows that the LPP1 and LPP1a over-expressing cells displayed significantly higher specific activity towards [^{32}P]-$PA_{(18:1/18:1)}$ compared to the LPP2 and LPP3 over-expressing cells under the assay conditions employed. However, it should be noted that due to the LPPs being multi-substrate enzymes, an estimation of relative expression levels could not be assumed directly from these results. However, these results do confirm that significant LPP activity was present in all stably transfected cell lines (see discussion – Section 4.3.1.). It was found that increasing levels of protein significantly reduced the specific activity of LPP1 transfected cells (Figure 4.2). This effect was also observed in the other LPP transfected HEK293 cells (results not shown). The assay conditions used for all activity measurements were therefore, optimised with respect to the amount of membrane protein added to each assay so that no more then 15% of the substrate was dephosphorylated during the assay. This was to minimise the possibility of product inhibition occurring. Therefore, in all experiments, the membrane protein concentration was maintained between 0.2 and 1μg per assay for the LPP1, LPP1a, LPP2 and LPP3 transfectants and assays were conducted for 5 minutes. However, in order to detect measurable LPP activity in the blank vector transfectants, between 4.5 and 6μg of membrane protein was used.

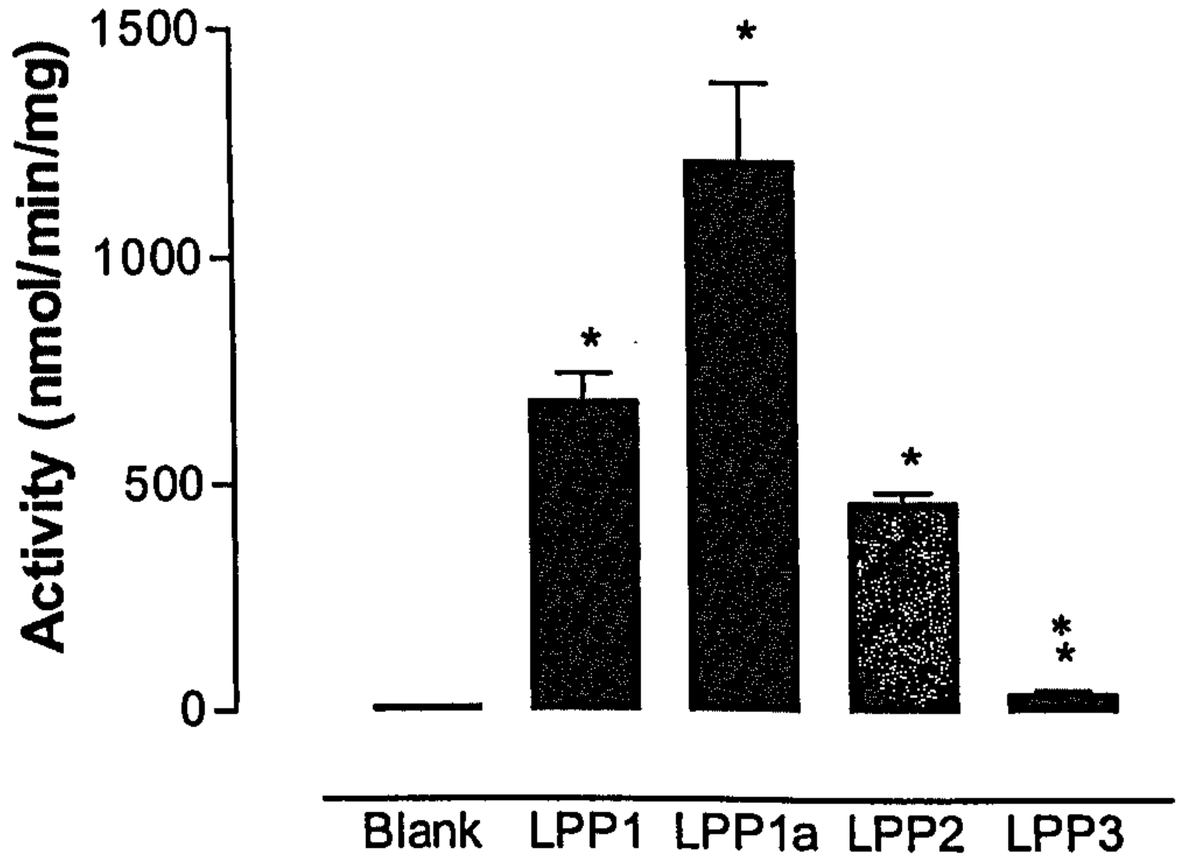


Figure 4.1: Total LPP activity in a crude membrane preparation derived from stably transfected HEK293 cells.

LPP activity was measured *in vitro* by incubation (5min, 30°C) of a crude membrane fraction (section 2.8.3.) with [^{32}P]-$PA_{(C18:1/18:1)}$ (500μM, prepared as described in section 2.5.) in a mixed micellar assay (section 2.6). The liberated ^{32}P, resulting from LPP activity, was separated from residual [^{32}P]-PA using a phase split and quantified by scintillation counting (Section 2.6). Specific activity was calculated using the membrane protein content that had been established previously by protein assay (Section 2.2.). Results are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3). * $p < 0.05$: Students T-test versus blank vector crude membranes.

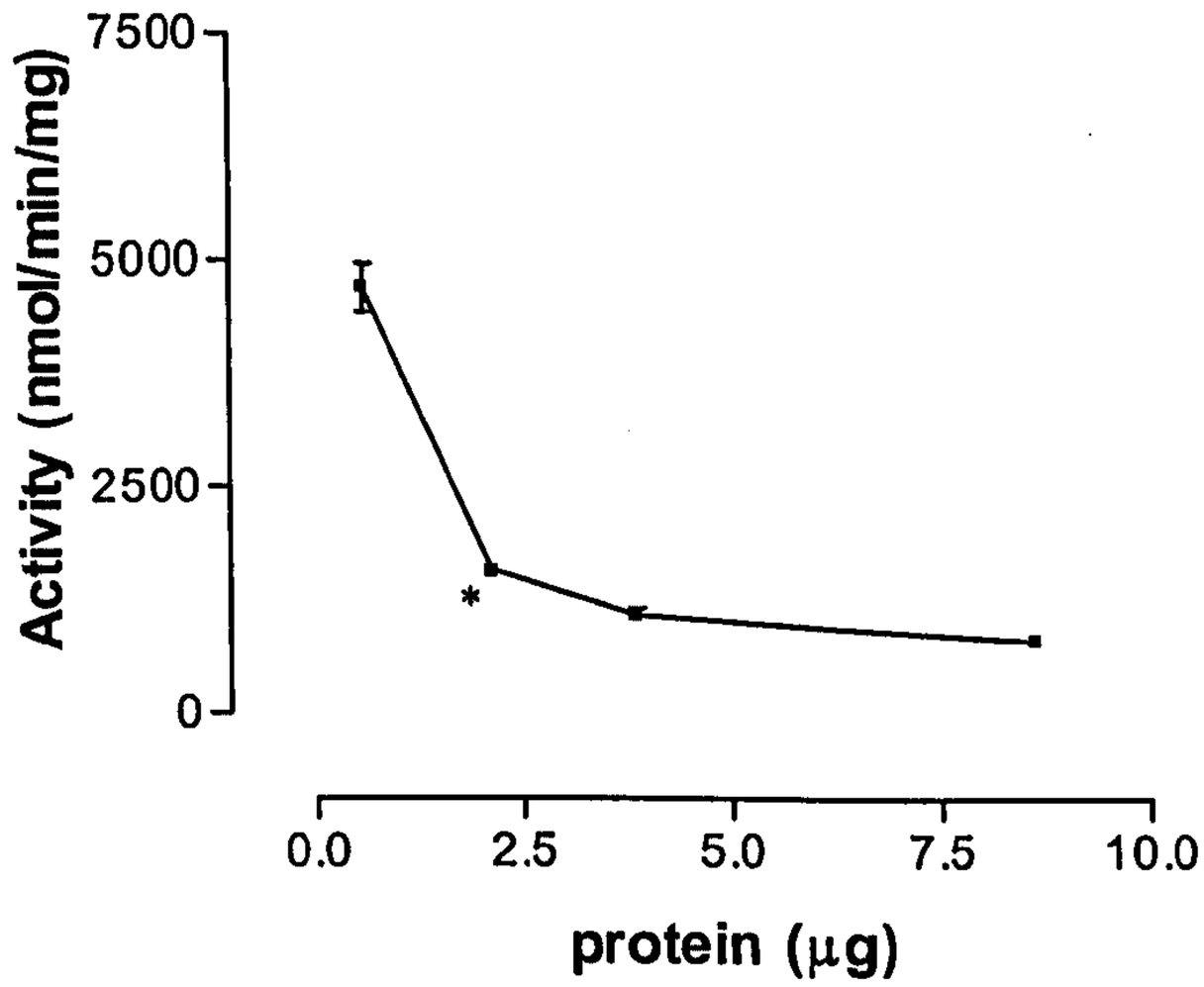


Figure 4.2: The effect of protein concentration on total LPP activity using a crude membrane preparation derived from HEK293 cells stably transfected with LPP1.

LPP activity was measured *in vitro* by incubation (5min, 30°C) of a crude membrane fraction (Section 2.8.3.) with [^{32}P]-dioleoyl $PA_{(C18:1/18:1)}$ (500μM, prepared as described in section 2.5.) in a mixed micellar assay (section 2.6.). The liberated ^{32}P, resulting from LPP activity, was separated from residual [^{32}P]-PA using a phase split and quantified by scintillation counting (Section 2.6.). Specific activity was calculated using the membrane protein content that had been established previously by protein assay (0.5 to 8.6μg protein/assay) (Section 2.2.). Results are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3). * $p < 0.05$: Students T-test versus specific activity at 0.5μg membrane protein/assay.

4.2.2. Kinetic analysis of LPP1, LPP1a, LPP2, and LPP3 against $PA_{(18:1/18:1)}$ in a single substrate assay

Mixed micelles of TX-100 and $PA_{(18:1/18:1)}$/ [^{32}P]-$PA_{(18:1/18:1)}$, were used to investigate the kinetics of LPP1, LPP1a, LPP2 and LPP3 activities in crude membranes derived from the cells stably overexpressing LPP1, LPP1a, LPP2 or LPP3, respectively. Initial rates of hydrolysis were measured as the concentration of $PA_{(18:1/18:1)}$ was increased while maintaining the PA:TX-100 ratio at a constant 1:10 (constant mole fraction of 0.091). A similar experiment was conducted for membranes derived from blank vector (pcDNA3.1 zeo(-)) transfected cells and the specific activities obtained subtracted from the data for each of the LPP overexpressing cell lines in order to minimise the effects of any endogenous, mixed LPP component. The data for each enzyme against each substrate was then analysed by using a curve-fit procedure of weighted non-linear regression to the Hill equation; $v = Vmax * A^h / (K_{0.5}{}^h + A^h)$; where V is the initial rate, Vmax is the apparent maximal activity of the enzyme, A is the substrate concentration, $K_{0.5}$ is the substrate concentration (A) at which v = 0.5Vmax (equivalent to Km in the Michaelis-Menten equation) and h is the Hill co-efficient. All curve-fit precedures were conducted using GraphPad Prism^{TM} (Version 2.01) unless otherwise stated. This enabled the calculation of the apparent Vmax and $K_{0.5}$ values for each of the LPP isoforms against $PA_{(18:1/18:1)}$ in a single substrate assay. The Vmax and $K_{0.5}$ values were then used to calculate the specificity constant (Vmax/ $K_{0.5}$) (Cornish-Bowden, 1995) as a measure of catalytic efficiency. This allows comparison of the specificity of the four enzymes for the different substrates as performed by Roberts et al., (1998). In addition to this analysis, data obtained was used to construct a Hanes plot for each LPP isoform against $PA_{(18:1/18:1)}$. The Hanes plot is used in the same way as the classical Lineweaver-Burke plot to display kinetic data. The Hanes plot was used as it provides a better means of presentation due to an uneven error distribution within the Lineweaver-Burke plot.

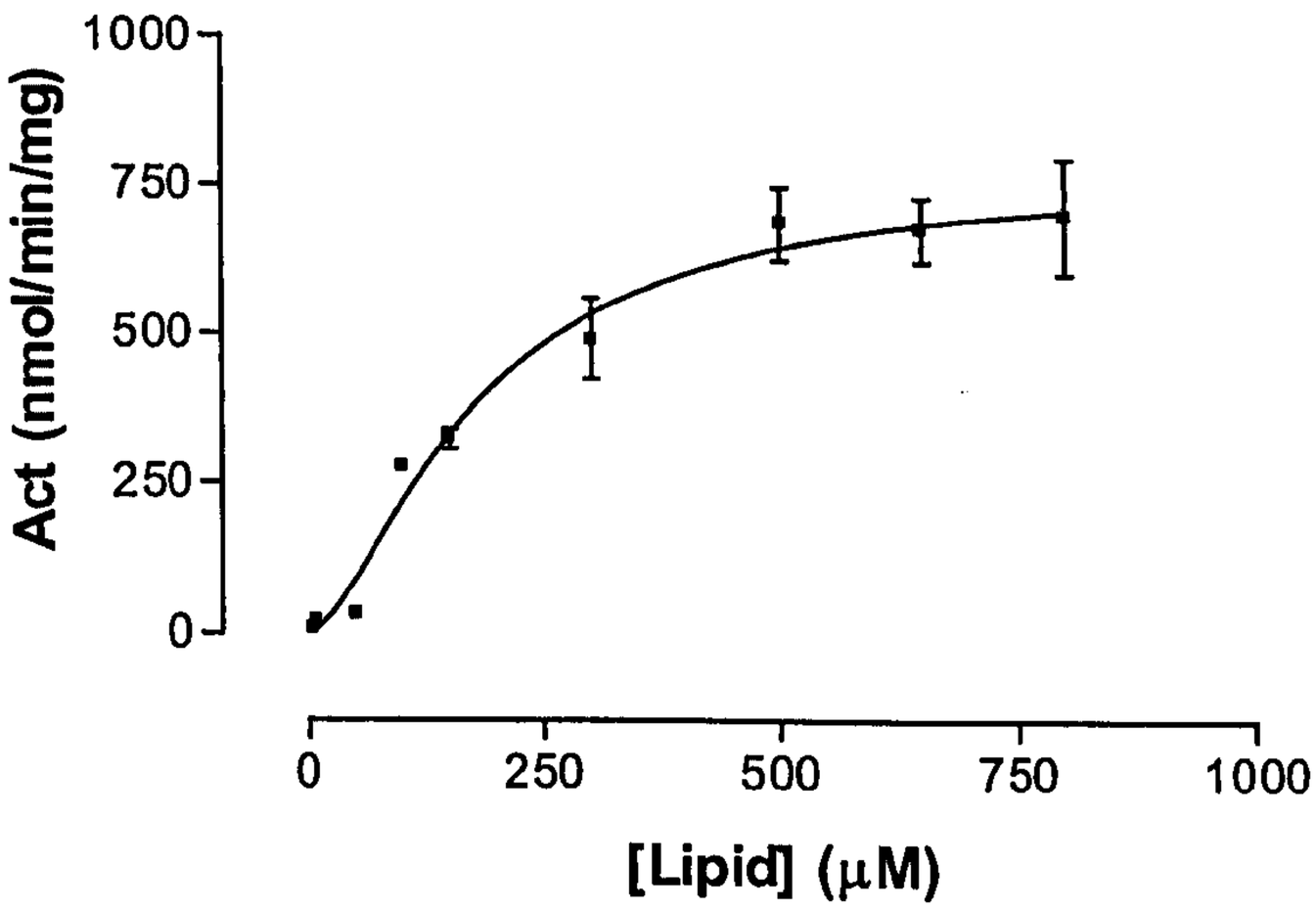
Figures 4.3A to 4.6A illustrate velocity versus substrate concentration plots for LPP1, LPP1a, LPP2 and LPP3 respectively, towards $PA_{(18:1/18:1)}$. Under the assay conditions employed, increasing the concentration of $PA_{(18:1/18:1)}$ in the assay, enhanced the activity of all LPP isoforms towards $PA_{(18:1/18:1)}$ and this activity

appeared to be saturable. Furthermore, a sigmoidal curve was apparent in the velocity versus substrate concentration plots obtained for LPP1, LPP1a and LPP2, but not LPP3. This suggested variation in the kinetics of the different LPPs under the assay conditions employed.

The kinetic constants (apparent Vmax and $K_{0.5}$) and Hill coefficients (h) obtained through the curve-fit procedure to the Hill equation, are detailed in Table 4.1. The apparent Vmax values for the LPPs against $PA_{(18:1/18:1)}$ are seen to vary considerably with values of 772, 1582, 492 and 60 for LPP1, LPP1a, LPP2 and LPP3 respectively. The apparent $K_{0.5}$ values however, show far less variablility with values of 181, 190,122 and 193μM for LPP1, LPP1a, LPP2 and LPP3 respectively. Furthermore, the h values obtained from the analysis were 1.58, 1.61, 2.1 and 0.99 for LPP1, LPP1a, LPP2 and LPP3 respectively. These h values suggest that only LPP3 follows typical Michaelis-Menten kinetics when $PA_{(18:1/18:1)}$ is the substrate and that the other LPPs display a degree of cooperativity. However, it is technically incorrect to use the h value as an estimate of the number of binding sites and, therefore, as direct evidence of cooperativity (see discussion – Section 4.3.3.). Thus, in order to investigate the possibility of cooperation further, Hanes plots of the data sets were constructed. Figures 4.3B to 4.6B show the Hanes plots for LPP1, LPP1a, LPP2 and LPP3 respectively. As shown, the Hanes plots for all LPPs except LPP3 are non-linear, and show a distinct upward turn in the plot at lower concentrations, which is indicative of positive substrate cooperativity (Dr Peter Birch, personal communication). Therefore, the data presented suggest that LPP1, LPP1a and LPP2 display positive substrate cooperativity towards $PA_{(18:1/18:1)}$ in a single substrate assay whereas LPP3 displays apparent Michaelis-Menten kinetics.

Table 4.1 also shows the specificity constants ($Vmax/K_{0.5}$) calculated for each of the LPPs towards $PA_{(18:1/18:1)}$. These were 4.27, 8.32, 4.04 and 0.31 for LPP1, LPP1a, LPP2 and LPP3 respectively. This therefore, suggests a rank order of ability to dephosphorylate $PA_{(18:1/18:1)}$ of LPP1a>LPP1≈LPP2>LPP3.

A



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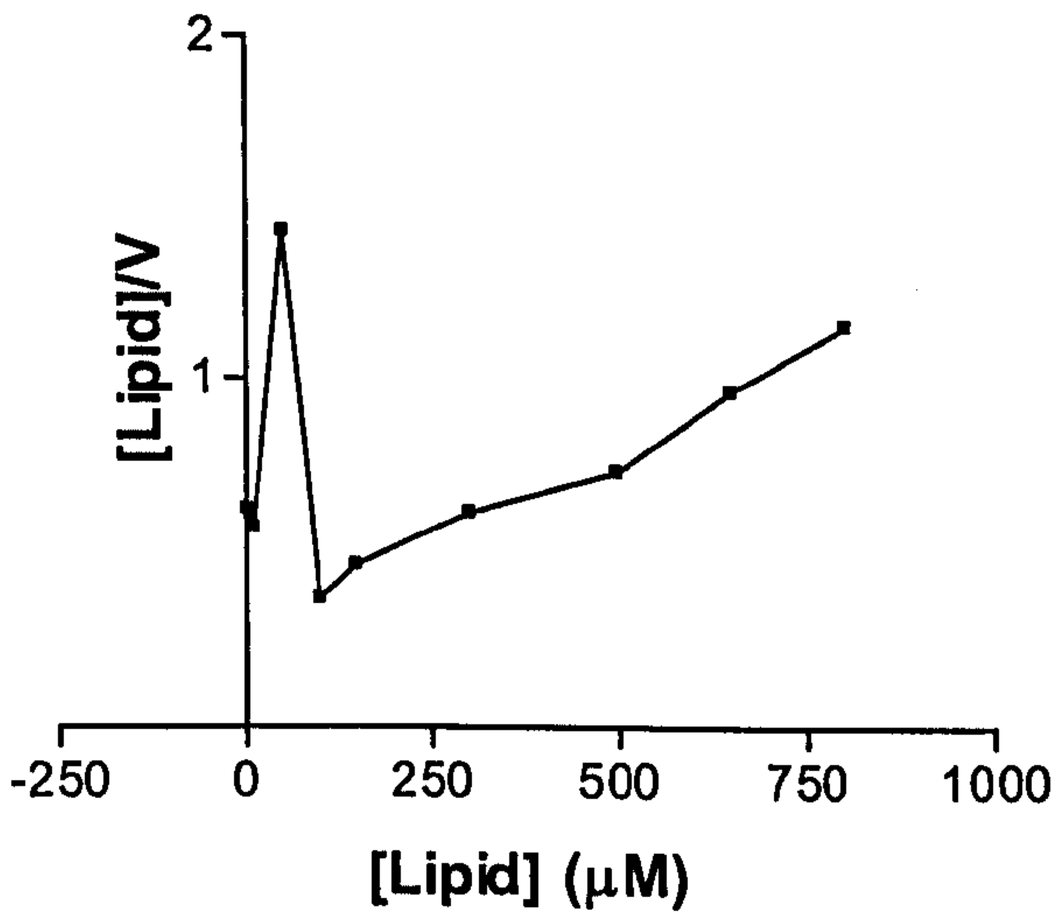
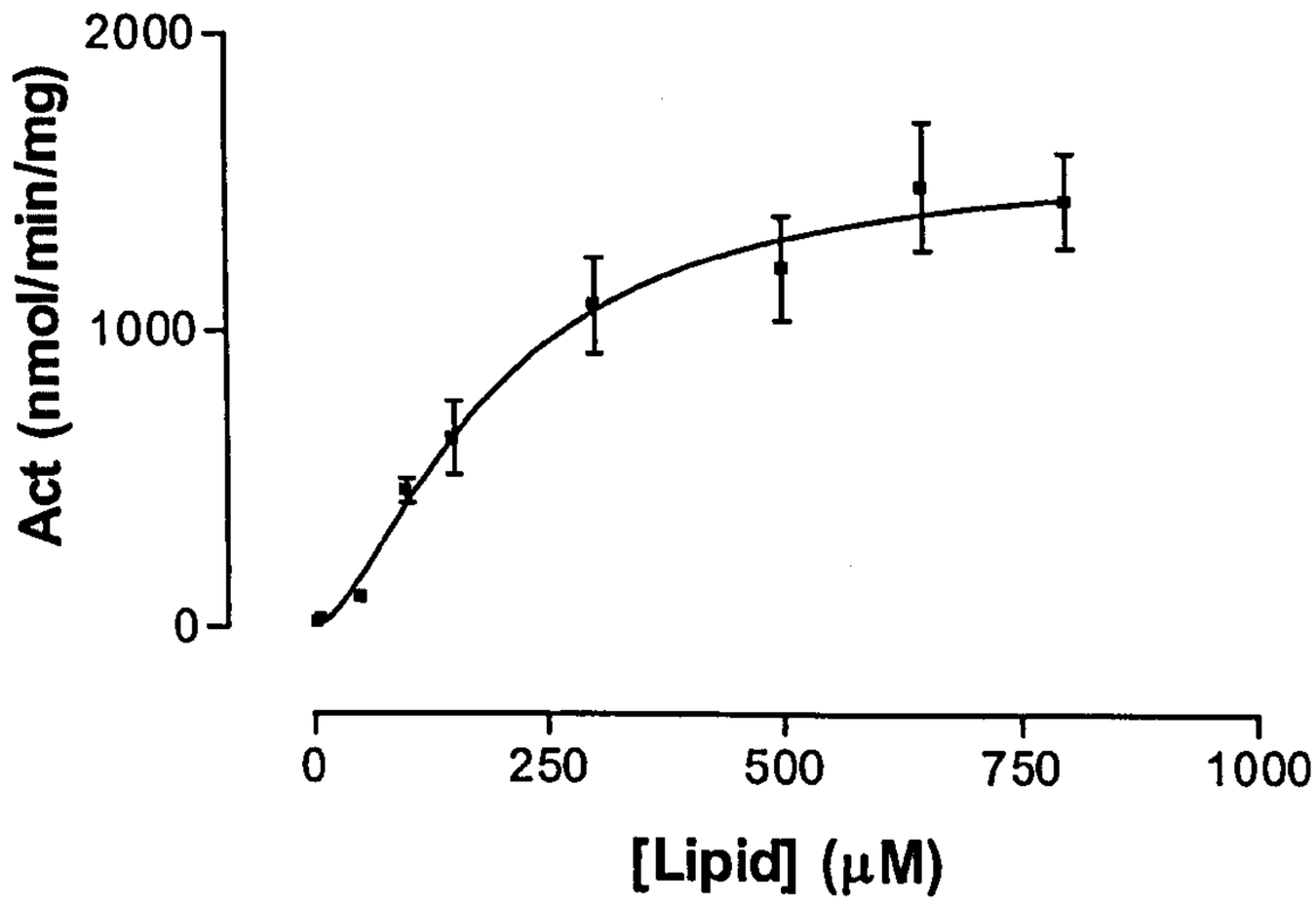


Figure 4.3: Substrate dependence (A) and Hanes plot (B) of LPP1 activity against PA $_{(18:1,18:1)}$.

LPP1 activity towards $PA_{(18:1/18:1)}$ was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



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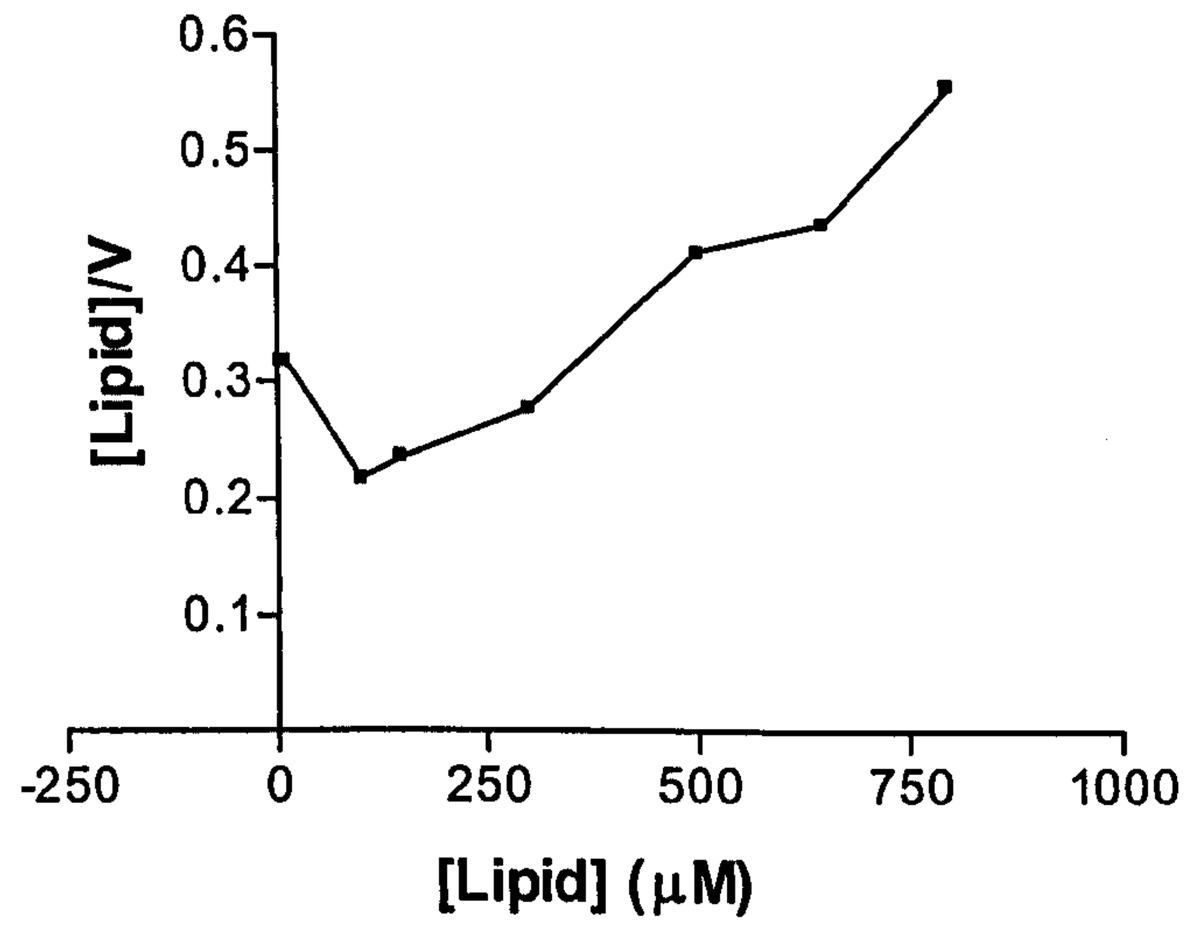
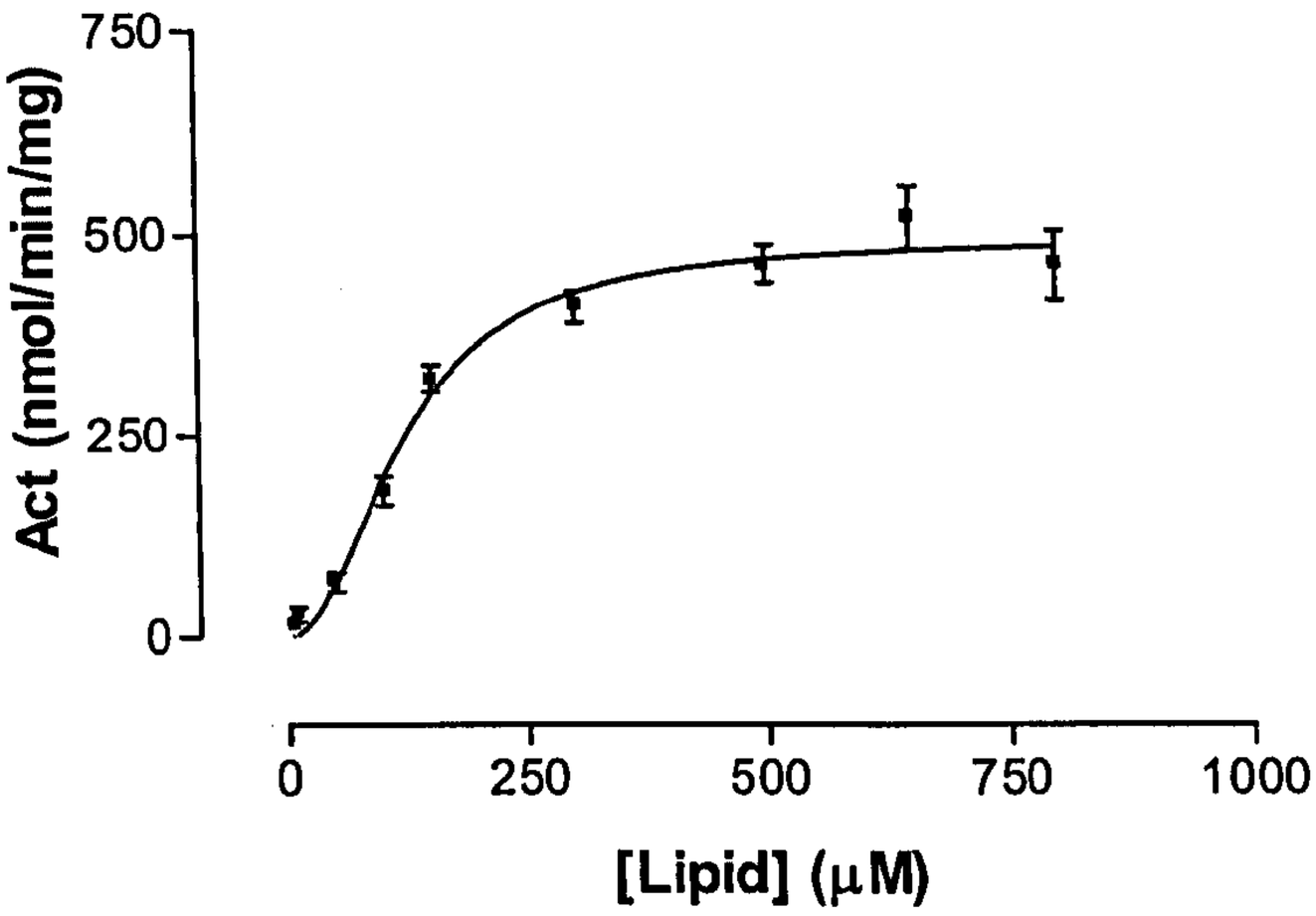


Figure 4.4: Substrate dependence (A) and Hanes plot (B) of LPP1a activity against PA $_{(18:1,18:1)}$.

LPP1a activity towards $PA_{(18:1/18:1)}$ was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1a as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



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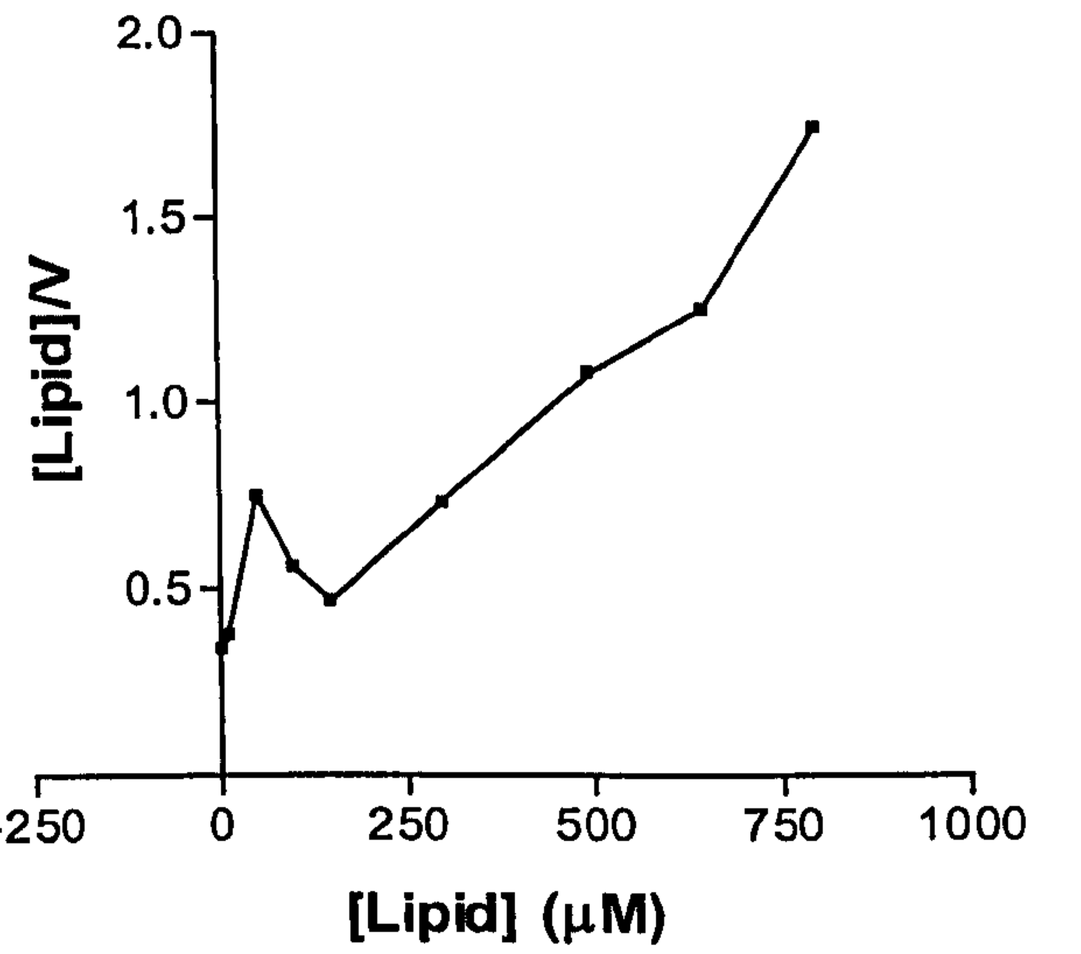
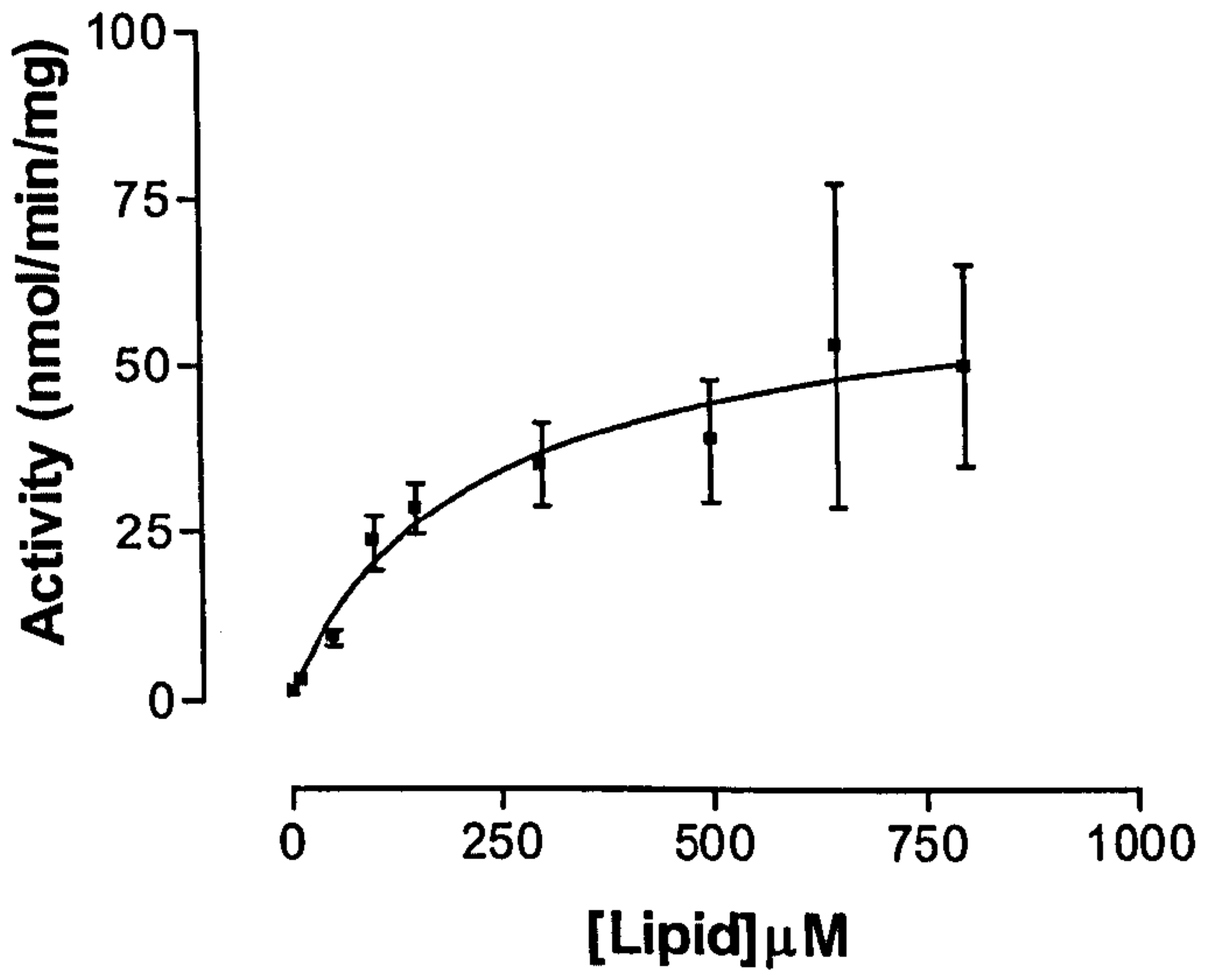


Figure 4.5: Substrate dependence (A) and Hanes plot (B) of LPP2 activity against PA $_{(18:1,18:1)}$.

LPP2 activity towards $PA_{(18:1/18:1)}$ was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP2 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



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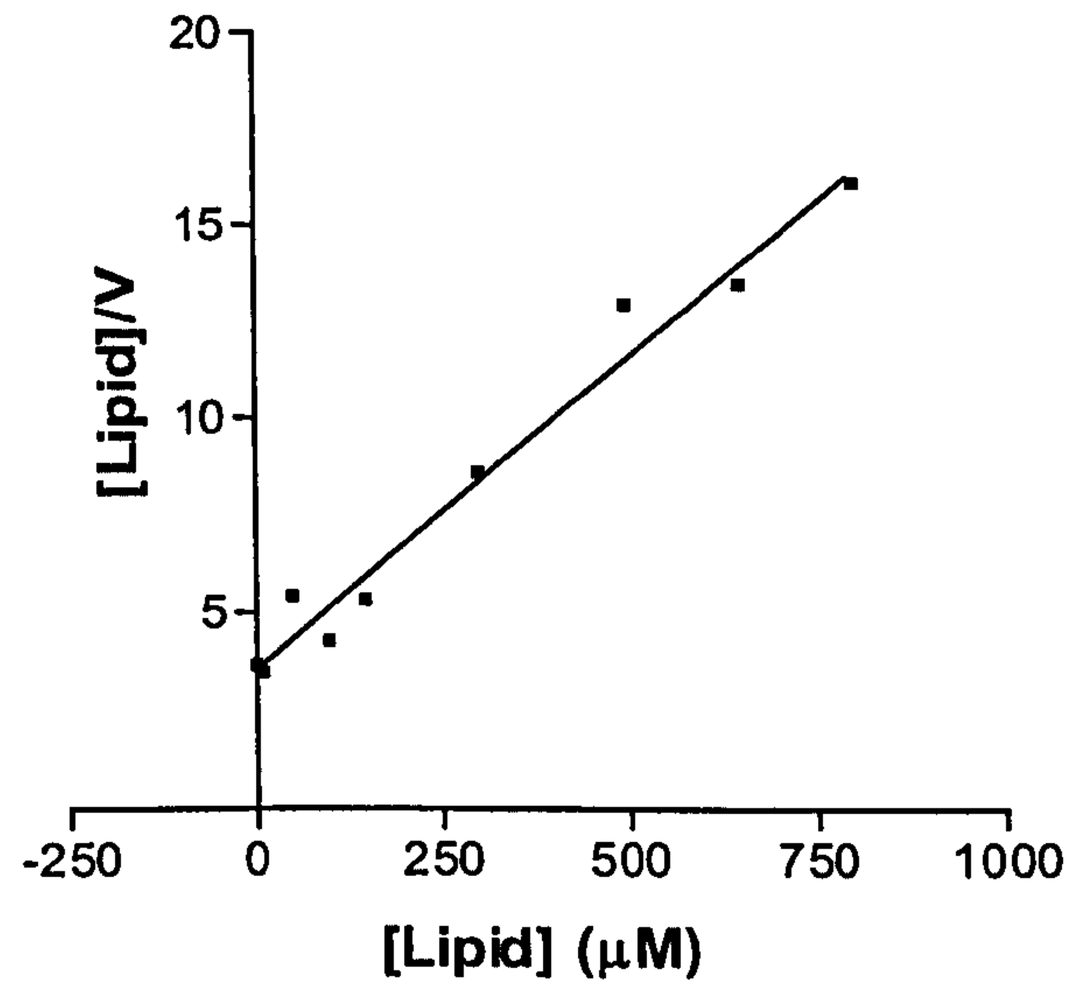


Figure 4.6: Substrate dependence (A) and Hanes plot (B) of LPP3 activity against PA $_{(18:1,18:1)}$.

LPP3 activity towards $PA_{(18:1/18:1)}$ was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP3 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

	Vmax (nmol/min/mg)	$K_{0.5}$ (μM)	Vmax/$K_{0.5}$	h
LPP1	772	181	4.27	1.58
LPP1a	1582	190	8.32	1.61
LPP2	492	122	4.04	2.1
LPP3	60	193	0.31	0.99

Table 4.1: Kinetic constants for LPP1, LPP1a, LPP2 and LPP3 against $PA_{(18:1/18:1)}$ presented in single substrate TX-100 micelles

LPP activity towards $PA_{(18:1/18:1)}$ was measured in crude membranes derived from HEK293 cells stably overexpressing LPP1, LPP1a, LPP2 or LPP3 as described in Section 2.6. Kinetic constants [apparent Vmax (nmol/min/mg), $K_{0.5}$ (μM)] and Hill coefficients (h) were derived by weighted, non-linear regression of the data presented in Figures 4.3 to 4.6 to the Hill equation [$v = Vmax * A^h / (K_{0.5}^h + A^h)$] using GraphPad Prism™ (version 2.01).

4.2.3. Kinetic analysis of LPP1, LPP1a, LPP2 and LPP3 towards several molecular species of PA presented in a mixed substrate assay with $PA_{(18:1/18:1)}$

The kinetics of the LPPs were investigated using a multi-substrate assay employing mixed micelles of TX-100, $PA_{(18:1/18:1)}$ and one of several alternative [^{32}P]-PA substrates ($PA_{(8:0/8:0)}$, $PA_{(18:0/20:4)}$, $PA_{(16:0/16:0)}$) and crude membranes derived from cells stably overexpressing LPP1, LPP1a, LPP2 or LPP3, respectively. Initial rates of hydrolysis of each alternative PA substrate were measured as the concentration of $PA_{(18:1/18:1)}$ was increased while maintaining the PA:TX-100 ratio at a constant 1:10 (constant mole fraction, 0.091). Again, similar experiments in all cases were conducted for membranes derived from blank vector (pcDNA3.1 zeo (-)) transfected cells and the initial rates obtained subtracted from the data for each of the LPP overexpressing cell lines. The data obtained for each enzyme was then analysed by a curvefit procedure of weighted non-linear regression to the Hill equation as described in section 4.2.2 and Hanes plots constructed.

<u>$PA_{(8:0/8:0)}$</u>

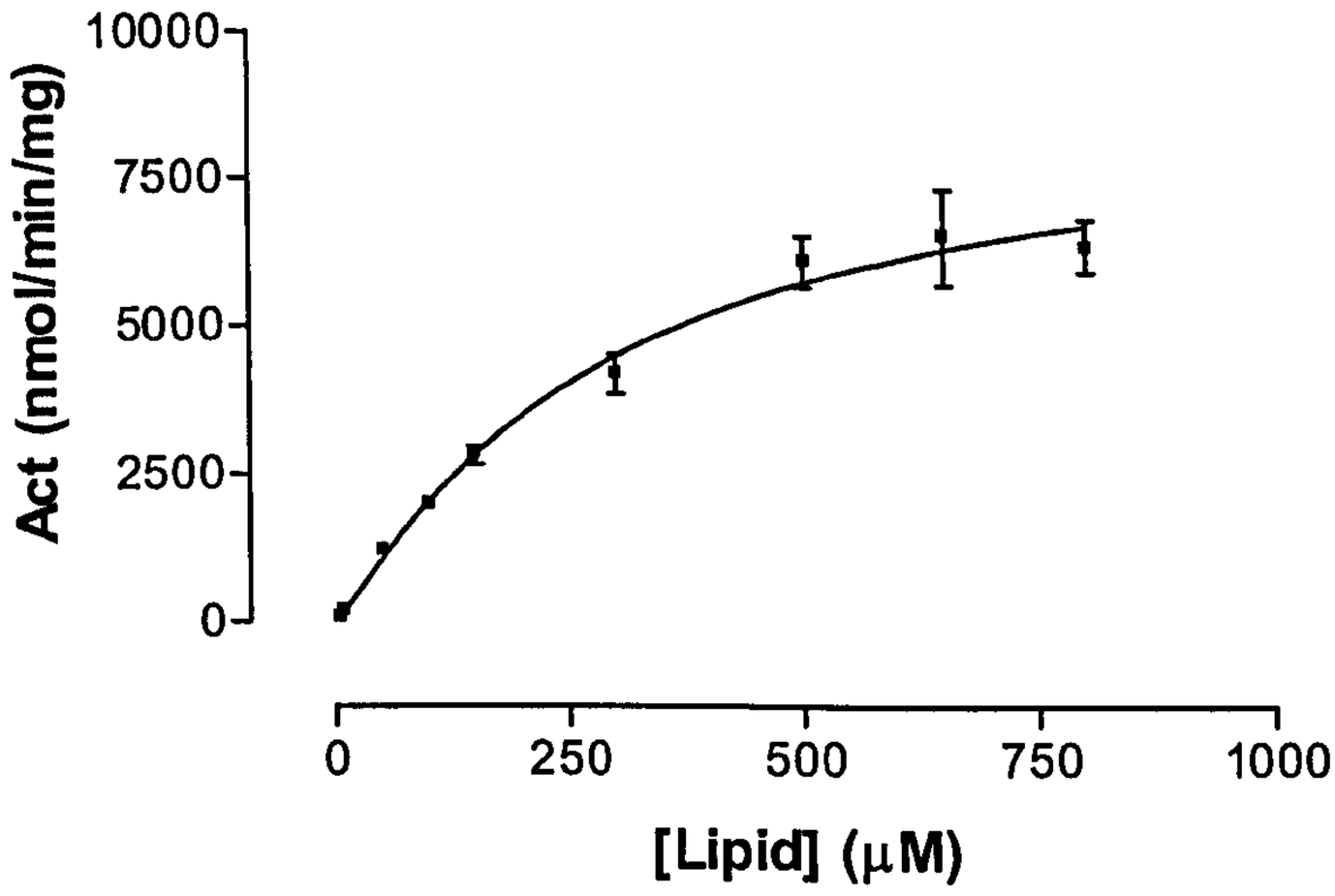
Figures 4.7A to 4.10A illustrate velocity versus substrate concentration plots for LPP1, LPP1a, LPP2 and LPP3 respectively towards [^{32}P]-$PA_{(8:0/8:0)}$ assayed in the presence of $PA_{(18:1/18:1)}$. Under the multi-substrate assay conditions employed, increasing the concentration of the LPP substrate $PA_{(18:1/18:1)}$ was found to enhance the activity of all LPP isoforms towards the alternative LPP substrate $PA_{(8:0/8:0)}$. In all cases, this activity appeared to be saturable, although this was less apparent for LPP3 (Figure 4.10A).

The kinetic constants (apparent Vmax and $K_{0.5}$) and h values obtained through the curvefitting to the Hill equation (section 4.2.2) for each of the LPP isoforms towards [^{32}P]-$PA_{(8:0/8:0)}$ are detailed in Table 4.2. As with the results for $PA_{(18:1/18:1)}$ in the single substrate assay, the apparent Vmax values vary between the different LPPs with values of 8768, 5495, 1878 and 1284 nmol/min/mg for LPP1, LPP1a, LPP2 and

LPP3 respectively. The apparent $K_{0.5}$ values also vary between the different LPP isoforms with values of 289, 197, 246 and 479μM for LPP1, LPP1a, LPP2 and LPP3 respectively. The h values were less variable for [^{32}P]-$PA_{(8:0/8:0)}$ than observed for $PA_{(18:1/18:1)}$ in the single substrate assay with values of 1.14, 1.18, 0.98 and 1.4 for LPP1, LPP1a, LPP2 and LPP3 respectively. These values therefore, again suggested the presence of substrate cooperativity within the LPP family towards [^{32}P]-$PA_{(8:0/8:0)}$. Hanes plots of the data obtained for LPP1, LPP1a, LPP2 and LPP3 were therefore, plotted and are shown in Figures 4.7B to 4.10B respectively. The Hanes plots for all LPP isoforms are non-linear and so confirm the presence of cooperativity with the characteristic upward turns observed for LPP1, LPP1a and LPP2. The Hanes plot for LPP3 differed in shape from the Hanes plots for the other three LPP isoforms and so suggested a potential difference in the mechanisms of action of LPP3 compared to the other LPPs under the conditions employed.

Table 4.2 also shows the $Vmax/K_{0.5}$ (specificity constants) for the LPPs against [^{32}P]-$PA_{(8:0/8:0)}$, these being 30.31, 27.92, 7.63 and 2.68 for LPP1, LPP1a, LPP2 and LPP3 respectively. These therefore demonstrate a considerably higher catalytic efficiency for LPP1 and LPP1a against [^{32}P]-$PA_{(8:0/8:0)}$ and a rank order of ability to dephosphorylate [^{32}P]-$PA_{(8:0/8:0)}$ under the assay conditions employed of LPP1≈LPP1a>LPP2>LPP3.

A



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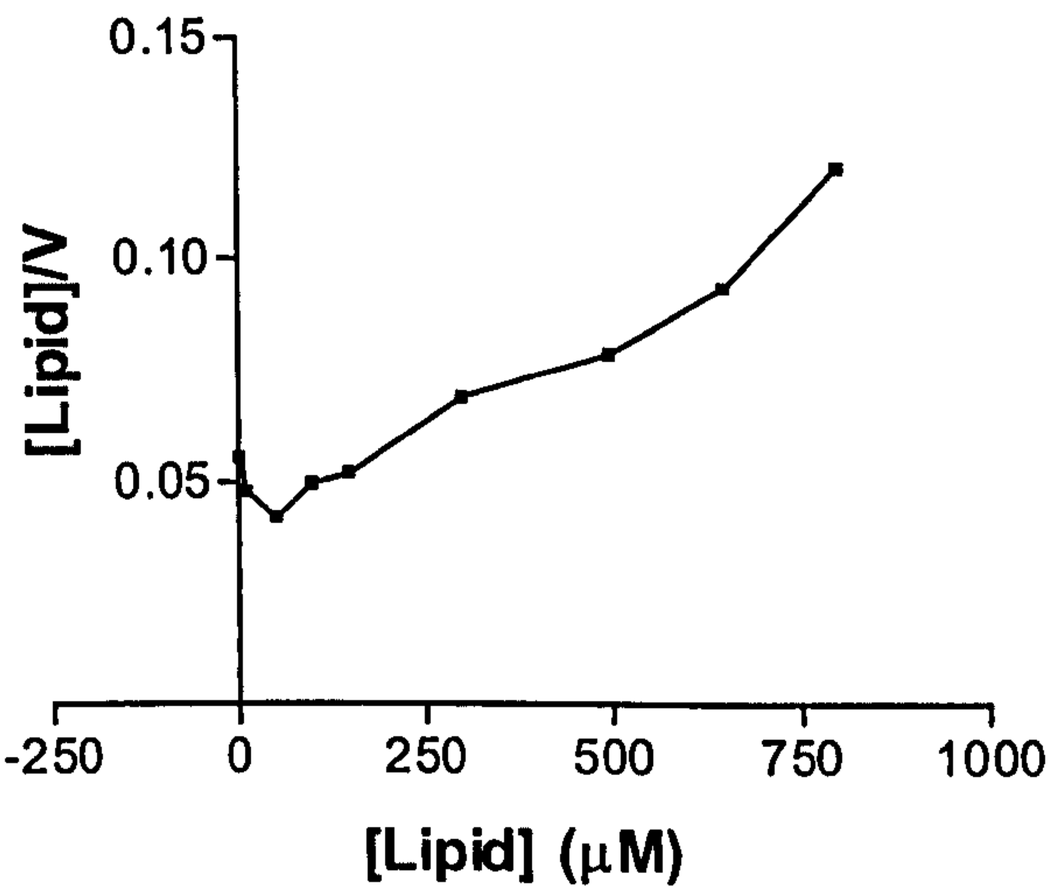
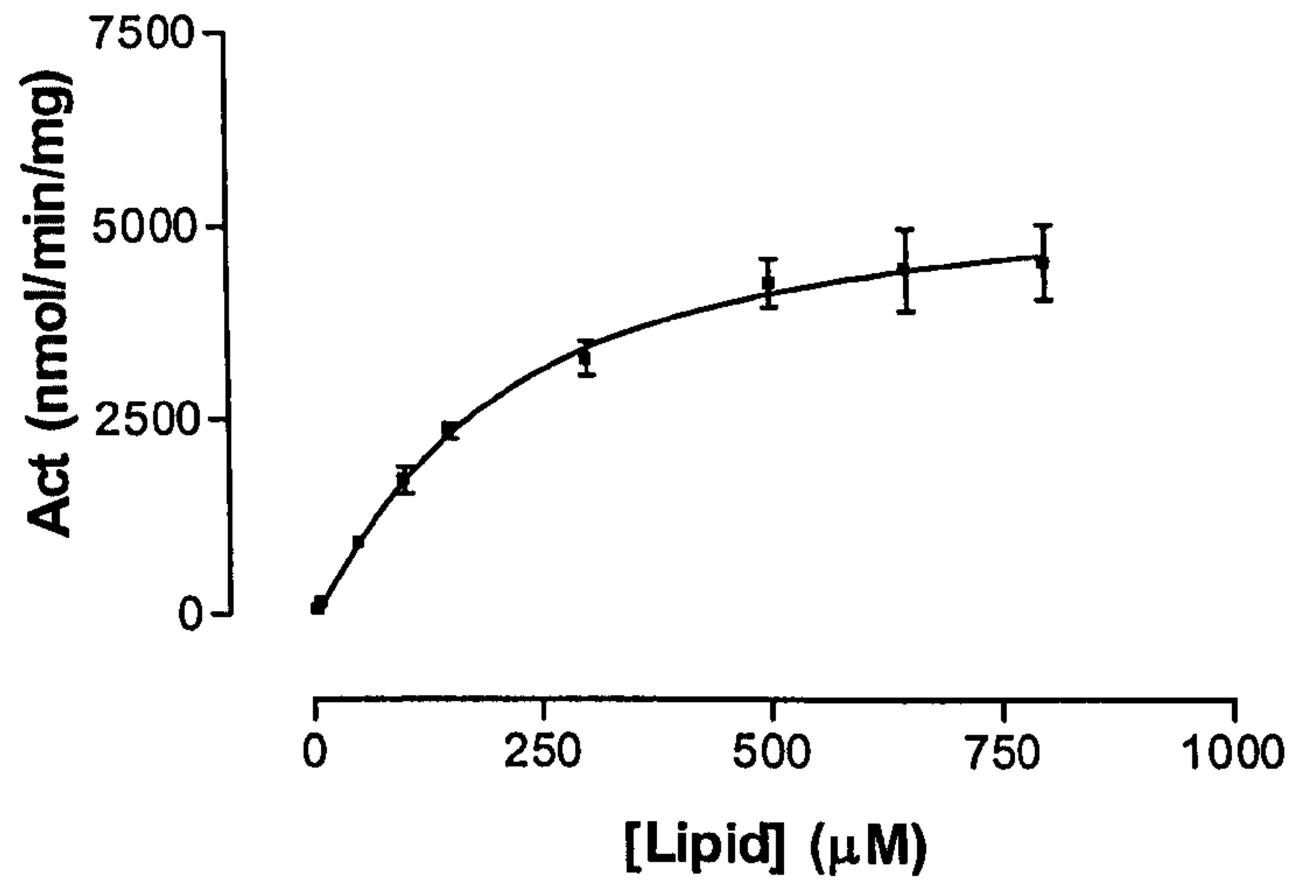


Figure 4.7: Substrate dependence (A) and Hanes plot (B) of LPP1 activity against [^{32}P]-PA $_{(8:0,8:0)}$.

LPP1 activity towards [^{32}P]-PA$_{(8:0/8:0)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



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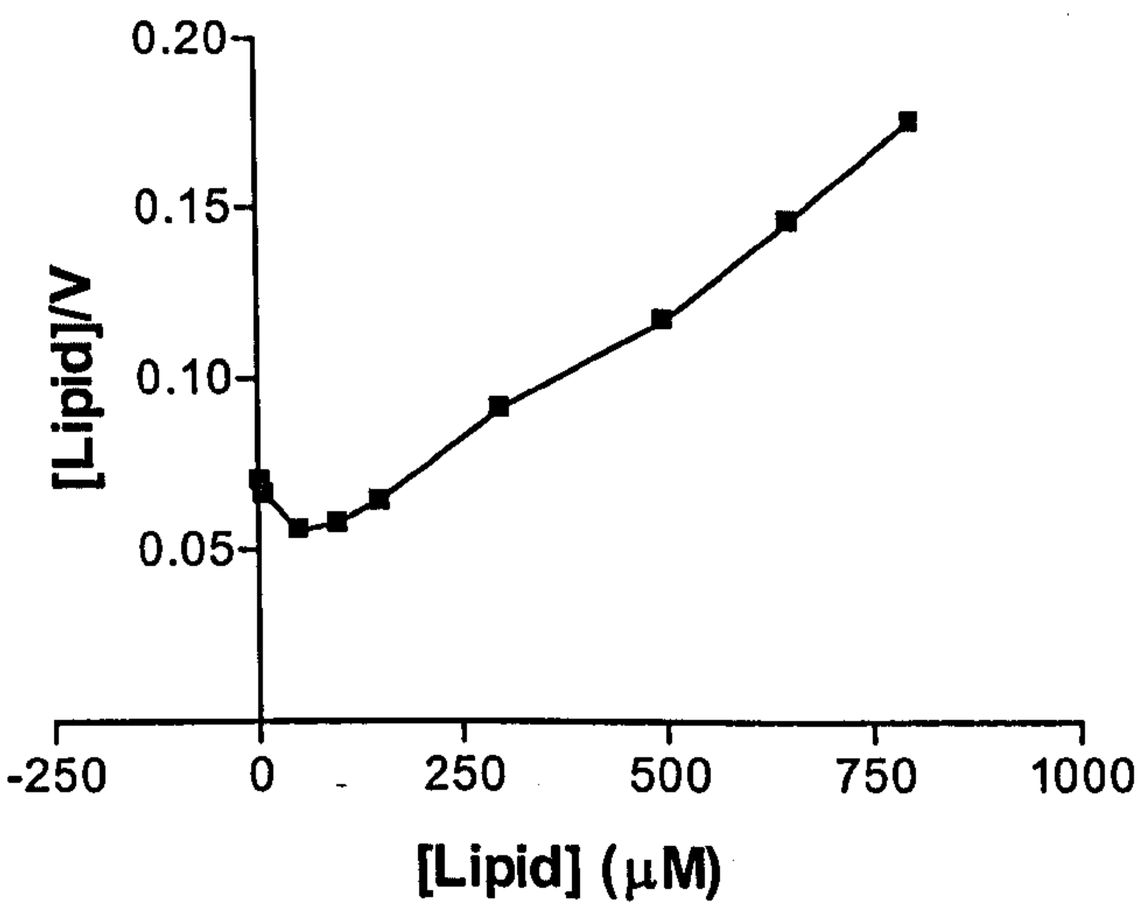
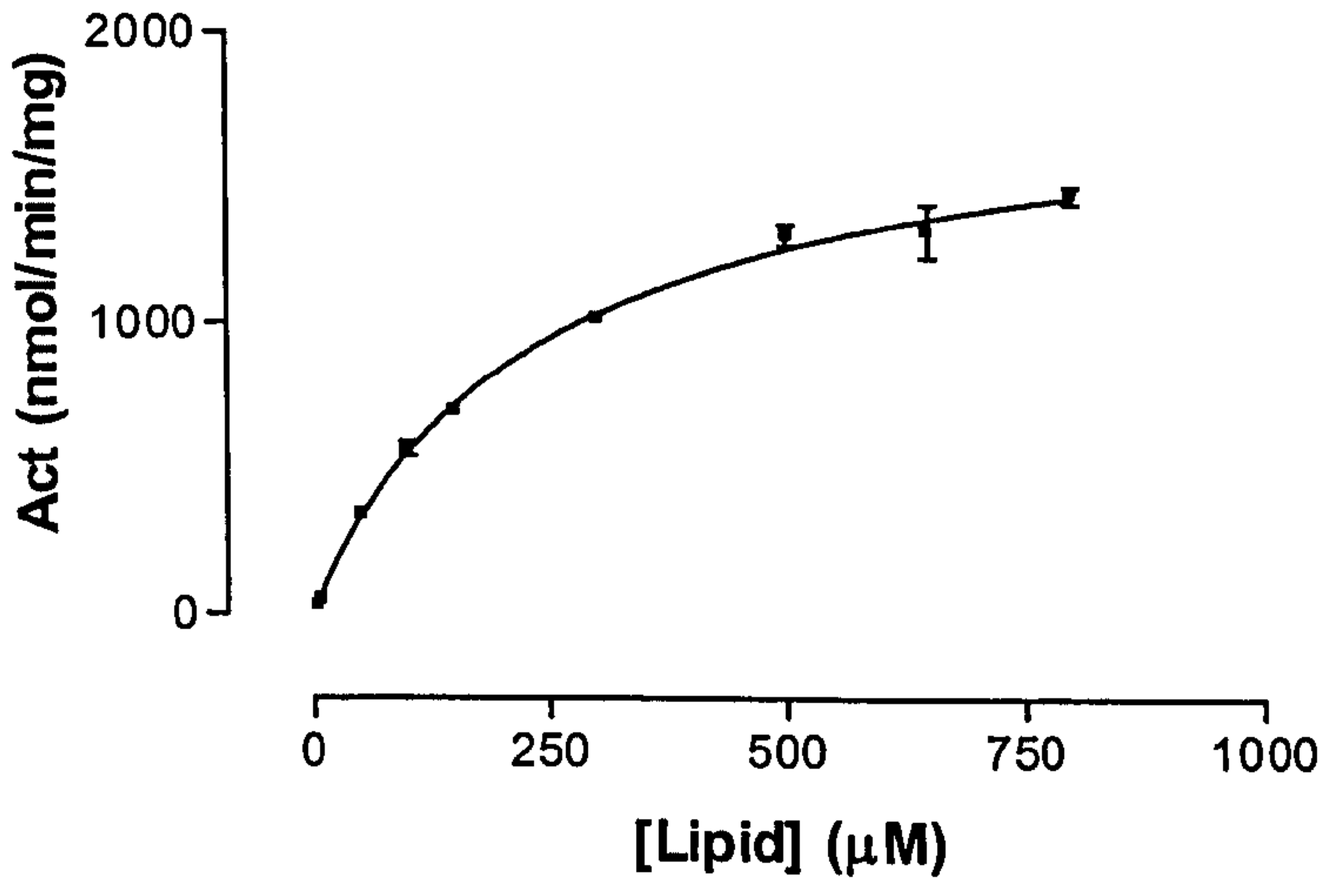


Figure 4.8: Substrate dependence (A) and Hanes plot (B) of LPP1a activity against [^{32}P]-PA $_{(8:0,8:0)}$.

LPP1a activity towards [^{32}P]-PA$_{(8:0/8:0)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1a as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



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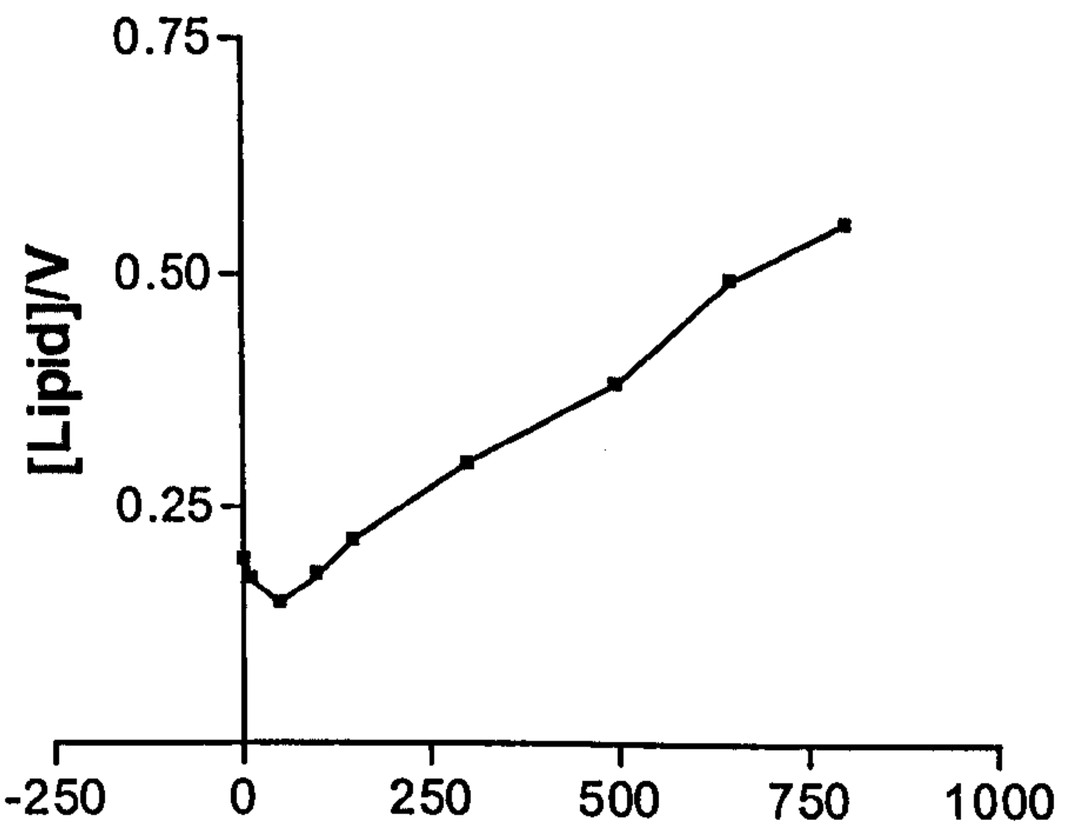


Figure 4.9: Substrate dependence (A) and Hanes plot (B) of LPP2 activity against [^{32}P]-PA $_{(8:0,8:0)}$.

LPP2 activity towards [^{32}P]-PA$_{(8:0/8:0)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP2 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

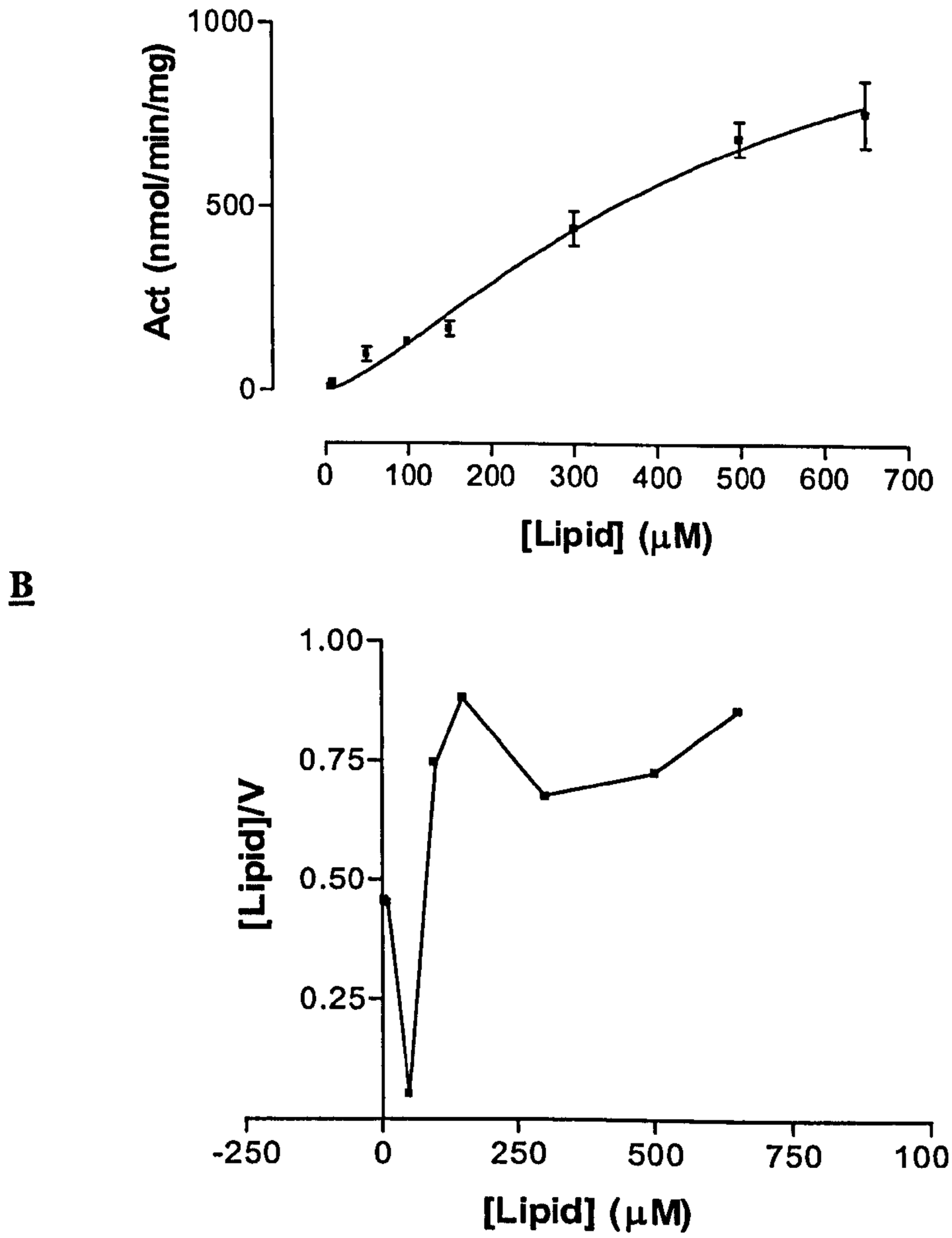


Figure 4.10: Substrate dependence (A) and hanes plot (B) of LPP3 activity against [^{32}P]-PA $_{(8:0,8:0)}$.

LPP3 activity towards [^{32}P]-PA$_{(8:0/8:0)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP3 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in A are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot B.

	Vmax (nmol/min/mg)	$K_{0.5}$ (μM)	Vmax/$K_{0.5}$	H
LPP1	8768	289	30.31	1.14
LPP1a	5495	197	27.92	1.18
LPP2	1878	246	7.63	0.98
LPP3	1284	479	2.68	1.4

Table 4.2: Kinetic constants for LPP1, LPP1a, LPP2 and LPP3 against $PA_{(8:0/8:0)}$ presented in multi-substrate TX-100 micelles with $PA_{(18:1/18:1)}$

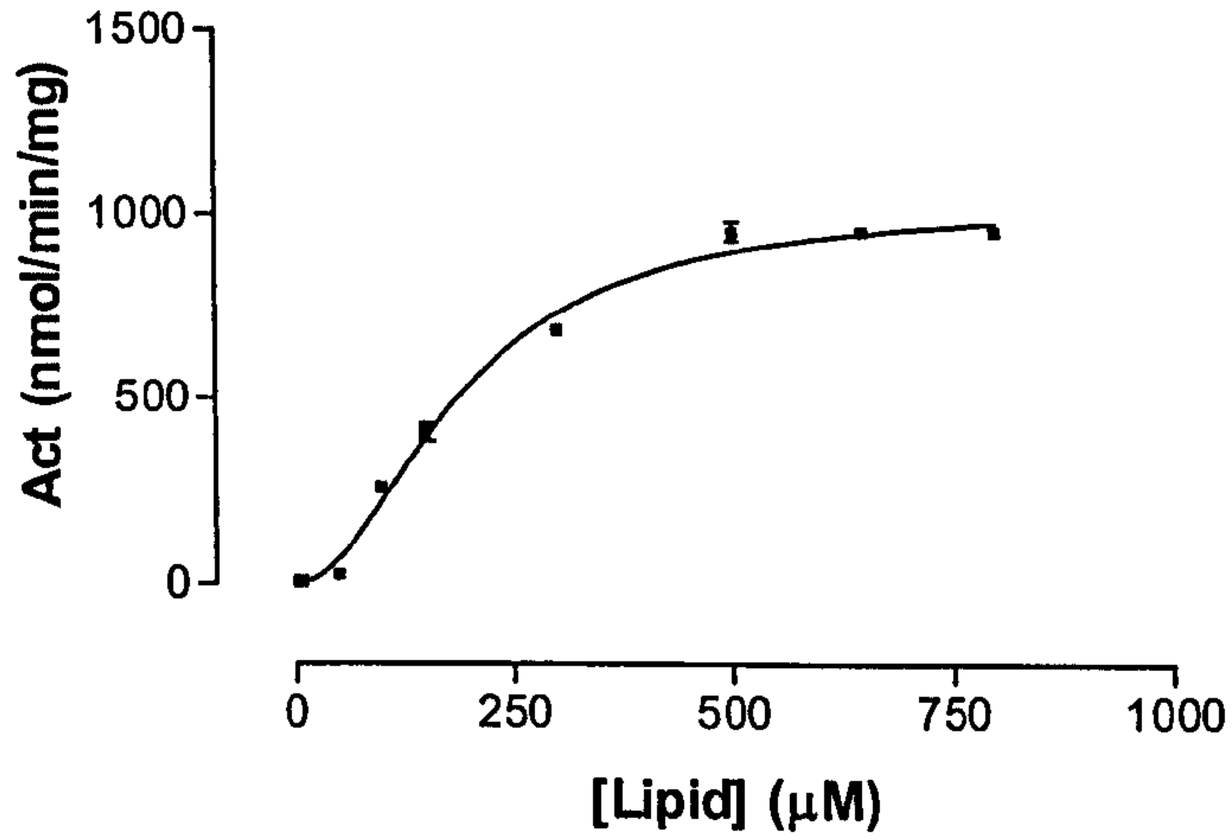
LPP activity towards [^{32}P]-$PA_{(8:0/8:0)}$ was measured in crude membranes derived from HEK293 cells stably overexpressing LPP1, LPP1a, LPP2 or LPP3 as described in Section 2.6. Kinetic constants [apparent Vmax (nmol/min/mg), $K_{0.5}$ (μM)] and Hill coefficients (h) were derived by weighted, non-linear regression of the data presented in Figures 4.7 to 4.10 to the Hill equation [$v = Vmax * A^h / (K_{0.5}^h + A^h)$] using GraphPad Prism™ (version 2.01).

$\underline{PA_{(18:0/20:4)}}$

Figures 4.11A to 4.13A, illustrate the velocity versus substrate concentration plots for LPP1, LPP1a and LPP2 respectively, towards $[^{32}P]$-$PA_{(18:0/20:4)}$ assayed in the presence of $PA_{(18:1/18:1)}$. Interestingly, no activity could be detected for LPP3 against $[^{32}P]$-$PA_{(18:0/20:4)}$ under the assay conditions used. However, increasing the concentration of $PA_{(18:1/18:1)}$ was found to enhance the activity of LPP1, LPP1a and LPP2 towards the alternative substrate $[^{32}P]$-$PA_{(18:0/20:4)}$ in a saturable manner. The velocity versus substrate concentration plots detailed in Figures 4.11A to 4.13A were also sigmoidal and so indicative of cooperative enzymes.

The kinetic constants (apparent Vmax, $K_{0.5}$) and h values obtained for each of the LPPs towards $[^{32}P]$-$PA_{(18:0/20:4)}$ are shown in Table 4.3. The apparent Vmax and $K_{0.5}$ values show a degree of variability with apparent Vmax values of 1032, 1218 and 365 nmol/min/mg and apparent $K_{0.5}$ values of 191, 154 and 137μM for LPP1, LPP1a and LPP2 respectively. The h values obtained show little variability with values of 1.97, 1.77 and 1.91 for LPP1, LPP1a and LPP2 respectively. These values were in agreement with the suggestion of cooperativity obtained from the sigmoidal velocity versus substrate plots. The Hanes plots shown in Figures 4.11B to 4.13B were also in agreement with the pattern of cooperativity suggested by the velocity versus substrate plots and the h values in that a marked upward turn in the plots for LPP1, LPP1a and LPP2 was apparent (Figures 4.11B to 4.13B respectively). The results indicate that, under the conditions used in this investigation, LPP1, LPP1a and LPP2 show a marked degree of positive substrate cooperativity towards $PA_{(18:0/20:4)}$. The specificity constants for the enzymes were calculated as 5.4, 7.91 and 2.66 for LPP1, LPP1a and LPP2 respectively (Table 4.3), suggesting a rank order of ability to dephosphorylate $PA_{(18:0/20:4)}$ of LPP1a>LPP1>LPP2. In notable contrast, LPP3 failed to dephosphorylate $PA_{(18:0/20:4)}$ under the assay conditions employed.

A



B

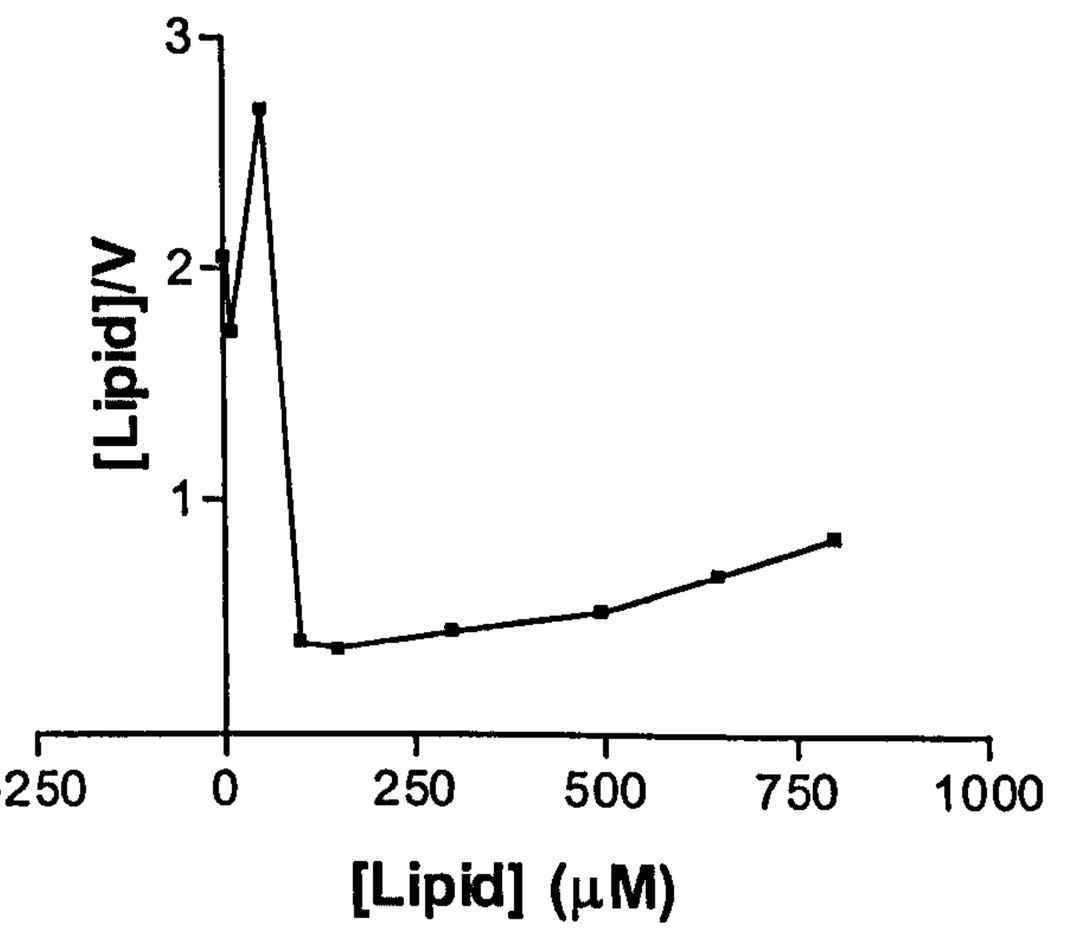
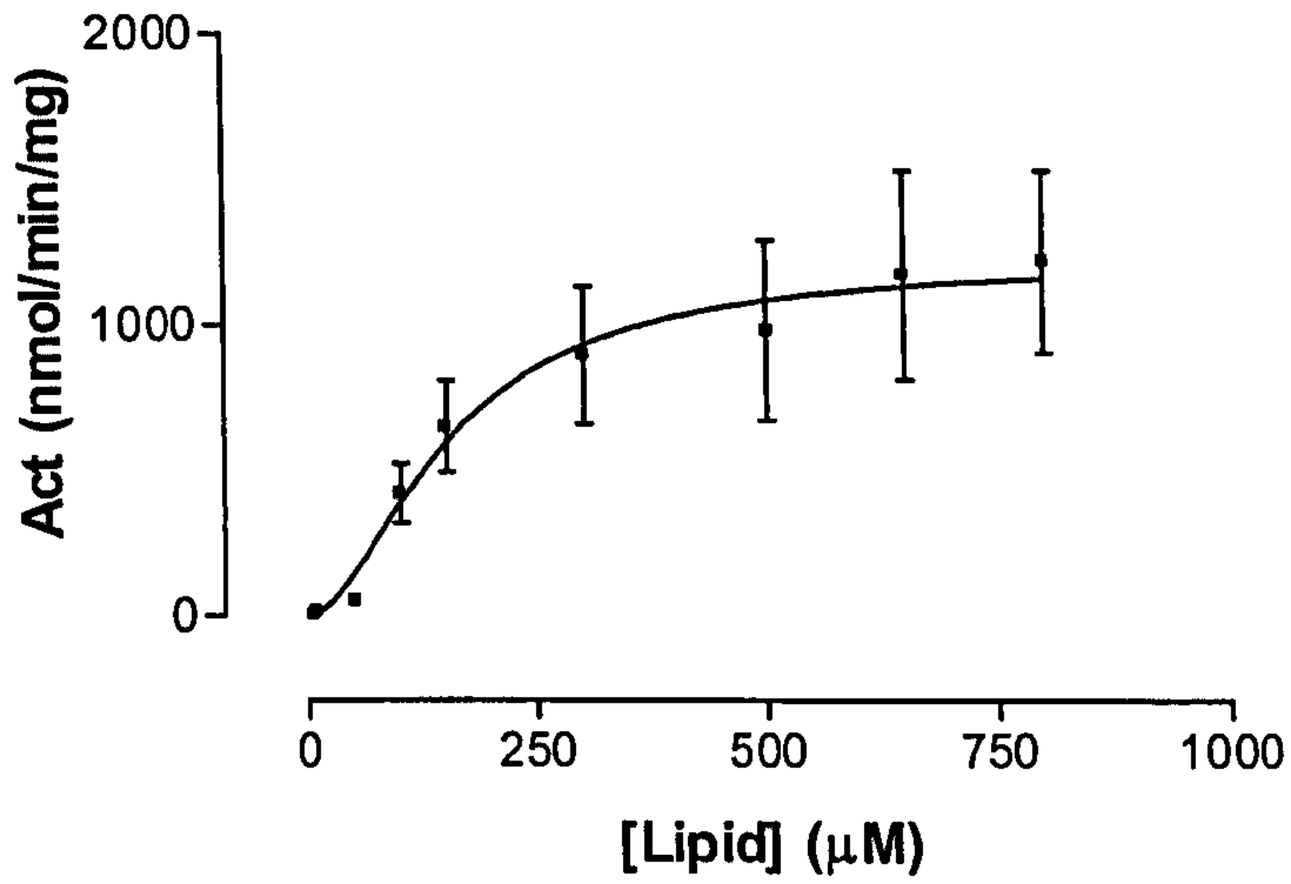


Figure 4.11: Substrate dependence (A) and Hanes plot (B) of LPP1 activity against [^{32}P]-PA $_{(18:0,20:4)}$.

LPP1 activity towards [^{32}P]-PA$_{(18:0/20:4)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



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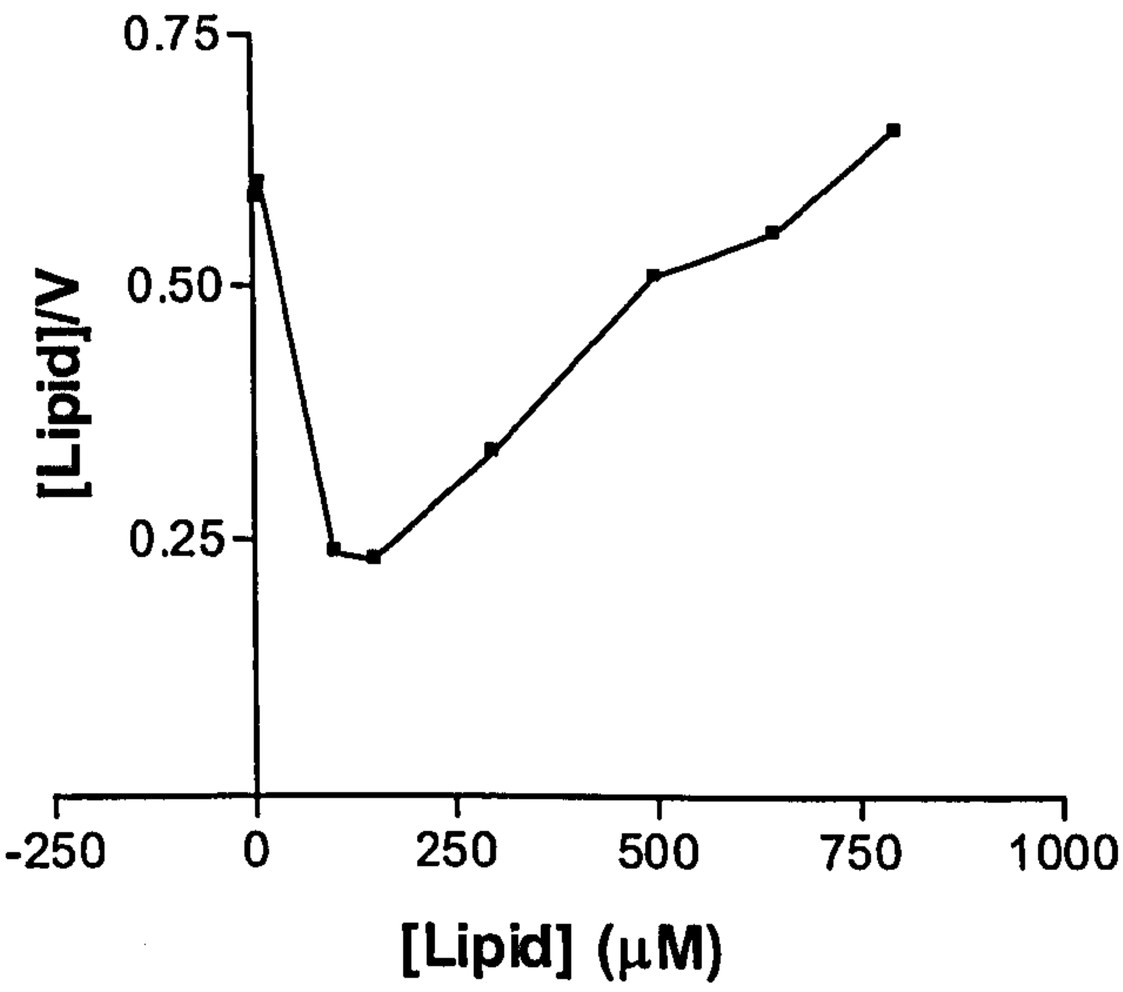
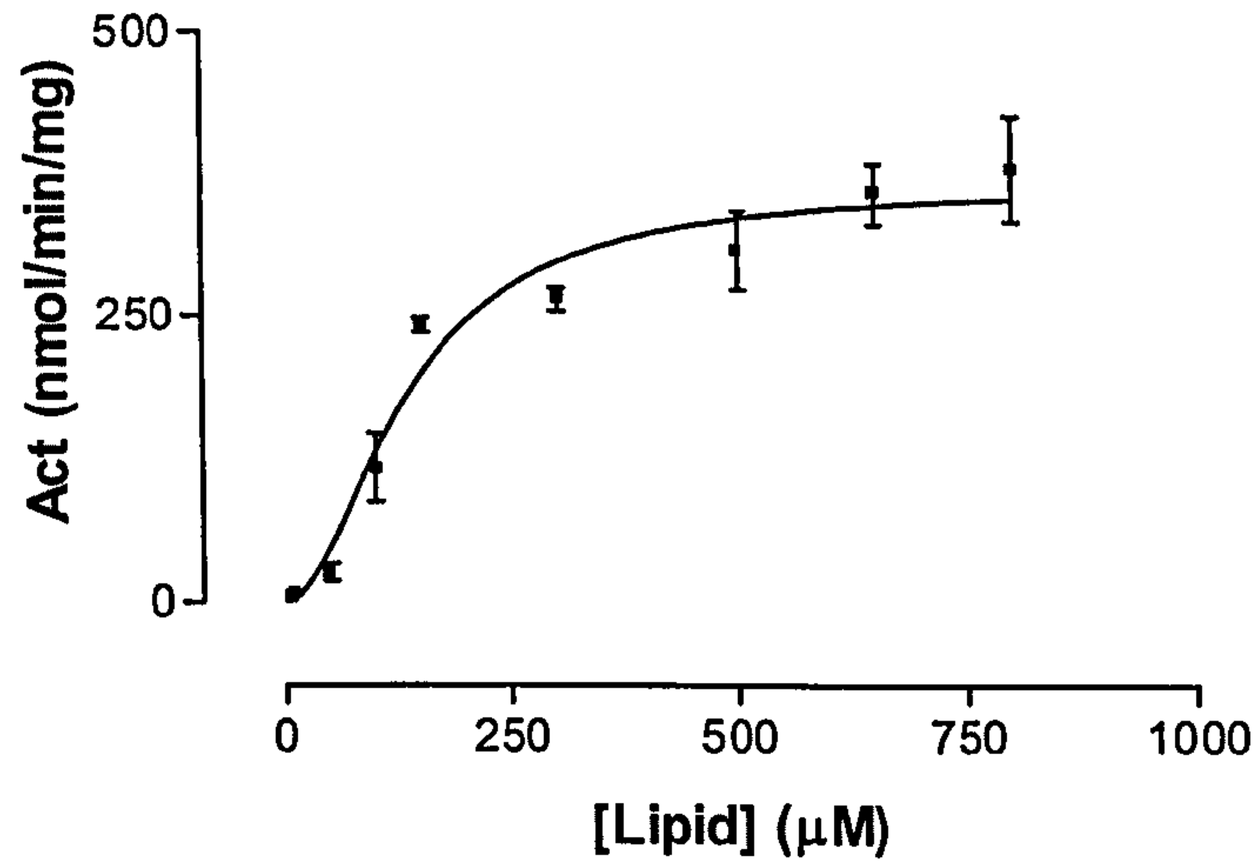


Figure 4.12: Substrate dependence (A) and Hanes plot (B) of LPP1a activity against [^{32}P]-PA $_{(18:0,20:4)}$.

LPP1a activity towards [^{32}P]-PA$_{(18:0/20:4)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1a as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



B

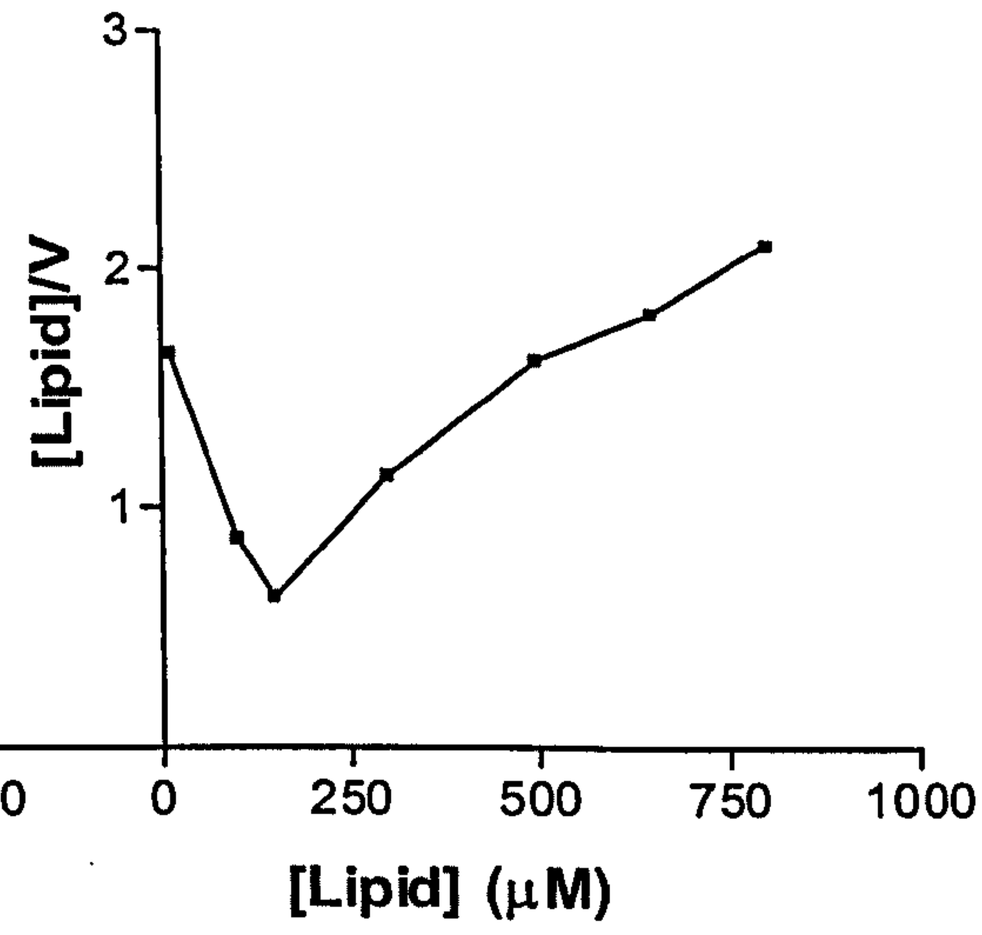


Figure 4.13: Substrate dependence (A) and Hanes plot (B) of LPP2 activity against [^{32}P]-PA $_{(18:0,20:4)}$.

LPP2 activity towards [^{32}P]-PA$_{(18:0/20:4)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP2 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

	Vmax (nmol/min/mg)	$K_{0.5}$ (μM)	Vmax/$K_{0.5}$	H
LPP1	1032	191	5.4	1.97
LPP1a	1218	154	7.91	1.77
LPP2	365	137	2.66	1.91
LPP3	N/A	N/A	N/A	N/A

Table 4.3: Kinetic constants for LPP1, LPP1a, LPP2 and LPP3 against [^{32}P]-$PA_{(18:0/20:4)}$ presented in mutisubstrate TX-100 micelles with $PA_{(18:1/18:1)}$

LPP activity towards [^{32}P]-$PA_{(18:0/20:4)}$ was measured in crude membranes derived from HEK293 cells stably overexpressing LPP1, LPP1a, LPP2 or LPP3 as described in Section 2.6. Kinetic constants [apparent Vmax (nmol/min/mg), $K_{0.5}$ (μM)] and Hill coefficients (h) were derived by weighted, non-linear regression of the data presented in Figures 4.11 to 4.13 to the Hill equation [$v = Vmax * A^h / (K_{0.5}^h + A^h)$] using GraphPad Prism™ (version 2.01). N/A – not applicable.

$\underline{PA_{(16:0/16:0)}}$

The velocity versus substrate concentration curves for LPP1, LPP1a and LPP2 against $[^{32}P]$-$PA_{(16:0/16:0)}$ assayed in the presence of $PA_{(18:1/18:1)}$ are shown in Figures 4.14A to 4.16A respectively. As with the results obtained for $PA_{(18:0/20:4)}$, no measurable activity was detected for LPP3 against $PA_{(16:0/16:0)}$. Furthermore, the velocity versus substrate curves for LPP1 and LPP1a are seen to have comparably shallow slopes when compared to the curves obtained for the LPPs against the previous PA substrates and also compared to the curve obtained for LPP2 against $PA_{(16:0/16:0)}$ (Figure 4.16A). This therefore, demonstrates a variation between LPP1 and LPP1a compared to LPP2 where, as shown in figure 4.16A, LPP2 produced a hyperbolic velocity versus substrate curve which is characteristic of an enzyme which follows typical Michaelis-Menten kinetics. Increasing the concentration of $PA_{(18:1/18:1)}$ in the assay, enhanced the activity of LPP1, LPP1a and LPP2 towards $[^{32}P]$-$PA_{(16:0/16:0)}$ in an apparently saturable manner although this was less obvious for LPP1 and LPP1a compared to LPP2.

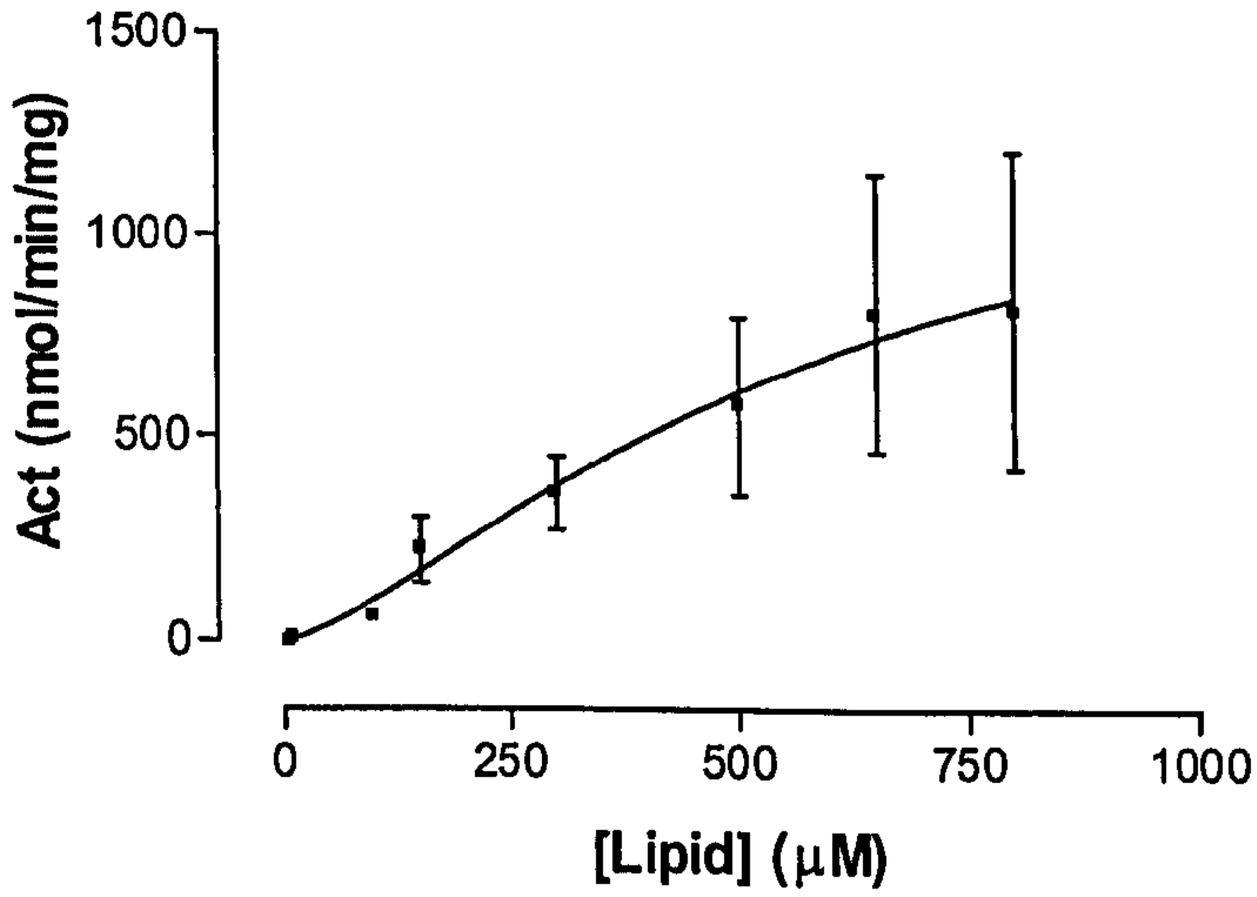
The kinetic constants (apparent Vmax and $K_{0.5}$) and h values obtained for LPP1, LPP1a and LPP2 against $[^{32}P]$-$PA_{(16:0/16:0)}$ are shown in Table 4.4. The apparent Vmax and $K_{0.5}$ values obtained for LPP1 and LPP1a are seen to be within similar ranges with apparent Vmax values of 1367 and 1480 nmol/min/mg and $K_{0.5}$ values of 577 and 417μM respectively. Furthermore, the h values are also seen to be similar with values of 1.47 and 1.32 for LPP1 and LPP1a respectively. The equivalent values are seen to be considerably different for LPP2 against $[^{32}P]$-$PA_{(16:0/16:0)}$ with an apparent Vmax of 46 nmol/min/mg, an apparent $K_{0.5}$ of 104μM and an h value of 0.86.

The Hanes plots obtained for LPP1, LPP1a and LPP2 against $[^{32}P]$-$PA_{(16:0/16:0)}$ are shown in Figures 4.14B to 4.16B respectively and these further highlight the potential differences between the kinetics of LPP1 and LPP1a compared to LPP2 under the conditions employed. The Hanes plots for LPP1 and LPP1a (Figures 4.14B and 4.15B respectively) display sharp upward turns in the plots suggesting the

presence of positive substrate cooperativity. In contrast, the Hanes plot for LPP2 (Figure 4.16B) is linear, indicative of typical Michaelis-Menten kinetics.

The specificity constants calculated suggest greater catalytic efficiency with respect to [^{32}P]-$PA_{(16:0/16:0)}$ for LPP1 and LPP1a compared to LPP2 with values of 2.37, 3.55 and 0.44 respectively (Table 4.4). Furthermore, these values suggest a rank order of ability to dephosphorylate $PA_{(16:0/16:0)}$ of LPP1a>LPP1>>LPP2.

A



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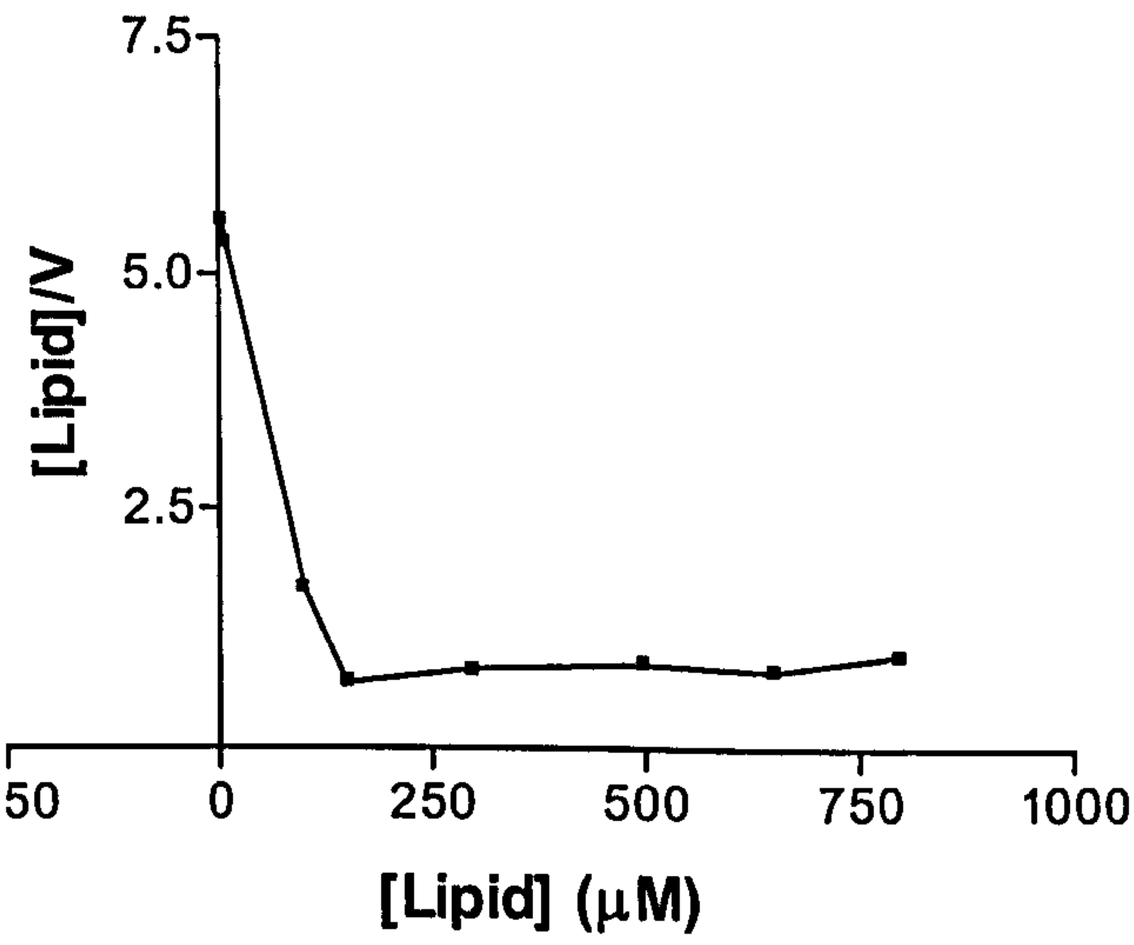
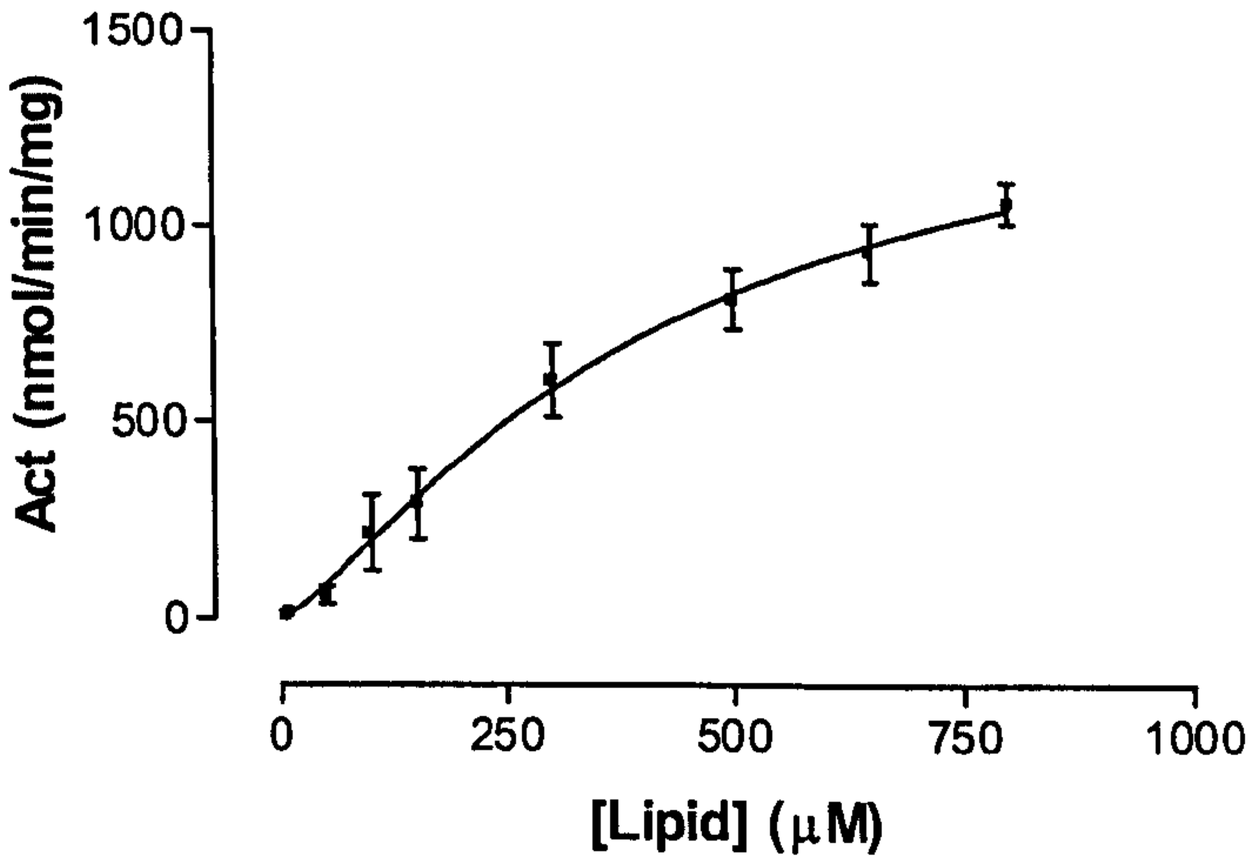


Figure 4.14: Substrate dependence (A) and Hanes plot (B) of LPP1 activity against [^{32}P]-PA $_{(16:0/16:0)}$.

LPP1 activity towards [^{32}P]-PA$_{(16:0/16:0)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



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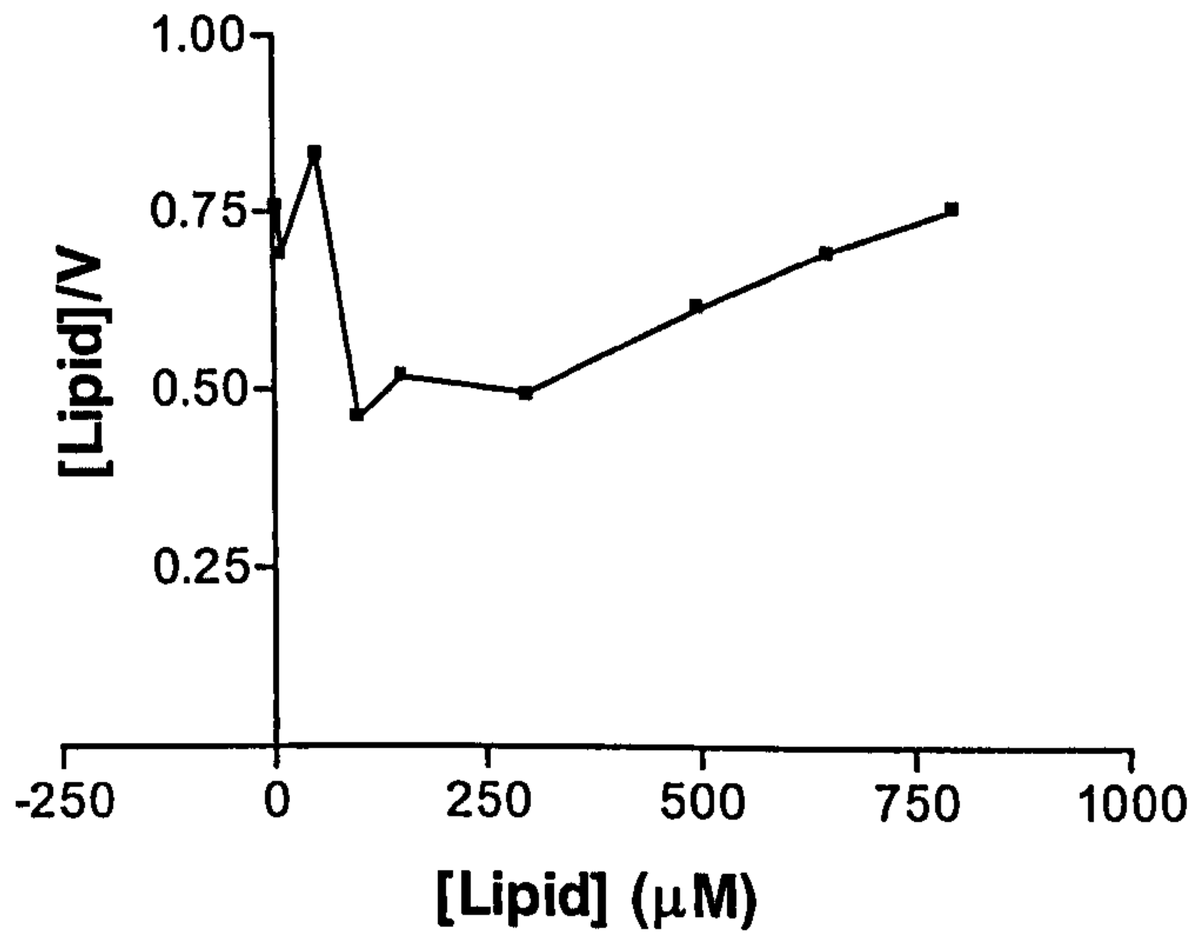


Figure 4.15: Substrate dependence (A) and Hanes plot (B) of LPP1a activity against [^{32}P]-PA $_{(16:0/16:0)}$.

LPP1a activity towards [^{32}P]-PA$_{(16:0/16:0)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1a as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

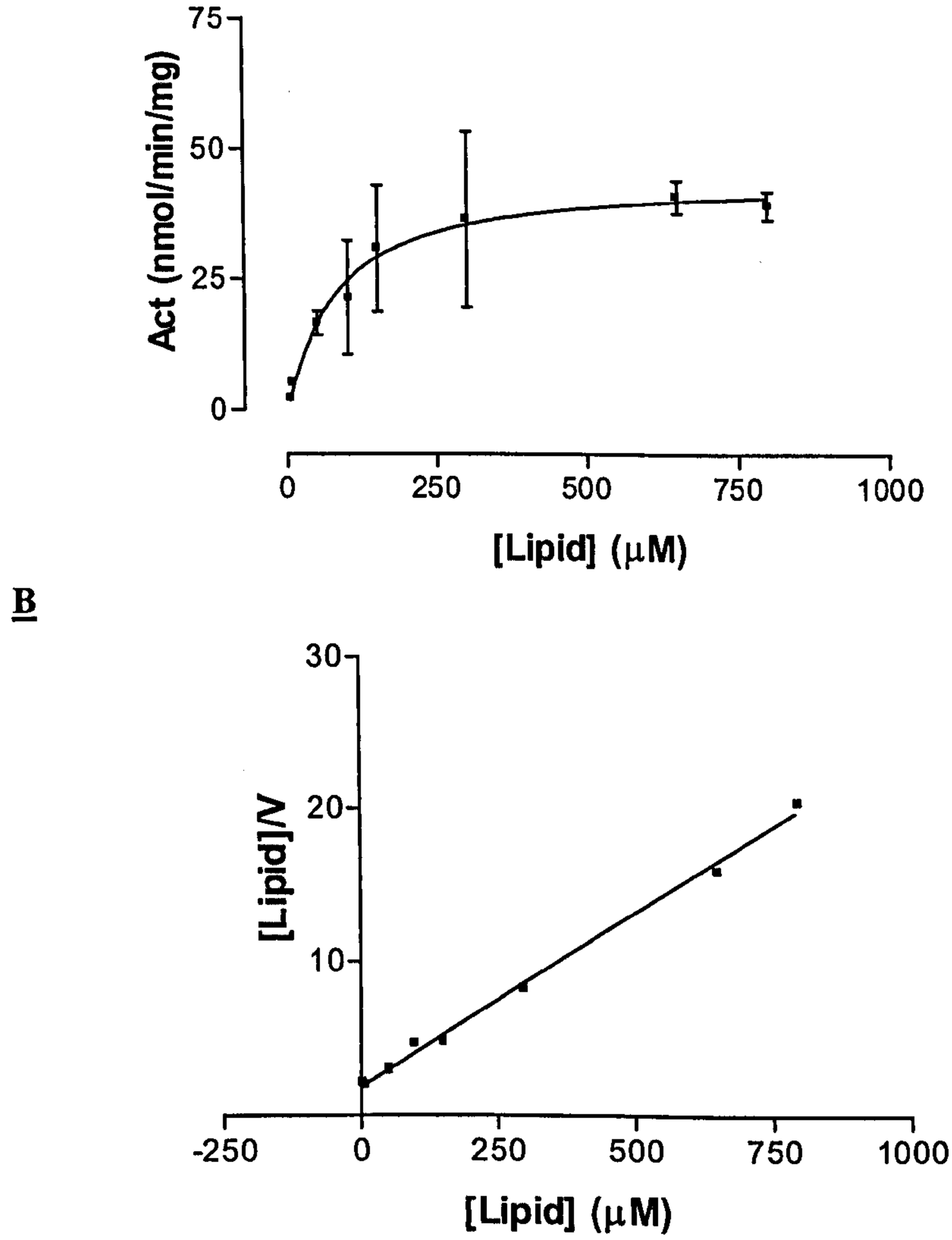


Figure 4.16: Substrate dependence (A) and Hanes plot (B) of LPP2 activity against [^{32}P]-PA $_{(16:0/16:0)}$.

LPP2 activity towards [^{32}P]-$PA_{(16:0/16:0)}$ was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP2 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

	Vmax (nmol/min/mg)	$K_{0.5}$ (μM)	Vmax/$K_{0.5}$	H
LPP1	1367	577	2.37	1.47
LPP1a	1480	417	3.55	1.32
LPP2	46	104	0.44	0.86
LPP3	N/A	N/A	N/A	N/A

Table 4.4: Kinetic constants for LPP1, LPP1a, LPP2 and LPP3 against $PA_{(16:0/16:0)}$ presented in mutisubstrate TX-100 micelles with $PA_{(18:1/18:1)}$

LPP activity towards $PA_{(16:0/16:0)}$ was measured in crude membranes derived from HEK293 cells stably transfected with LPP1, LPP1a, LPP2 and LPP3 as described in Section 2.6. Kinetic constants [apparent Vmax (nmol/min/mg), $K_{0.5}$ (μM)] and Hill coefficients (h) were derived by weighted, non-linear regression of the data presented in figures 4.14 to 4.16 to the Hill equation [$v = Vmax * A^h / (K_{0.5}^h + A^h)$] using GraphPad Prism™ (version 2.01).

4.2.4. Kinetic analysis of LPP1, LPP1a, LPP2 and LPP3 towards the alternative LPP substrates $LPA_{(18:1)}$, C8-C1P and S1P presented in a mixed substrate assay with $PA_{(18:1/18:1)}$

The LPPs, in addition to dephosphorylating PA, are known to hydrolyse LPA, C1P and S1P (for reviews, see Brindley & Waggoner, 1998, Waggoner et al., 1999). Therefore, the kinetic analysis of the LPPs using the multi-substrate assay system with $PA_{(18:1/18:1)}$, was extended to investigate the kinetics of the LPPs towards $LPA_{(18:1)}$, C8-C1P and S1P. Initial rates of hydrolysis of each alternative substrate were measured therefore, as the concentration of $PA_{(18:1/18:1)}$ was increased while maintaining the lipid:TX-100 ratio at a constant 1:10 (constant mole fraction 0.091). As with the previous substrates investigated, similar measurements were obtained for blank vector transfected cells and the initial rates subtracted from the data obtained for each of the LPP overexpressing cell lines. The data sets were then analysed in the same manner as described in section 4.2.2. for $PA_{(18:1/18:1)}$.

<u>$LPA_{(18:1)}$</u>

The velocity versus substrate concentration curves for LPP1, LPP1a, LPP2 and LPP3 against [^{32}P]-$LPA_{(18:1)}$ assayed in the presence of $PA_{(18:1/18:1)}$ are shown in Figures 4.17A to 4.20A respectively. Under the assay condition used, increasing the concentration of $PA_{(18:1/18:1)}$ was found to enhance the hydrolysis of the alternative substrate $LPA_{(18:1)}$ by all four LPP isoforms. In the cases of LPP2 and LPP3, this activity appeared saturable and the velocity versus substrate curves are seen to be slightly sigmoidal in shape indicating the presence of substrate cooperativity. In contrast, the data obtained against [^{32}P]-$LPA_{(18:1)}$ for LPP1 and LPP1a displayed an interesting feature which could not be applied to the Hill equation to fit a curve. As shown in Figures 4.17A and 4.18A for LPP1 and LPP1a respectively, at high substrate concentrations, an apparent inhibition of activity was observed. This excess substrate inhibition is normally not observed or relevant when substrate concentrations are kept at or below their likely physiological concentrations (Cornish-Bowden, 1995, Dr P. Birch – personal communication). However, this

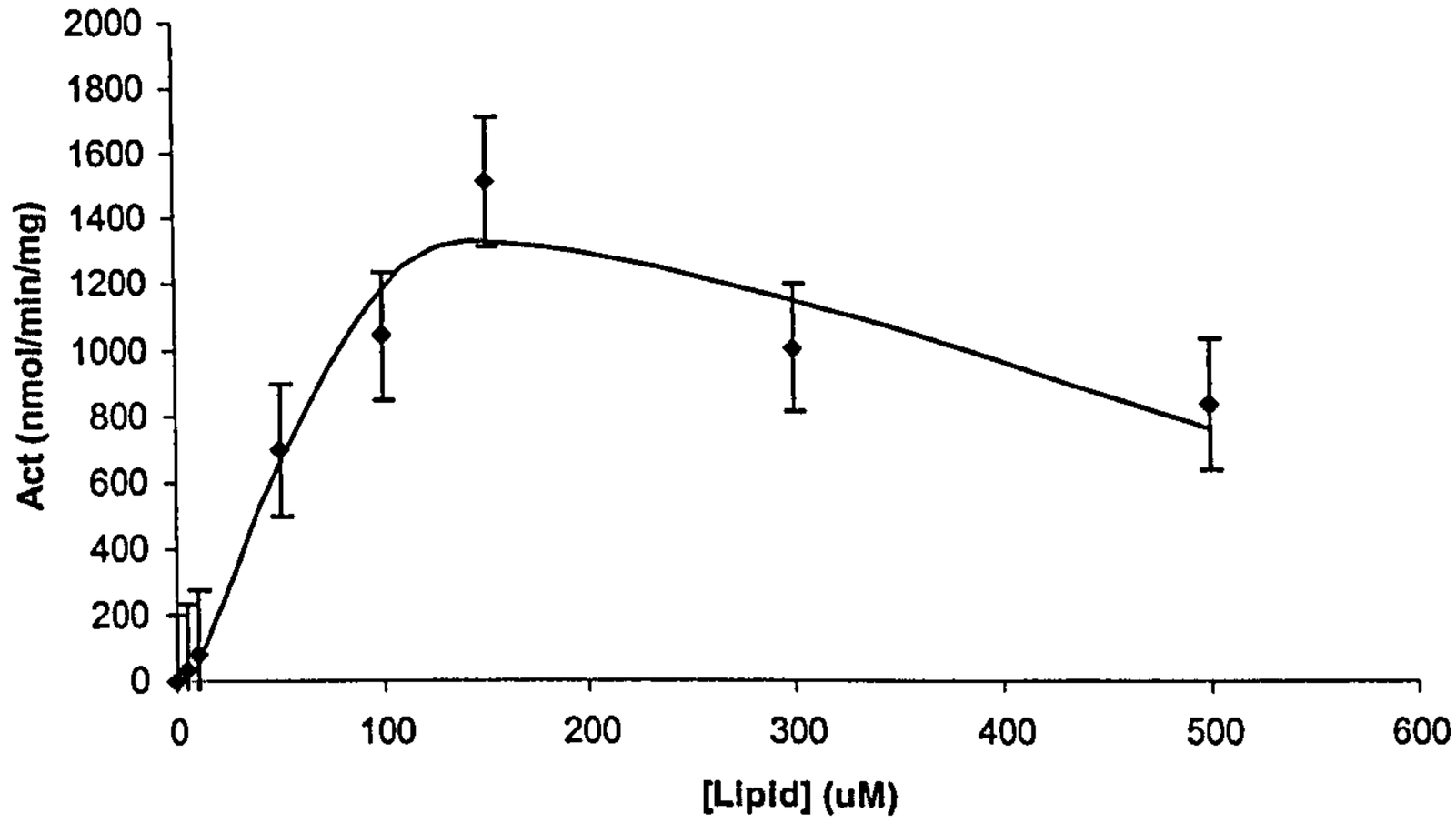
factor complicates the kinetic analysis of data sets such that an inhibitor constant needs to be applied to the data analysis. Therefore, for these data sets, curvefit analysis was conducted using the solver add-in of Microsoft® Excel 1997 to the equation $v = Vmax * A^h / [K_{0.5}{}^h + A^h (1 + A^h/K_{is})]$; where all variables are identical to those in the Hill equation (section 4.2.2.) but where K_{is} represents the apparent constant for inhibition by the substrates (Dr P. Birch – personal communication; Cornish-Bowden, 1995). The K_{is} values may be of limited relevance physiologically due to the high concentrations of substrate required to induce the effect. However, as shown in figures 4.17A and 4.18A, this equation provides a reasonable explanation (i.e. a combination of cooperativity and substrate inhibition) of the data sets and enabled a curve to be fitted to the data and the kinetic constants to be derived. Thus, there is a considerable difference between LPP1 and LPP1a compared to LPP2 and LPP3 with respect to the hydrolysis of $[^{32}P]$-$LPA_{(18:1)}$ under the assay conditions employed in this study.

The kinetic constants (apparent Vmax and $K_{0.5}$) and h values obtained for each of the LPPs against $[^{32}P]$-$LPA_{(18:1)}$ are shown in Table 4.5. The apparent Vmax values vary considerably with values of 1786, 2116, 4279 and 727 nmol/min/mg for LPP1, LPP1a, LPP2 and LPP3 respectively. In addition, a similar variability was observed in the $K_{0.5}$ values of 66, 28, 187 and 246μM for LPP1, LPP1a, LPP2 and LPP3 respectively. The h values obtained for LPP1, LPP1a, LPP2 and LPP3 were 1.91, 1.92, 1.48 and 1.42 for LPP1, LPP1a, LPP2 and LPP3 respectively, again suggesting the presence of cooperativity. In addition to these values, inhibitor constants (K_{is}) of 109688 and 109686μM were obtained for LPP1 and LPP1a respectively.

The Hanes plots obtained using the data for LPP1, LPP1a, LPP2 and LPP3 against $[^{32}P]$-$LPA_{(18:1)}$ are shown in Figures 4.17B to 4.20B respectively. The Hanes plots obtained for all the LPPs are non-linear and further indicate that the LPPs do not follow typical Michaelis-Menten kinetics to $LPA_{(18:1)}$ but do in fact display positive substrate cooperativity. However, the Hanes plot obtained for LPP1a against $[^{32}P]$-$LPA_{(18:1)}$ does not show a marked upward turn in the plot but shows a slight curve. It is possible that the Hanes plot would be linear if the excess substrate inhibition

component of the data was removed and so LPP1a may actually display more typical Michaelis-Menten kinetics towards $LPA_{(18:1)}$ but this is being disguised by the excess substrate inhibition. The specificity constants are 27.2, 76.67, 22.8 and 2.95 for LPP1, LPP1a, LPP2 and LPP3 respectively (Table 4.5) suggesting a very high catalytic efficiency for LPP1a towards $LPA_{(18:1)}$ compared to the other LPPs and an apparent rank order of ability to dephosphorylate $LPA_{(18:1)}$ of LPP1a>>LPP1≈LPP2>>LPP3.

A



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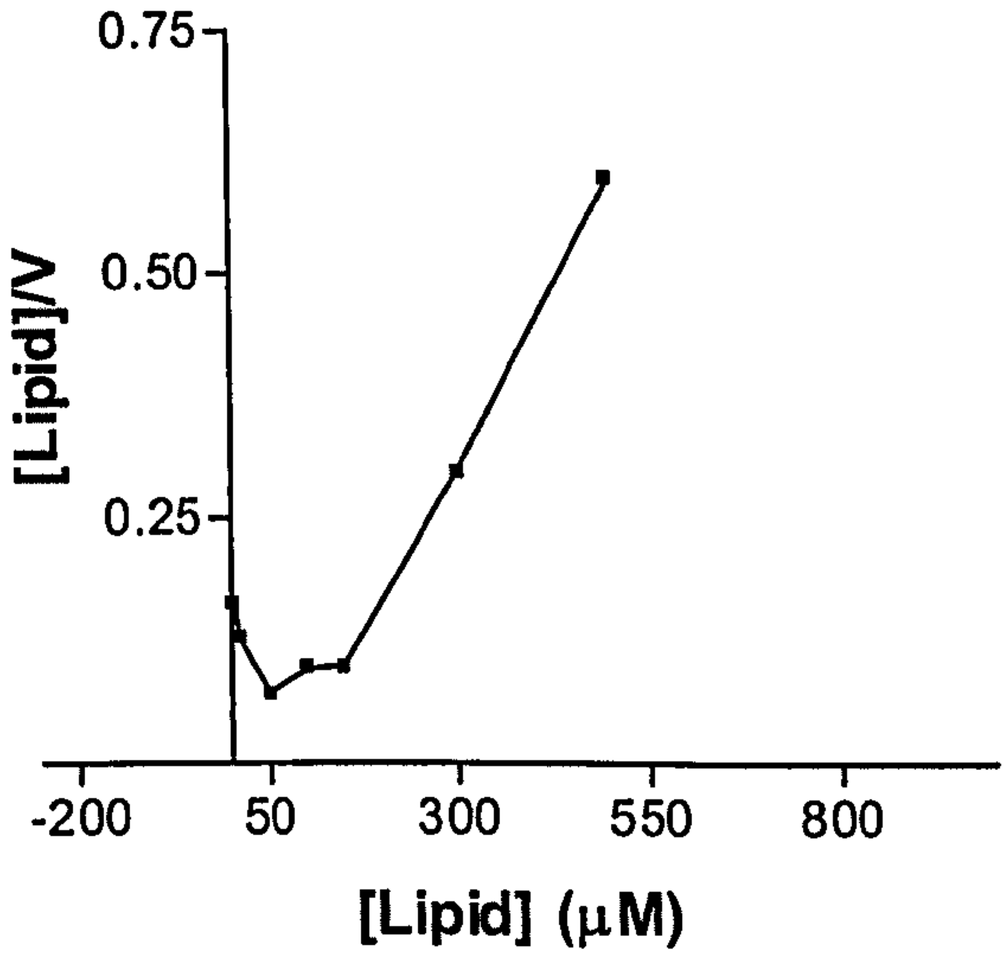
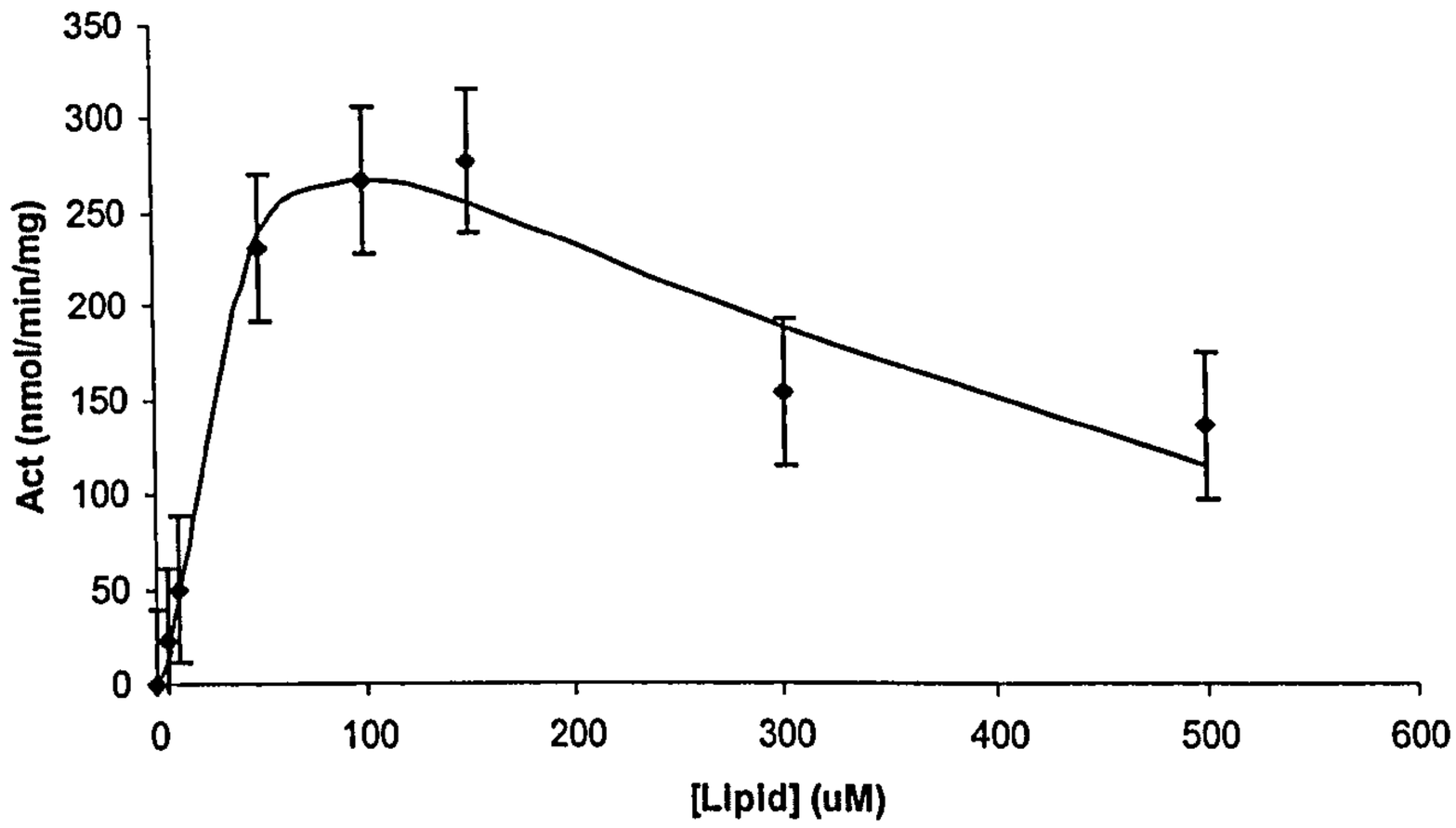


Figure 4.17: Substrate dependence (A) and Hanes plot (B) of LPP1 activity against [^{32}P]-LPA $_{(18:1)}$.

LPP1 activity towards [^{32}P]-LPA$_{(18:1)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



B

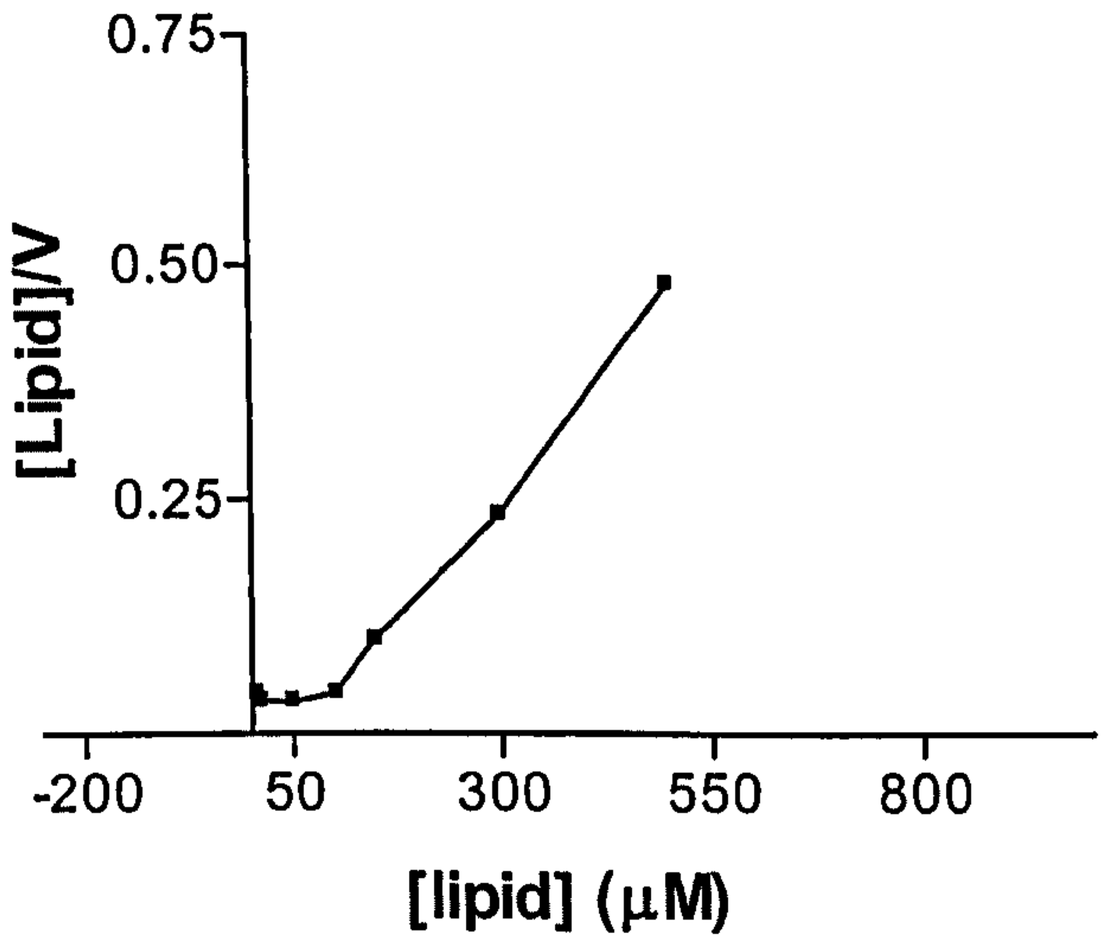
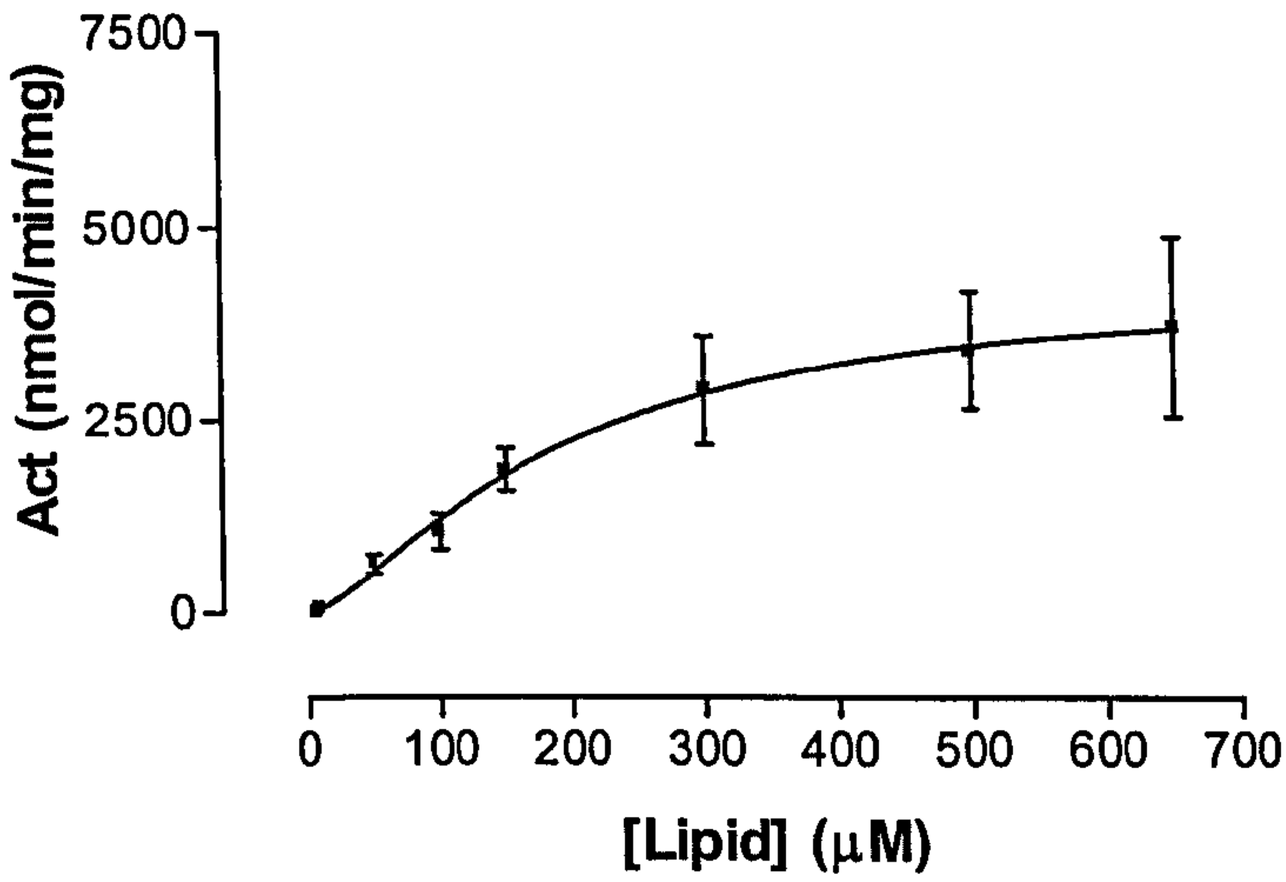


Figure 4.18: Substrate dependence (A) and Hanes plot (B) of LPP1a activity against [^{32}P]-LPA $_{(18:1)}$.

LPP1a activity towards [^{32}P]-LPA$_{(18:1)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1a as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



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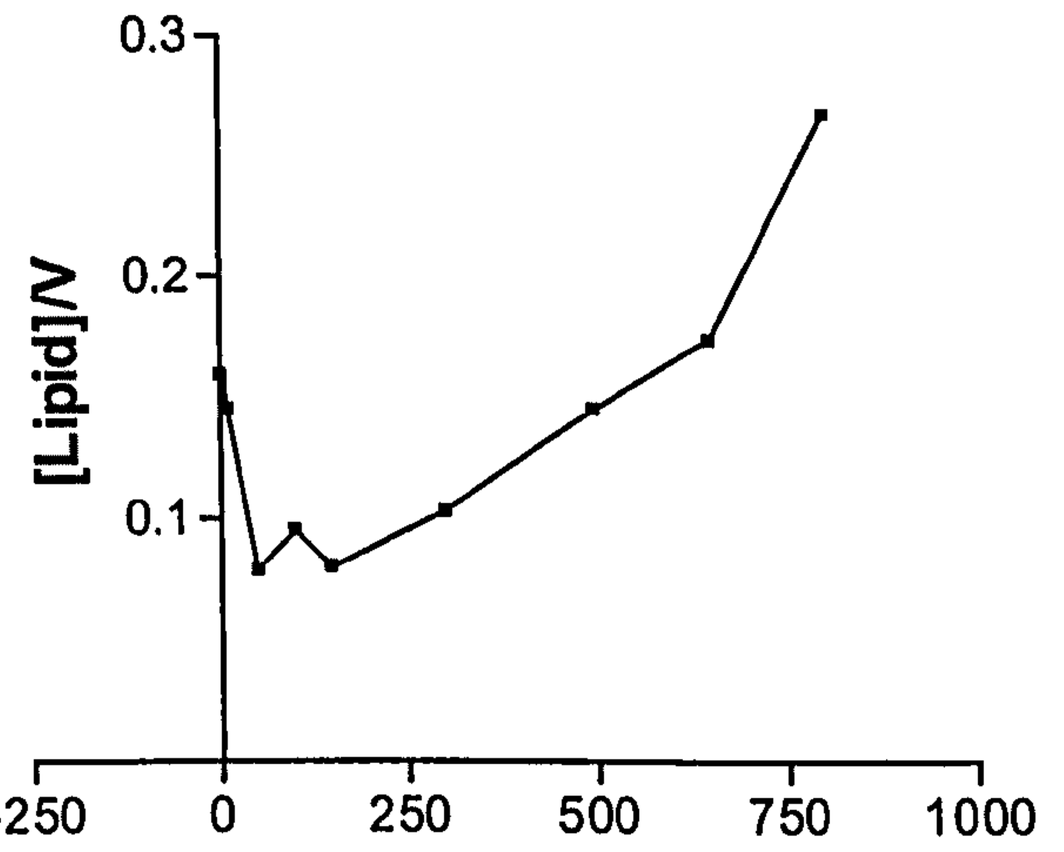
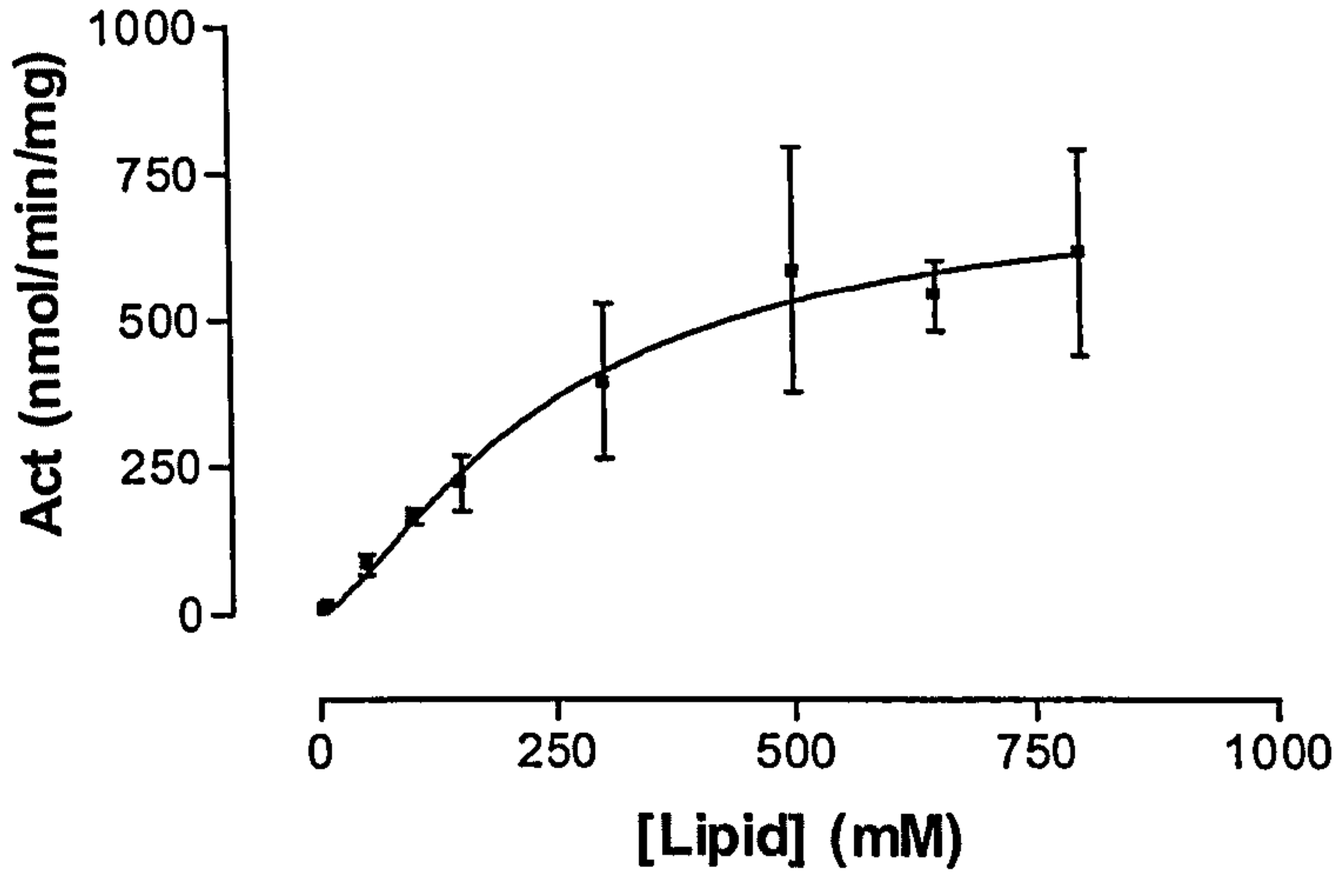


Figure 4.19: Substrate dependence (A) and Hanes plot (B) of LPP2 activity against [^{32}P]-LPA $_{(18:1)}$.

LPP2 activity towards [^{32}P]-LPA$_{(18:1)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP2 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



B

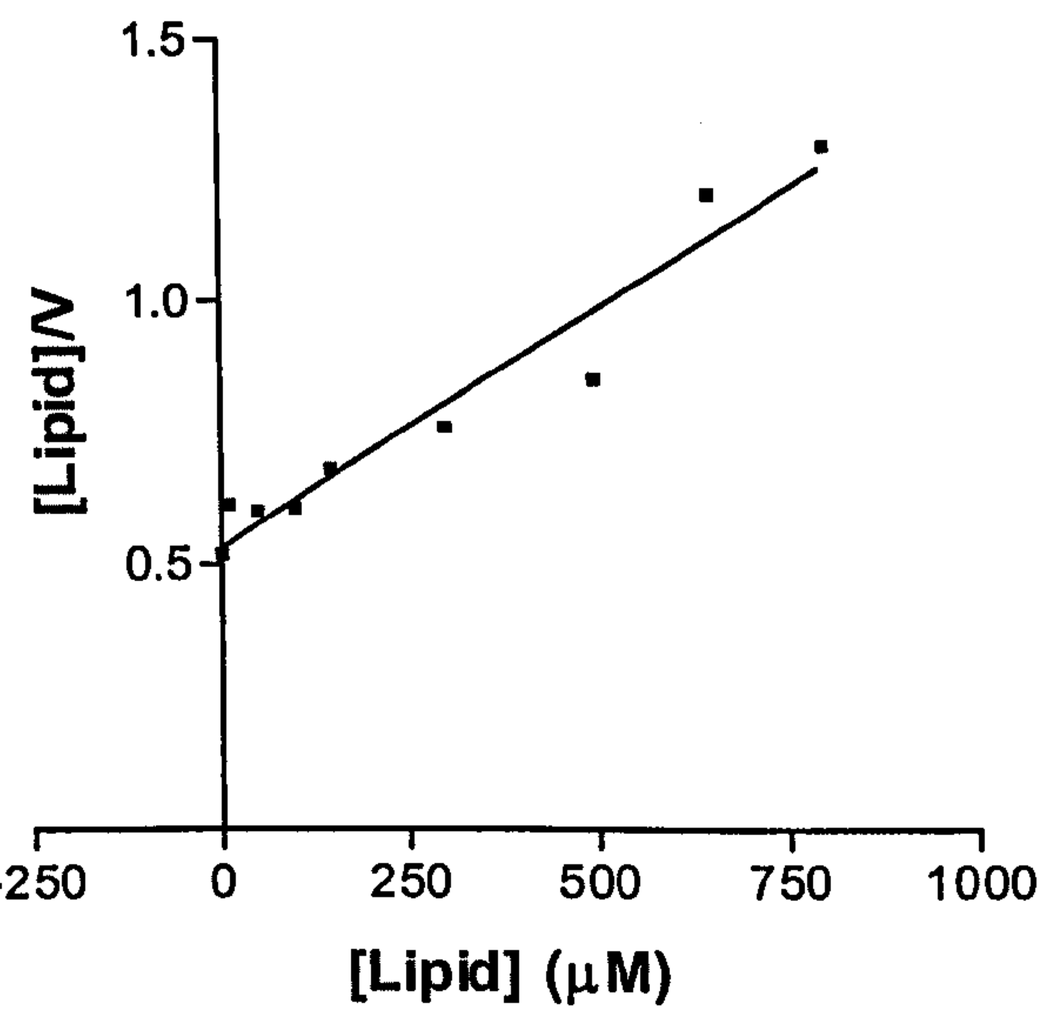


Figure 4.20: Substrate dependence (A) and Hanes plot (B) of LPP3 activity against [^{32}P]-LPA $_{(18:1)}$.

LPP3 activity towards [^{32}P]-LPA$_{(18:1)}$ was measured *in vitro* against increasing concentrations of PA$_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP3 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

	Vmax (nmol/min/mg)	$K_{0.5}$ (μM)	Vmax/$K_{0.5}$	H	K_{is} (μM)
LPP1	1786	66	27.2	1.91	109688
LPP1a	2116	28	76.67	1.92	109686
LPP2	4279	187	22.88	1.48	N/A
LPP3	727	246	2.95	1.42	N/A

Table 4.5: Kinetic constants for LPP1, LPP1a, LPP2 and LPP3 against [^{32}P]-$LPA_{(18:1)}$ presented in multi-substrate TX-100 micelles with $PA_{(18:1/18:1)}$

LPP activity towards [^{32}P]-$LPA_{(18:1)}$ was measured in crude membranes derived from HEK293 cells stably overexpressing LPP1, LPP1a, LPP2 and LPP3 as described in Section 2.6. Kinetic constants for LPP2 and LPP3 [apparent Vmax (nmol/min/mg), $K_{0.5}$ (μM)] and Hill coefficients (h) were derived by weighted, non-linear regression of the data presented in Figures 4.19 and 4.20 to the Hill equation [$v = Vmax * A^h / (K_{0.5}^h + A^h)$] using GraphPad Prism™ (version 2.01). However, the kinetic constants for LPP1 and LPP1a were derived by weighted, non-linear regression of the data presented in Figures 4.17 and 4.18 to the equation $v = Vmax * A^h / [K_{0.5}^h + A^h (1 + A^h/K_{is})]$ using Microsoft® Excel 1997. N/A – not applicable.

C8-ceramide 1-phosphate

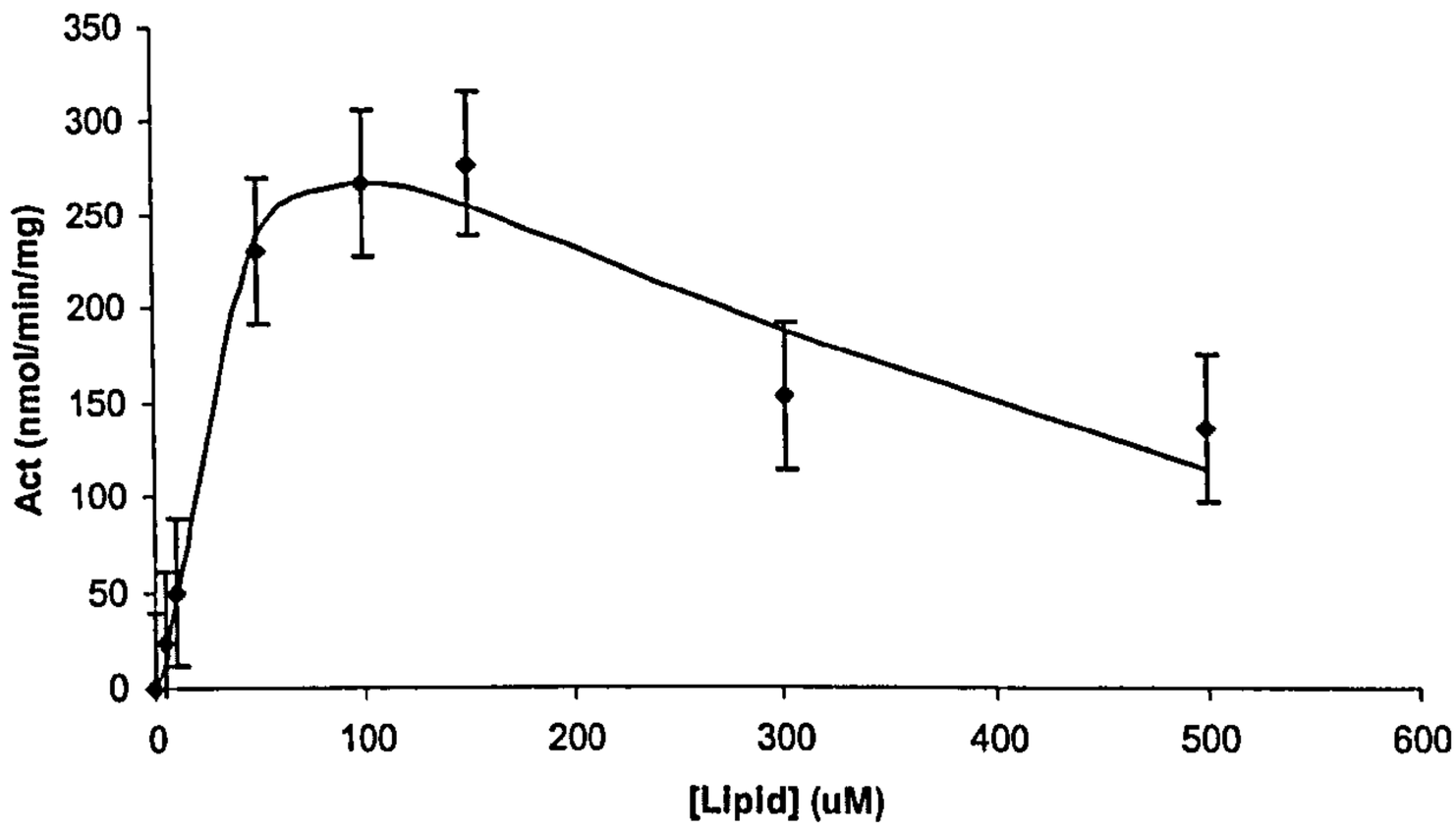
Figures 4.21A to 4.24A, show the velocity versus substrate concentration curves obtained for LPP1, LPP1a, LPP2 and LPP3 respectively towards [^{32}P]-C8-C1P assayed in the presence of $PA_{(18:1/18:1)}$. As with all other substrates tested, except [^{32}P]-$PA_{(18:0/20:4)}$ and [^{32}P]-$PA_{(16:0/16:0)}$ for LPP3, increasing the concentration of the LPP substrate $PA_{(18:1/18:1)}$ in the assay, enhanced the activity of all LPP isoforms towards the alternative LPP substrate [^{32}P]-C8-C1P. As with the results obtained for LPP1 and LPP1a against $LPA_{(18:1)}$, there is clear evidence for excess substrate inhibition for LPP1 against C8-C1P. However, during the curve-fitting procedure, it was found that in addition to the data for LPP1, the data for LPP1a did not fit to the Hill equation and so both sets of data required an inhibitor constant to be applied as was performed for LPP1 and LPP1a against $LPA_{(18:1)}$. It is noted that the experimental errors are consistently large in the data set for LPP1a and this may be responsible for the inability to fit the data to the Hill equation. In contrast, LPP2 and LPP3 activities against [^{32}P]-C8-C1P increased with increasing $PA_{(18:1/18:1)}$ concentration and appeared saturable. Furthermore, the velocity versus substrate curve obtained for LPP3 was sigmoidal in nature (Figure 4.24A).

The kinetic constants (apparent Vmax, $K_{0.5}$ and, where appropriate K_{is}) and h values obtained for each of the LPPs against C8-C1P are shown in Table 4.6. The apparent Vmax values were 304, 2116, 2333 and 398 nmol/min/mg for LPP1, LPP1a, LPP2 and LPP3 respectively. Therefore, the Vmax values obtained were comparable between LPP1 and LPP3 but substantially larger for LPP1a and LPP2 which were also of similar values. The apparent $K_{0.5}$ values were found to be 24, 53, 155 and 192μM and the h values were 1.67, 1.92, 0.89 and 1.86 for LPP1, LPP1a, LPP2 and LPP3, respectively. In addition to these values, inhibitor constants (K_{is}) were derived from the data sets for LPP1 and LPP1a against C8-C1P and found to be 109674 and 109695μM, respectively.

The Hanes plots obtained using the data for LPP1, LPP1a, LPP2 and LPP3 against C8-C1P are shown in Figures 4.21B to 4.24B respectively. The Hanes plots are non-

linear for LPP1, LPP1a and LPP3 but linear for LPP2. This suggests the presence of substrate cooperativity for LPP1, LPP1a and LPP3 towards [^{32}P]-C8-C1P but that LPP2 follows more typical Michaelis-Menten kinetics, under the assay conditions used in this study. However, the Hanes plot obtained for LPP1 against [^{32}P]-C8-C1P is similar to that obtained for LPP1a against [^{32}P]-LPA$_{(18:1)}$ (Figure 4.18B) in that it does not show an marked upward turn in the plot but just a slight curve. It is possible that the Hanes plot would be linear if the excess substrate inhibition component of the data was removed and so LPP1 may display more typical Michaelis-Menten kinetics as with LPP2 which is masked in a Hanes plot due to the excess substrate inhibition. In addition, the specificity constants (Vmax/$K_{0.5}$) obtained are 12.46, 51.55, 15.01 and 2.07 for LPP1, LPP1a, LPP2 and LPP3 respectively (Table 4.6), suggesting that as with [^{32}P]-LPA$_{(18:1)}$, LPP1a displayed very high catalytic efficiency for [^{32}P]-C8-C1P compared to the other LPP isoforms. Furthermore, this indicated a rank order of ability to dephisphorylate C8-C1P of LPP1a>>LPP2≈LPP1>>LPP3.

A



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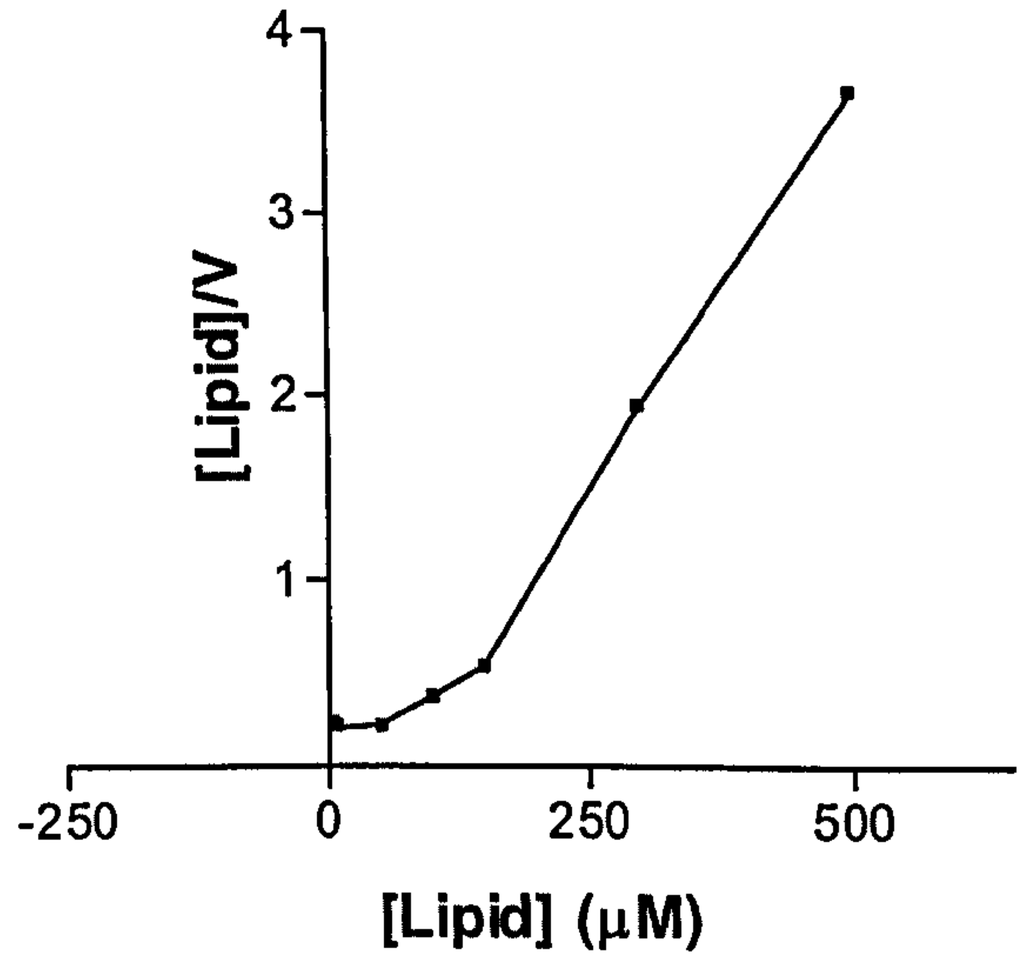
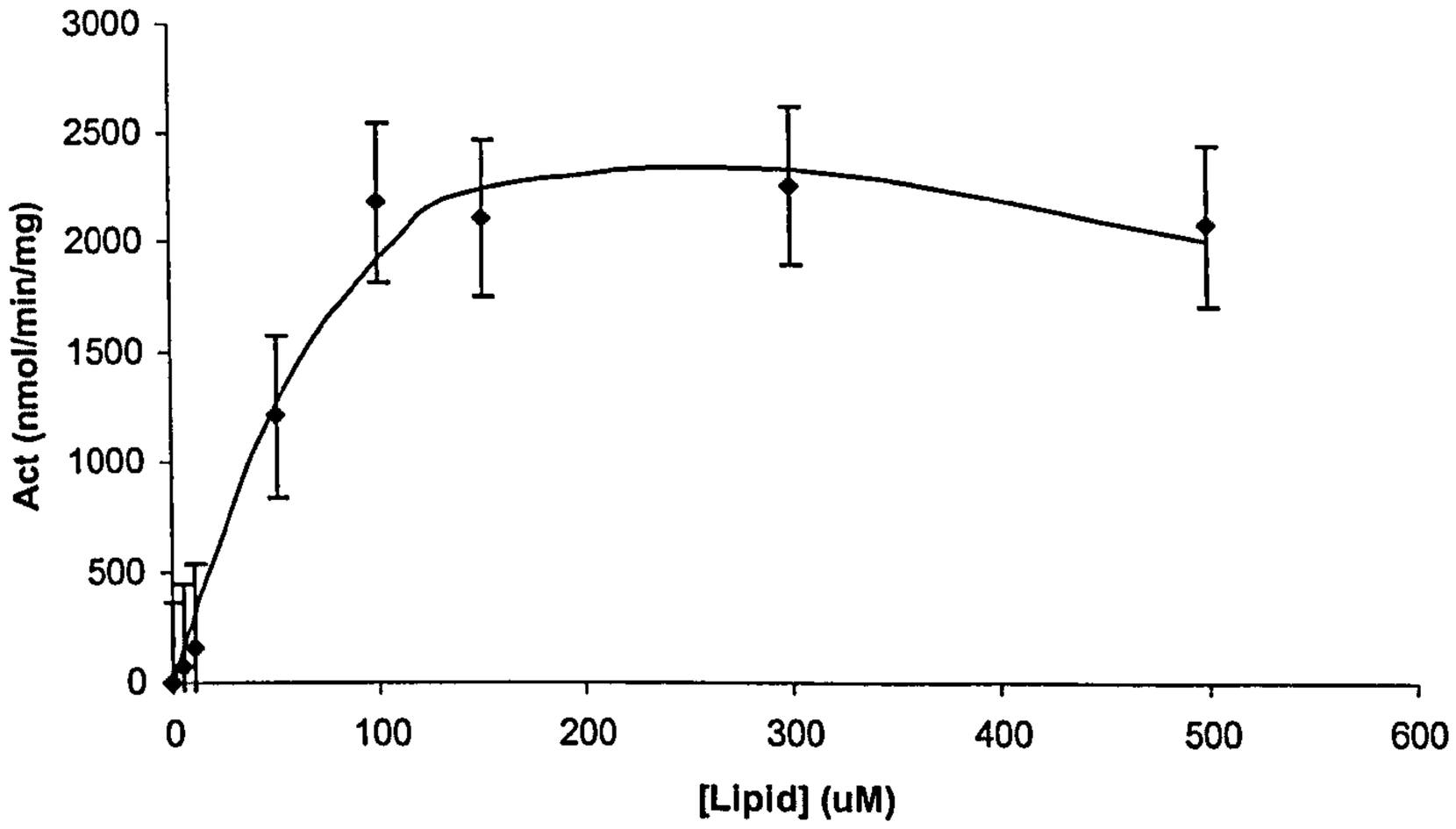


Figure 4.21: Substrate dependence (A) and Hanes plot (B) of LPP1 activity against [^{32}P]-C8-C1P.

LPP1 activity towards [^{32}P]-C8-C1P was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



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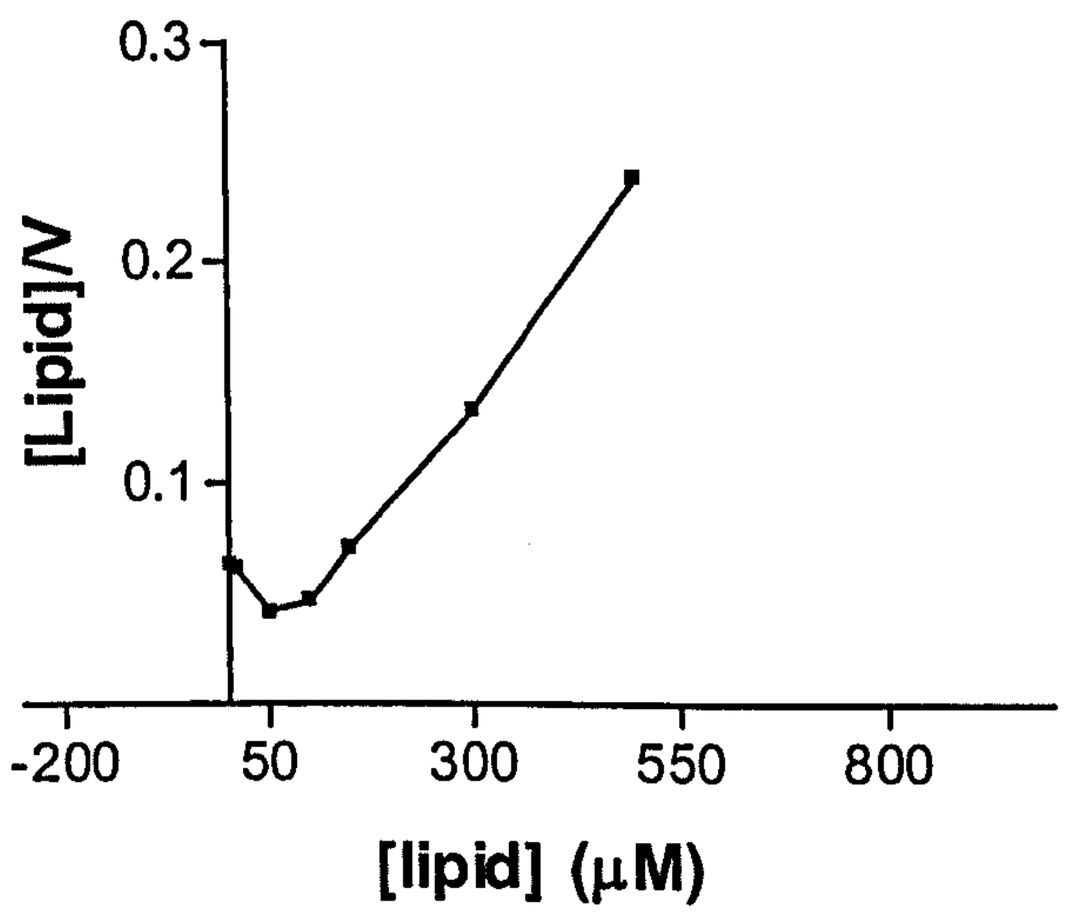


Figure 4.22: Substrate dependence (A) and Hanes plot (B) of LPP1a activity against [^{32}P]-C8-C1P

LPP1a activity towards [^{32}P]-C8-C1P was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1a as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

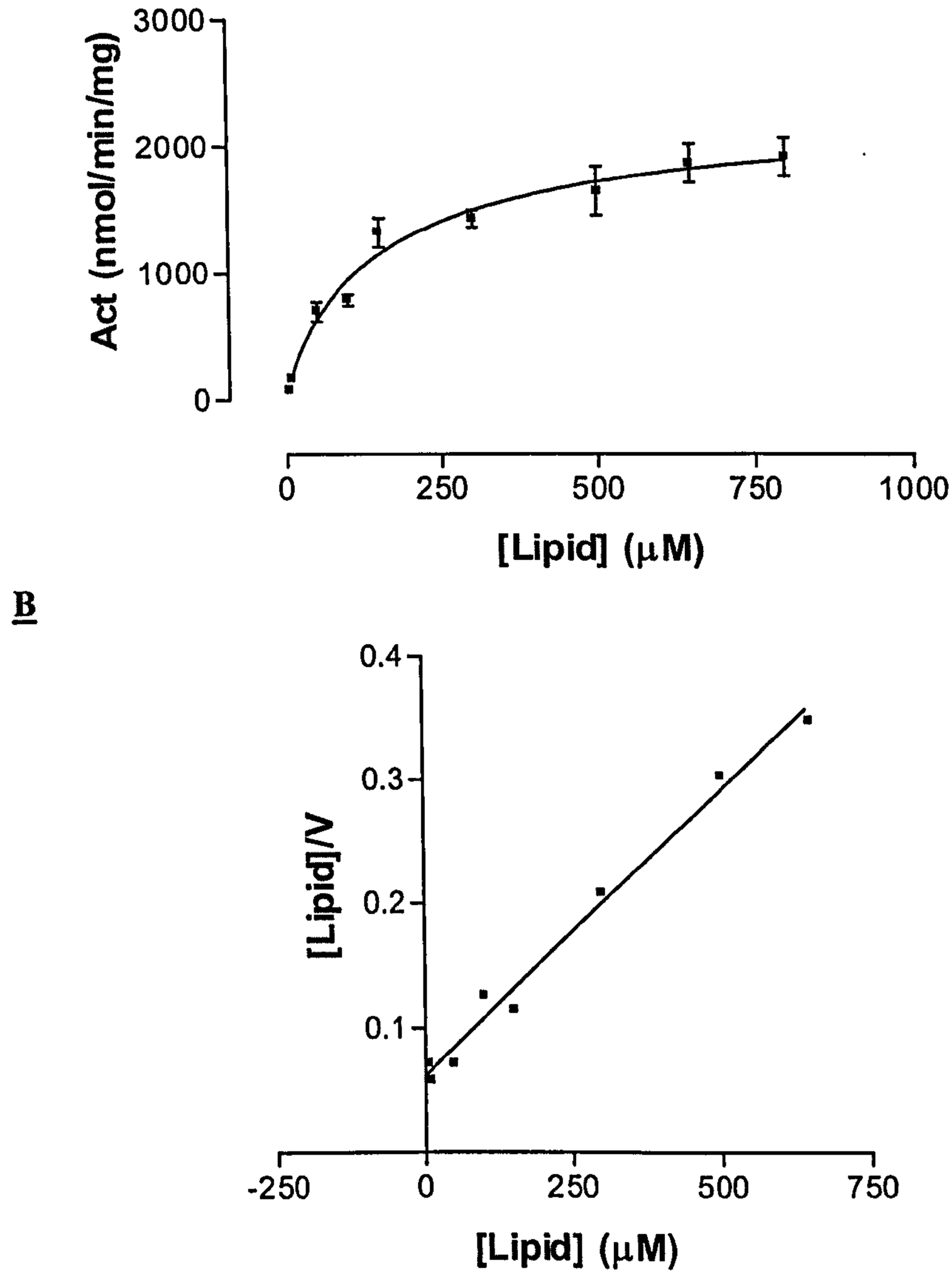


Figure 4.23: Substrate dependence (A) and Hanes plot (B) of LPP2 activity against [^{32}P]-C8-C1P

LPP2 activity towards [^{32}P]-C8-C1P was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP2 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

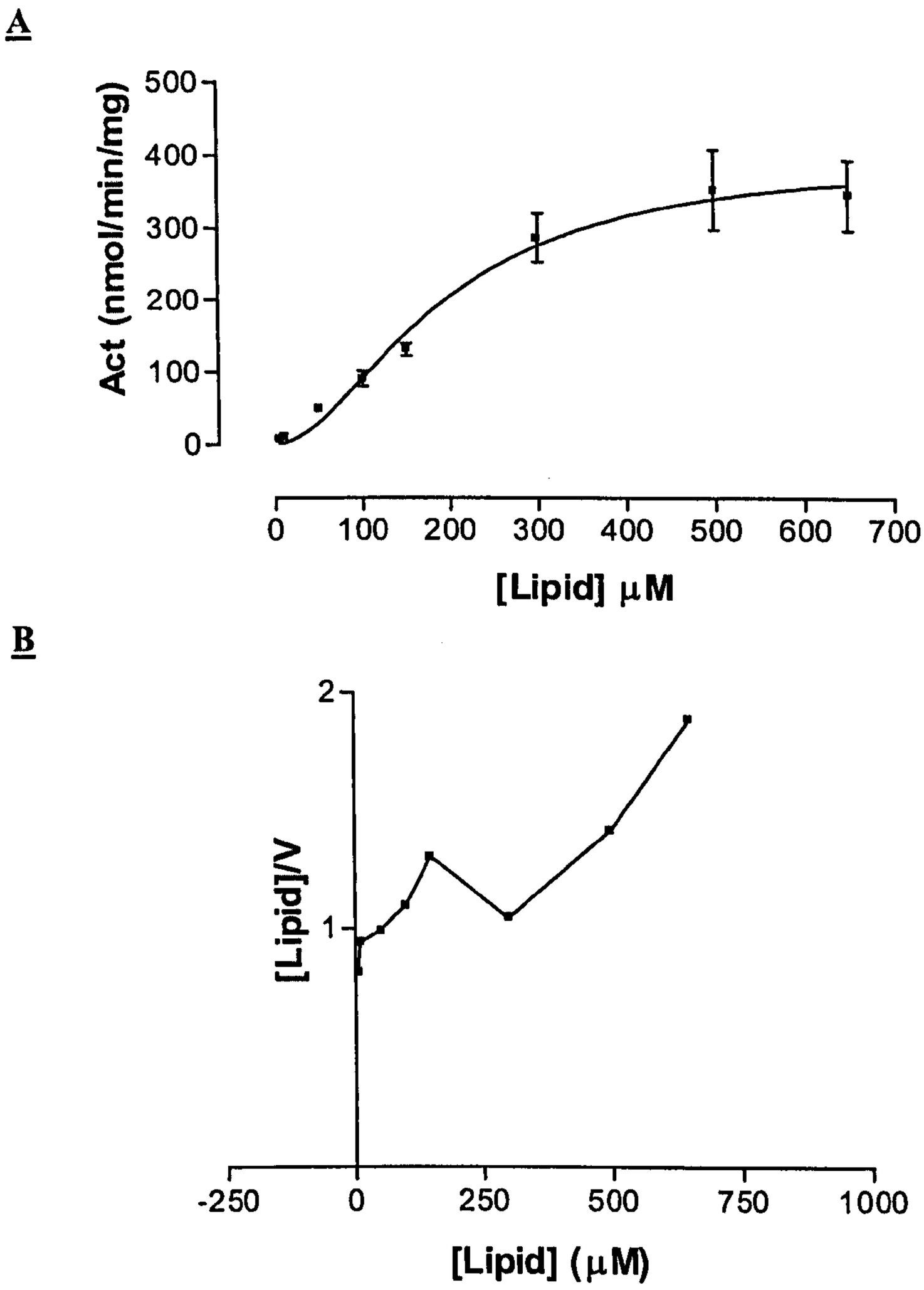


Figure 4.24: Substrate dependence (A) and Hanes plot (B) of LPP3 activity against [^{32}P]-C8-C1P

LPP3 activity towards [^{32}P]-C8-C1P was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP3 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

	Vmax (nmol/min/mg)	$K_{0.5}$ (μM)	Vmax/$K_{0.5}$	H	K_{is} (μM)
LPP1	304	24	12.46	1.95	109674
LPP1a	2712	53	51.55	1.67	109695
LPP2	2333	155	15.01	0.89	N/A
LPP3	398	192	2.07	1.86	N/A

Table 4.6: Kinetic constants for LPP1, LPP1a, LPP2 and LPP3 against [^{32}P]-C8-C1P presented in multi-substrate TX-100 micelles with $PA_{(18:1/18:1)}$

LPP activity towards [^{32}P]-C8-C1P was measured in crude membranes derived from HEK293 cells stably overexpressing LPP1, LPP1a, LPP2 or LPP3 as described in Section 2.6. Kinetic constants for LPP2 and LPP3 [apparent Vmax (nmol/min/mg), $K_{0.5}$ (μM)] and Hill coefficients (h) were derived by weighted, non-linear regression of the data presented in Figures 4.19 and 4.20 to the Hill equation [$v = Vmax * A^h / (K_{0.5}^h + A^h)$] using GraphPad Prism™ (version 2.01). However, the kinetic constants for LPP1 and LPP1a were derived by weighted, non-linear regression of the data presented in Figures 4.17 and 4.18 to the equation $v = Vmax * A^h / [K_{0.5}^h + A^h (1 + A^h/K_{is})]$ using Microsoft® Excel 1997. N/A – not applicable.

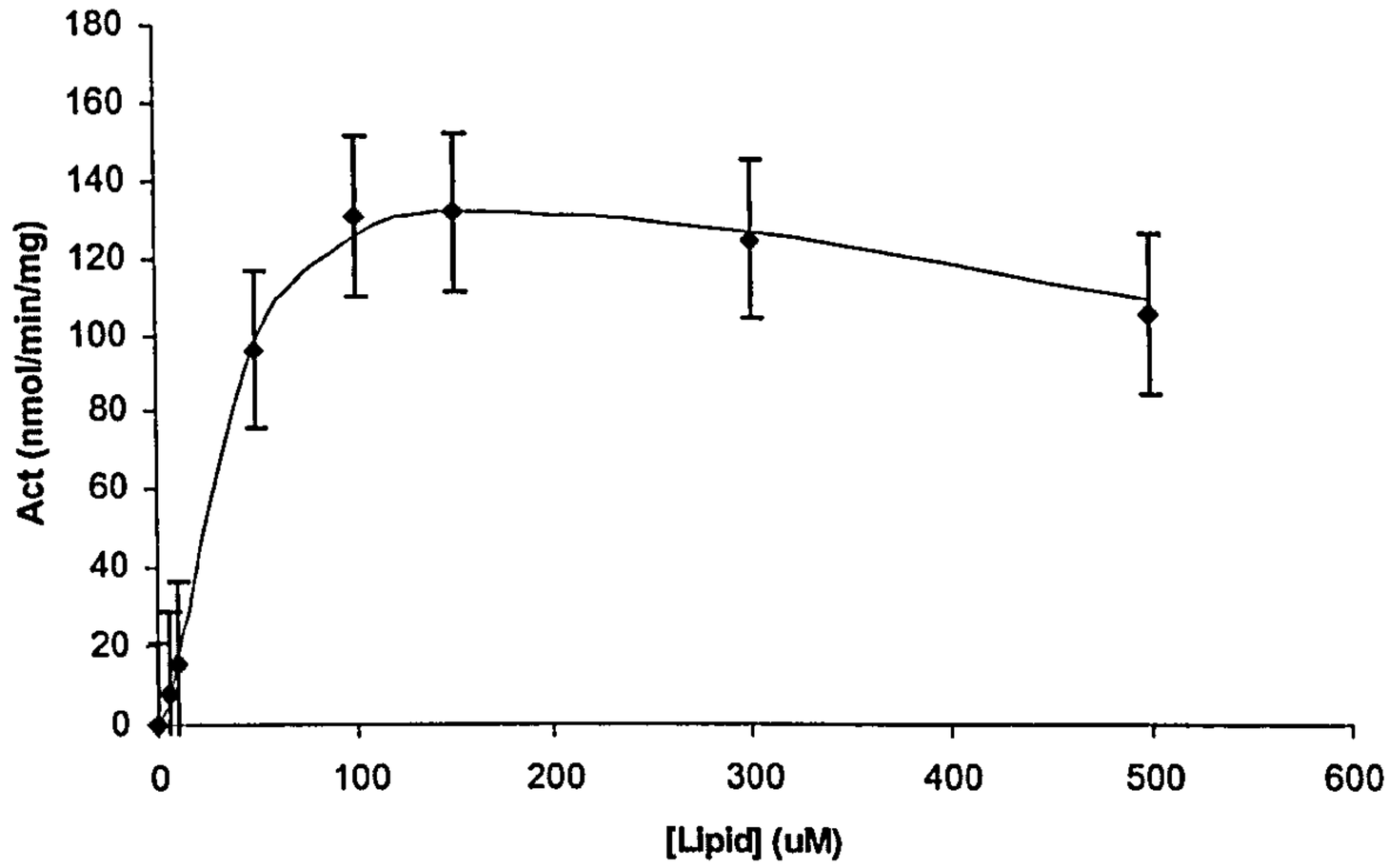
Sphingosine 1-phosphate

The velocity versus substrate concentration curves for LPP1, LPP1a, LPP2 and LPP3 towards [^{32}P]-S1P, assayed in the presence of $PA_{(18:1/18:1)}$ are shown in Figures 4.25A to 4.28A respectively. Again, under the assay conditions employed, increasing the concentration of $PA_{(18:1/18:1)}$ in the assay, enhanced the activity of all LPP isoforms towards the alternative substrate, S1P. In the case of LPP1a, LPP2 and LPP3. this activity appeared to be saturable. However, the data for LPP1 against S1P was found to exhibit excess substrate inhibition in a similar manner to that observed for LPP1 and LPP1a against [^{32}P]-$LPA_{(18:1)}$ and [^{32}P]-C8-C1P. Therefore, this curve was fitted to the equation which included an inhibitor constant as described earlier.

The kinetic constants (apparent Vmax, $K_{0.5}$ and where appropriate K_{is}) and h values obtained for each of the LPPs against S1P are shown in Table 4.7. The apparent Vmax values for LPP1, LPP1a, LPP2 and LPP3 were 148, 104, 1898 and 6.93 nmol/min/mg respectively. The $K_{0.5}$ values were 33, 223, 218 and 20μM for LPP1, LPP1a, LPP2 and LPP3 respectively. There were therefore, similarities in the $K_{0.5}$ values for LPP1 and LPP3 but also for LPP1a and LPP2. The h values derived from the curve-fit procedures were 1.7, 0.71, 1.19 and 1.17 for LPP1, LPP1a, LPP2 and LPP3 respectively. In addition to these values, the apparent substrate inhibitor constant K_{is} for LPP1 was 109674μM, i.e. similar to those obtained previously.

The Hanes plots obtained from the data for LPP1, LPP1a, LPP2 and LPP3 against [^{32}P]-S1P are shown in Figures 4.25B to 4.28B respectively. The Hanes plots for LPP1 (Figure 4.25B) and LPP2 (Figure 4.27B) are non-linear, suggesting the presence of positive substrate cooperativity. In contrast, the Hanes plots for LPP1a (Figure 4.26B) and LPP3 (Figure 4.28B) are linear suggesting that these two LPPs follow more typical Michaelis-Menten kinetics towards [^{32}P]-S1P, under the assay conditions employed in this study. The specificity constants ($V_{max}/K_{0.5}$) for LPP1, LPP1a, LPP2 and LPP3 were calculted to be 4.43, 0.47, 8.71 and 0.34 respectively. This suggests a rank order of ability to dephosphorylate [^{32}P]-S1P of LPP2>LPP1>>LPP1a≈LPP3.

A



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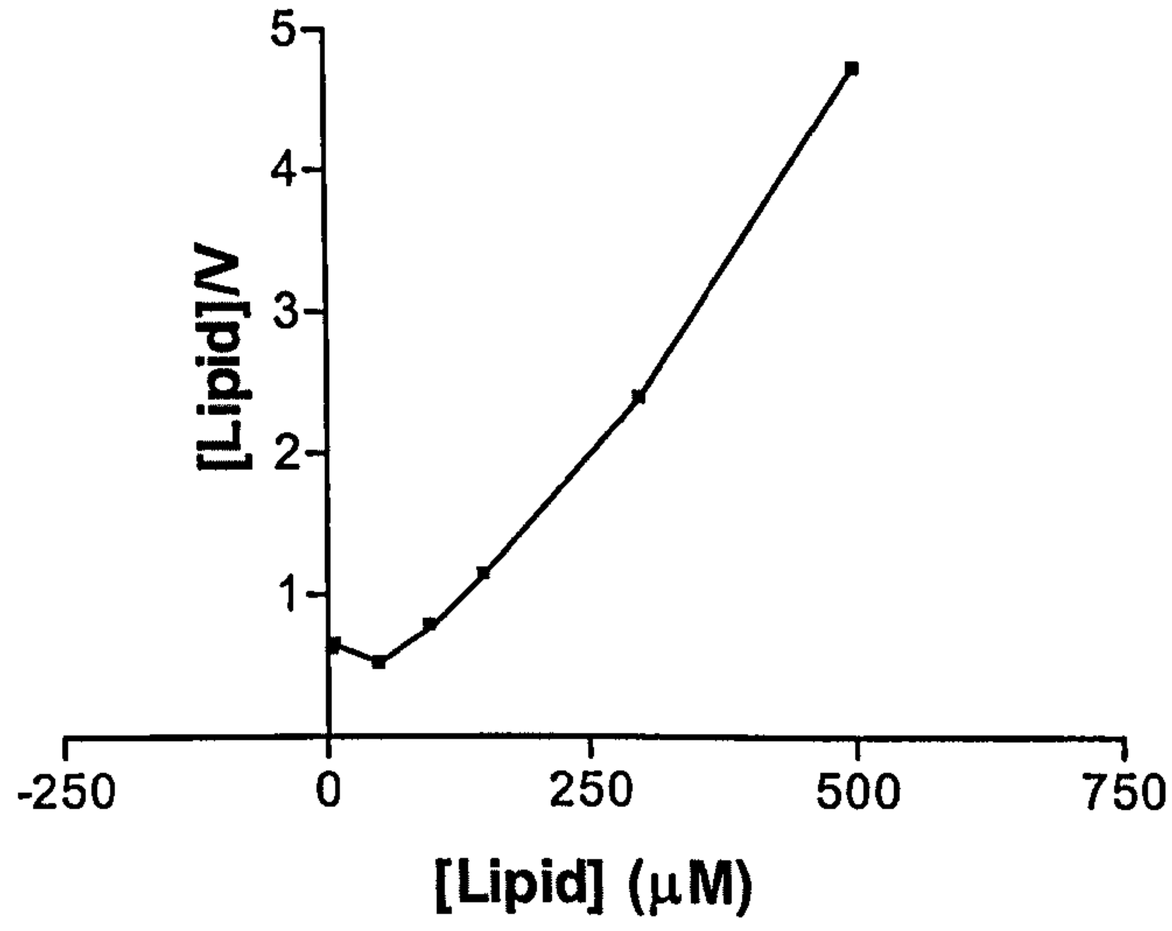
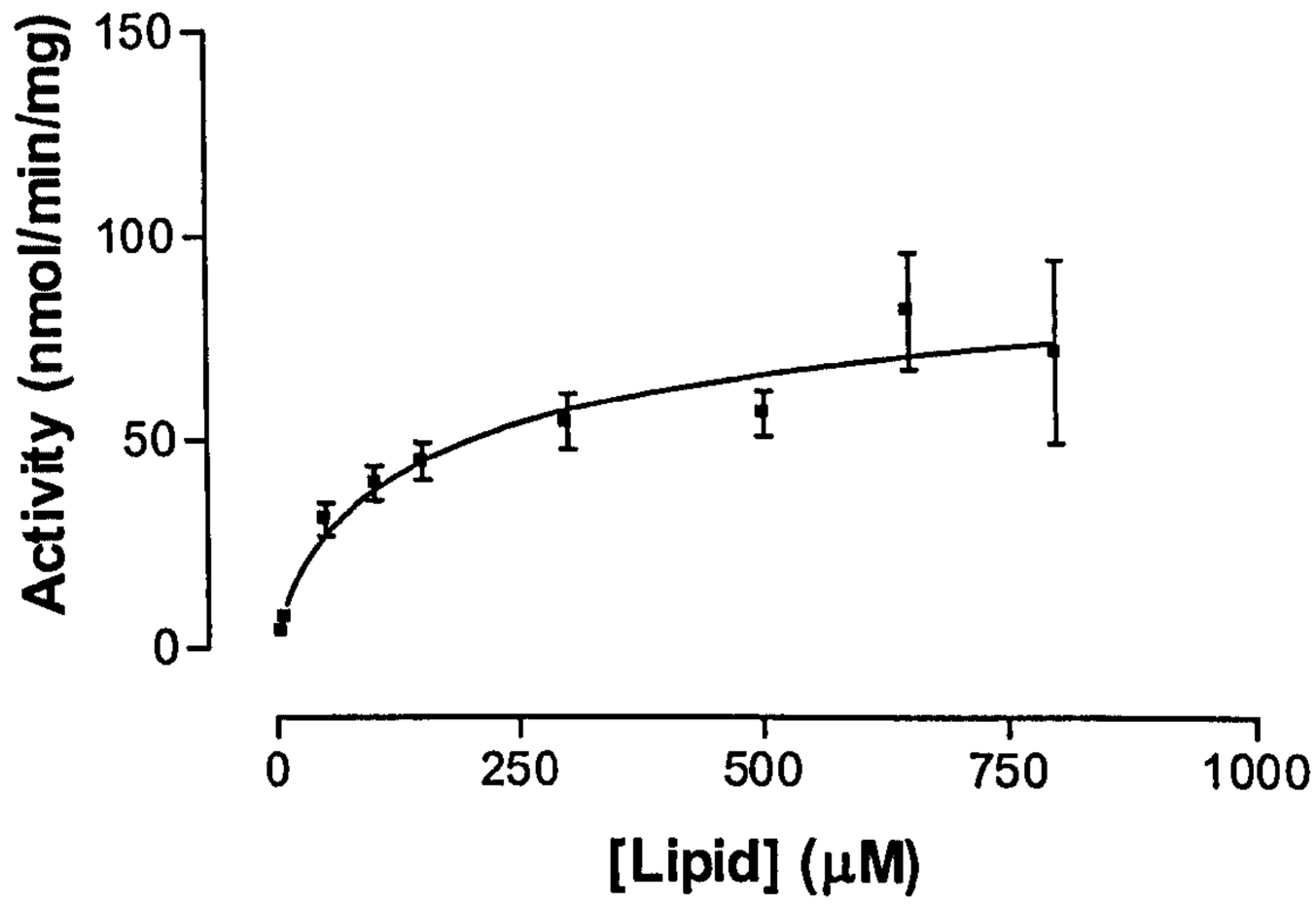


Figure 4.25: Substrate dependence (A) and Hanes plot (B) of LPP1 activity against [^{32}P]-S1P

LPP1 activity towards [^{32}P]-S1P was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

A



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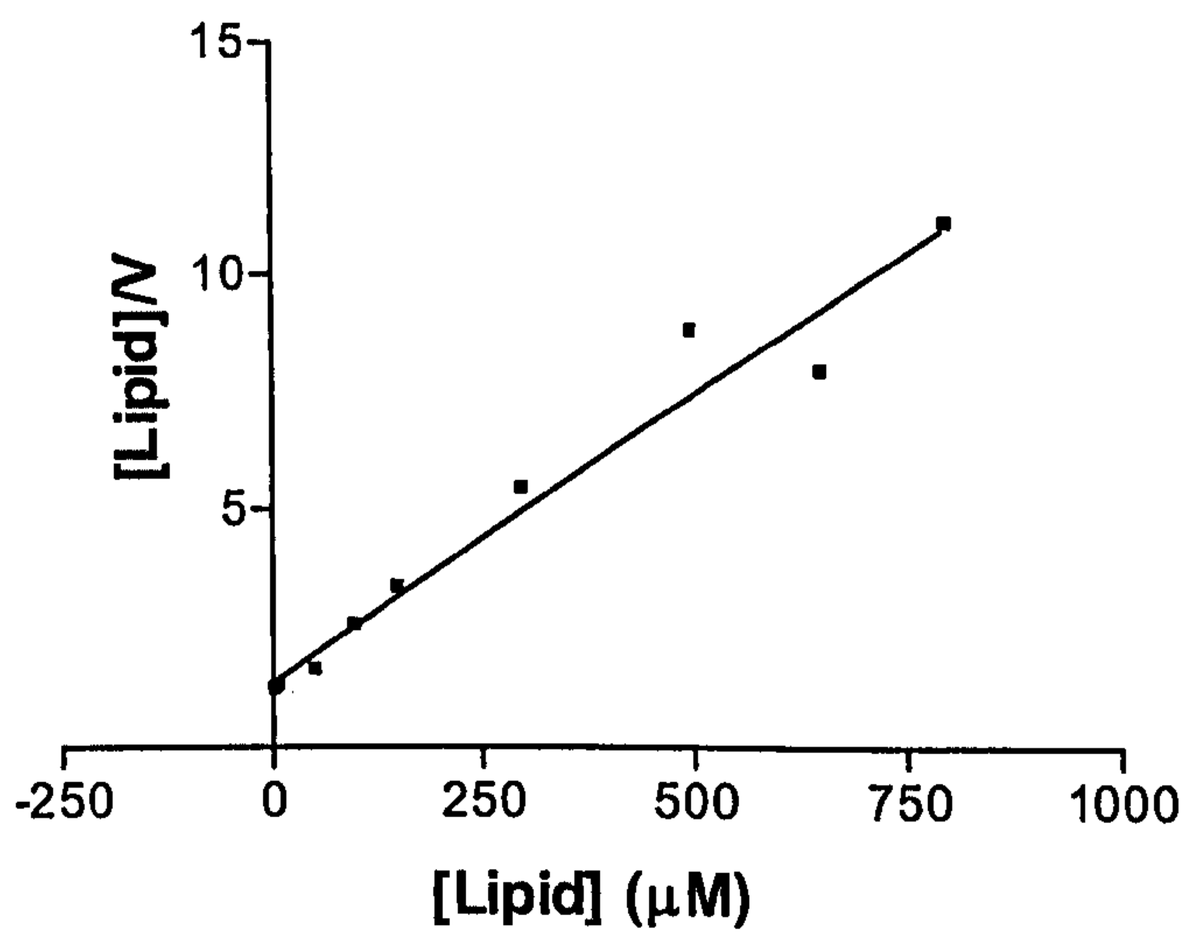

Figure 4.26: Substrate dependence (A) and Hanes plot (B) of LPP1a activity against [^{32}P]-S1P

LPP1a activity towards [^{32}P]-S1P was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP1a as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

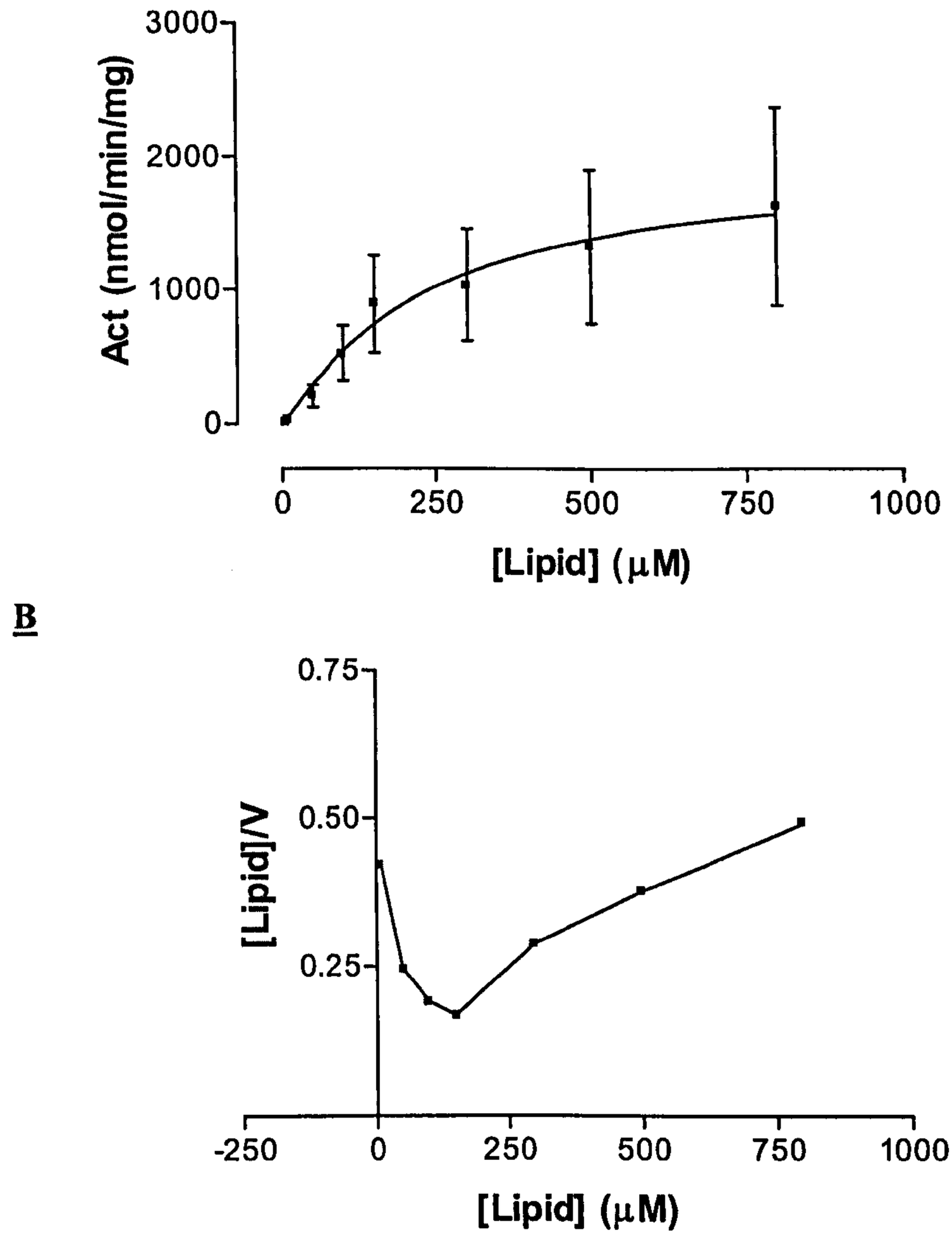

Figure 4.27: Substrate dependence (A) and Hanes plot (B) of LPP2 activity against [^{32}P]-S1P

LPP2 activity towards [^{32}P]-S1P was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP2 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

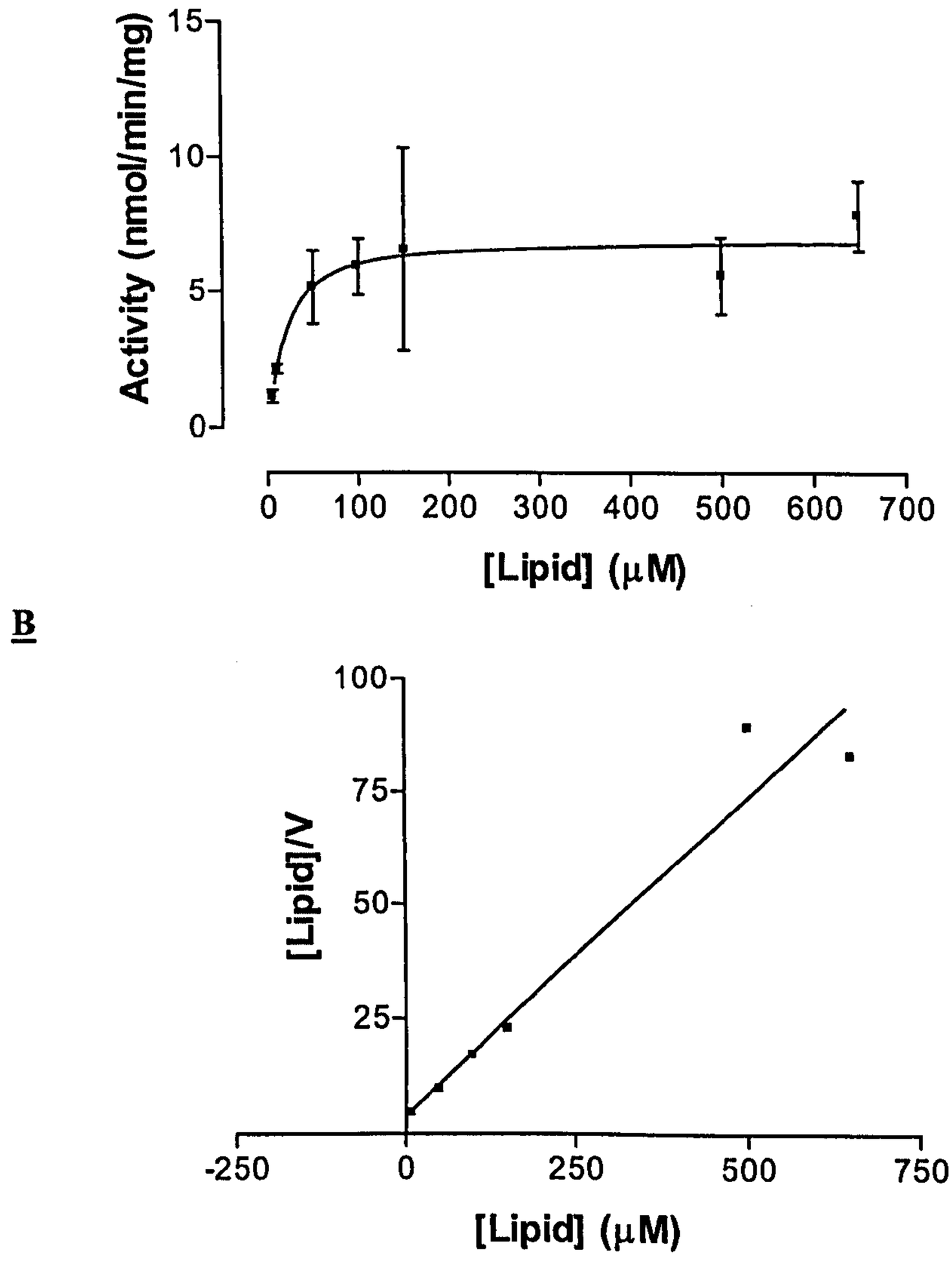

Figure 4.28: Substrate dependence (A) and Hanes plot (B) of LPP3 activity against [^{32}P]-S1P

LPP3 activity towards [^{32}P]-S1P was measured *in vitro* against increasing concentrations of $PA_{(18:1/18:1)}$ using crude membranes derived from HEK293 cells stably overexpressing LPP3 as described in 2.6. Values of endogenous LPP activity have been subtracted from the data presented. Results in **A** are expressed as nmol/min/mg membrane protein, mean +/-SEM (n=3) and the mean values used to derive the Hanes plot **B**.

	Vmax (nmol/min/mg)	$K_{0.5}$ (μM)	$Vmax/K_{0.5}$	H	K_{is} (μM)
LPP1	148	33	4.43	1.7	109674
LPP1a	104	223	0.47	0.71	N/A
LPP2	1898	218	8.71	1.19	N/A
LPP3	6.93	20	0.34	1.17	N/A

Table 4.7: Kinetic constants for LPP1, LPP1a, LPP2 and LPP3 against [^{32}P]-S1P presented in multi-substrate TX-100 micelles with $PA_{(18:1/18:1)}$

LPP activity towards [^{32}P]-S1P was measured in crude membranes derived from HEK293 cells stably overexpressing LPP1, LPP1a, LPP2 and LPP3 as described in Section 2.6. Kinetic constants for LPP1a, LPP2 and LPP3 [apparent Vmax (nmol/min/mg), $K_{0.5}$ (μM)] and Hill coefficients (h) were derived by weighted, non-linear regression of the data presented in Figures 4.26 to 4.28 to the Hill equation [$v = Vmax * A^h / (K_{0.5}^h + A^h)$] using GraphPad Prism™ (version 2.01). However, the kinetic constants for LPP1 were derived by weighted, non-linear regression of the data presented in Figures 4.25 to the equation $v = Vmax * A^h / [K_{0.5}^h + A^h (1 + A^h/K_{is})]$ using Microsoft® Excel 1997. N/A – not applicable.

4.3. Discussion

4.3.1. Overexpression of the LPPs

gpLPP1, gpLPP1a, hLPP2 and hLPP3 have been stably overexpressed in HEK293 cells. This is evident from comparison of the apparent Vmax values obtained. It is noteworthy that, the apparent Vmax values are dependent on the concentration of enzyme in an assay and therefore, are better referred to as the '*limiting rate*' and are not fundamental properties of the enzyme itself (Cornish-Bowden, 1995). However, the *limiting rate* is still a measure of maximal activity and is referred to as Vmax in most instances of kinetic equations such as the Michaelis-Menten equation unless the exact enzyme concentration is known. Large increases in specific LPP activity in membranes derived from the LPP transfected cell lines compared to the blank vector transfected cells indicate high levels of expression of the LPPs (Figure 4.1). For example, analysis of the activity levels shown in Figure 4.1 towards 500μM $PA_{(18:1/18:1)}$ revealed 100-fold, 177-fold, 68-fold and 6-fold increases in LPP activity for the LPP1, LPP1a, LPP2 and LPP3 transfected cell lines, respectively. However, if the results obtained from the measurement of LPP activity in the multi-substrate assay towards 500μM $PA_{(8:0/8:0)}$ were used for the same purpose, the apparent fold increases in activity would become 130-fold, 91-fold, 28-fold and 15-fold for LPP1, LPP1a, LPP2 and LPP3, respectively. Moreover, using similar values obtained using C8-C1P in the multi-substrate assay, the apparent fold increases would be 17-fold, 262-fold, 207-fold and 44-fold for LPP1, LPP1a, LPP2 and LPP3, respectively. Therefore, depending on the substrate and assay conditions used, the apparent expression levels could be over-, or indeed, under-estimated. It is therefore, not possible to estimate the relative levels of expression of each LPP from the results presented, due to the fact that the LPPs represent a multi-substrate enzyme family and no single assay condition can be presumed to reflect the endogenous environment of each LPP isoform. Despite these limitations, a comparison of the Vmax values obtained here for $PA_{(18:1/18:1)}$ with those obtained by other groups, using a single substrate (PA) assay, can be used to estimate the relative expression levels of LPPs in this study compared to those published previously. Thus, we have attained

apparently high levels of overexpression when compared to that achieved by other groups. Roberts and colleagues (1998) reported a 7-fold, 15-fold and 7-fold increase in activity (measured using an unspecified PA species) for hLPP1, hLPP2 and hLPP3 respectively when the isoforms were transiently expressed in HEK293. Similar increases in activity following overexpression have also been reported by Hooks and colleagues (6-fold, 7 fold and 4-fold for LPP1, LPP2 and LPP3, respectively, against an unspecified PA species) and also Kai and colleagues (9-fold and 7-fold for LPP1 and LPP3, respectively, against an unspecified PA species), (Hooks et al., 1998, Kai et al., 1997). The reason for the apparent differences between the present study and those published previously is that stable transfectants have been used in this study compared to transient transfections in the others. In the present study, cells were selected for overexpression of the desired gene and it is expected that every cell in the population will be overexpressing the gene of interest. This contrasts with transient transfections where, depending on the transfection efficiency, only a proportion of cells in an overall population will be overexpressing the gene of interest with the remainder of the cells expressing only endogenous levels of LPP activity. However, Roberts and colleagues also overexpressed hLPP1, hLPP2 and hLPP3 in Sf9 insect cells and reported 1140-fold, 540-fold and 460-fold increases in LPP activity, respectively (Roberts et al., 1998).

The kinetic analysis of LPP isoforms would clearly be best conducted using purified enzyme preparations. However, we believe that the high levels of activity which were obtained in our system and the subsequent subtraction of any endogenous, mixed LPP component, allows us to provide a good approximation of the properties of the individual LPP isoforms. However, we cannot exclude the possibility that the enzymes may be affected by other proteins/factors within the assays conducted.

During the optimisation of the assay using $PA_{(18:1/18:1)}$ as a substrate, it was found that the LPP specific activity significantly decreased as protein levels in the assay were increased (Figure 4.2). Two possible explanations for this effect can be considered. Firstly, high protein levels in the assay may have disrupted the detergent micelles within the assay due to the high levels of endogenous lipids being added. Since, LPP

assays were conducted while maintaining the detergent to lipid ratio at 10:1, the high levels of endogenous lipid and protein may have led to incorrect presentation of the substrates to the enzymes or a dilution of the surface concentration of the substrate. Secondly, it could be indicative of product inhibition by the DG produced during the assay. In support of this idea are the findings of Tolan (1997) who demonstrated a concentration dependent inhibition of LPP activity from GPASM cells by DG. In this study it was reported that LPP activity towards PA was reduced by around 70% when equimolar concentrations of DG were incorporated into the assay. In relation to this, at the high protein levels, it was found that around 65% of the added radiolabelled $PA_{(18:1/18:1)}$ had been hydrolysed (results not shown). Therefore, the DG levels in the assay would have been significantly elevated and could explain the results shown in Figure 4.2. As detailed, all assays were optimised so that at no point was more than 15% of the added substrate hydrolysed during the assay.

4.3.2. Kinetic analysis of LPP1, LPP1a, LPP2 and LPP3 against $PA_{(18:1/18:1)}$ in a single substrate assay

The initial investigation of the kinetics of LPP1, LPP1a, LPP2 and LPP3 involved the use of a single substrate assay system composed entirely of $PA_{(18:1/18:1)}$. As detailed, the data obtained in the experiments for each LPP isoform were used to derive the apparent kinetic parameters through a curve-fitting procedure to the Hill equation [$v = Vmax * A^h / (K_{0.5} + A^h)$] (Section 4.2.2). The Hill equation was developed as a model for cooperativity and the Hill coefficient (h), is now widely used as an index of cooperativity. Thus, if h=1, the Hill equation becomes the equivalent of the Michaelis-Menten equation and there is no cooperativity. However, if h>1, possible positive cooperativity exists and, if h<1, possible negative cooperativity exists (Cornish-Bowden, 1995). However, as described in more detail later (Section 4.3.4), this is not strictly correct. Infact, Hill himself denied the presence of any physical meaning for the value h and considered his equation purely empirical (Cornish-Bowden, 1995). Therefore, during this study, conclusions on the presence of cooperativity were based on the velocity versus substrate concentration plots and Hanes plots derived from the data.

The results of the present study towards $PA_{(18:1/18:1)}$ can be compared to the results reported for hLPP1, hLPP2 and hLPP3 by Roberts and colleagues (1998) and also by Hooks and colleagues (1998) as single substrate assays were employed in each case. The higher Vmax values calculated for LPP1 compared to LPP2 and LPP3 by these groups are similar to the results in this study (Table 4.1). However, the Km values reported by Hooks and colleagues (1998) (though technically not Km values) were 98, 100 and 150μM for LPP1, LPP2 and LPP3 respectively. These are somewhat lower but of a similar order to the $K_{0.5}$ values reported here which are 180, 122 and 193μM for LPP1, LPP2 and LPP3, respectively. In contrast, Roberts and colleagues (1998) report their Km values in terms of mol% and so can not be directly compared with either of the other studies. In fact, an important difference in the study conducted by Roberts and colleagues is that they measured the activity of the LPP isoforms as the surface concentration of the substrate was increased relative to the detergent (Roberts et al., 1998). In contrast, the present study and that of Hooks and colleagues (1998) maintained a constant lipid to detergent ratio while increasing the substrate concentration.

The specificity constants ($Vmax/K_{0.5}$) (Table 4.8) which combine the apparent ability of an enzyme to dephosphorylate a substrate (Vmax), with the apparent affinity of the enzyme for a substrate ($K_{0.5}$) provide a measure of catalytic specificity towards a particular substrate (Cornish-Bowden, 1995). Therefore, using the specificity constants, it is evident that LPP1a displayed the highest catalytic efficiency towards $PA_{(18:1/18:1)}$ compared to the other isoforms and LPP3 by far the least, in a single substrate assay system. Interestingly, the study by Roberts and colleagues (1998) detailed specificity constants ($Vmax/K_{0.5}$) which suggested a rank order of catalytic efficiency towards PA of LPP3>LPP2>LPP1. This contrasts with that reported for all species of PA reported in this study where LPP1 and LPP1a isoforms were seen to display the highest catalytic efficiency for all PA substrates tested. Furthermore, calculation of the specificity constants using the Vmax and Km values reported by Hooks and colleagues (1998), reveals an apparent rank order of catalytic efficiency towards PA of LPP1>LPP2>LPP3 which is identical to the findings of this study.

	LPP1	LPP1a	LPP2	LPP3
$PA_{18:1/18:1}$	4.27	8.32	4.04	0.31
$PA_{8:0/8:0}$	30.31	27.92	7.63	2.68
$PA_{18:0/20:4}$	5.4	7.91	2.66	N/A
$PA_{16:0/16:0}$	2.37	3.55	0.44	N/A
$LPA_{18:1}$	27.2	76.67	22.88	2.95
C8-C1P	12.46	51.55	15.01	2.07
S1P	4.43	0.47	8.71	0.34

Table 4.8: Specificity constants calculated for LPP1, LPP1a, LPP2 and LPP3 against each LPP substrate tested

Specificity constants ($Vmax/K_{0.5}$) were calculated from the Vmax and $K_{0.5}$ values derived from the data obtained for each LPP against each LPP substrate. N/A – not applicable.

Therefore, the assay procedure utilised for these types of studies appears critical in determining the results which are obtained.

4.3.3. Kinetic analysis of LPP1, LPP1a, LPP2 and LPP3 against alternative LPP substrates in a multi-substrate assay system

A multi-substrate assay system utilising mixed micelles of TX-100 and $PA_{(18:1/18:1)}$ against a fixed, trace concentration of an alternative [^{32}P]-labelled substrate was used to probe the effects of $PA_{(18:1/18:1)}$ on the kinetics of the LPP isoforms towards several alternative LPP substrates. This is the first study in which this approach has been adopted. Previous reports of mixed substrate assays have examined the inhibitory effects of alternative LPP substrates such as LPA and C1P on the dephosphorylation of [^{32}P]-PA. For example, English and colleagues tested the effects of adding several different PA and LPA species at a concentration of 200μM, on neutrophil 'ecto'-LPP activity against [^{32}P]- $PA_{(8:0/8:0)}$ and found that various PA and LPA species acted as competitive inhibitors (English et al., 1997). However, the different PA and LPA species were found to cause differing degrees of inhibition suggesting that the acyl-chain composition was important in determining the effects. Interestingly, the authors reported that even though $PA_{(18:1/18:1)}$ was a relatively poor substrate for the 'ecto'-LPP activity, it was a surprisingly strong inhibitor (English et al., 1997). Furthermore, several LPA species were also found to be strong inhibitors of the 'ecto'-LPP activity towards [^{32}P]-$PA_{(8:0/8:0)}$ and longer chain, saturated PA species were found to be typically non-competitive (English et al., 1997). Similar observations were made by Fleming and Yeaman (1995b) using rat liver LPP and LPA and by Tolan (1997), using LPP activity from GPASM and LPA or S1P, where LPA was a significantly stronger inhibitor of LPP activity against [^{32}P]-PA. Interestingly, C1P was found to have little effect on the LPP activity towards PA in the latter study (Tolan, 1997). Importantly, these studies looked at the effects of alternative substrates on PA hydrolysis relative to an apparently maximal LPP activity measured in the absence of the second substrate. Therefore, these investigations were specifically studying the *inhibition*, rather than the *kinetics* as in this study. A more detailed study of the effects of LPA, C1P and S1P on the ability

of rat liver LPP activity to dephosphorylate PA was conducted by Waggoner and colleagues (1996). In this study, hydrolysis of [^{32}P]-PA at various concentrations was decreased in a concentration dependent manner by LPA, C1P or S1P which were found to be competitive inhibitors (Waggoner et al., 1996). In contrast, Kanoh and colleagues (1992) reported that LPP activity from porcine thymus membranes was unaffected by LPA.

Significantly, in the present study, $PA_{(18:1/18:1)}$ was found to enhance the activity of the LPP isoforms towards all substrates tested with differing levels of positive substrate cooperativity being evident in many cases. The only substrates which were not hydrolysed in the presence of $PA_{(18:1/18:1)}$ were $PA_{(18:0/20:4)}$ and $PA_{(16:0/16:0)}$ by LPP3. However, whether the inability of LPP3 to dephosphorylate either of these PA substrates reflects differences in the presentation of the substrates within the TX-100 micelles, or is a reflection of longer or polyunsaturated fatty acid side chains being simply, 'less preferred' substrates for this LPP isoform remains to be shown.

Using the specificity constants ($Vmax/K_{0.5}$) (Table 4.8) as a measure of the catalytic specificity, the results of the present study show that for all LPP isoforms, $PA_{(8:0/8:0)}$ was hydrolysed more readily than the other PA substrates. However, $PA_{(8:0/8:0)}$ is not a naturally occuring PA molecular species and so the physiological relevance of these results is limited. However, of the other PA species tested, $PA_{(18:1/18:1)}$ appeared to be hydrolysed to comparable levels as $PA_{(18:0/20:4)}$ suggesting similar specificities for LPP1, LPP1a and LPP2 towards these two species of PA. As detailed above, LPP3 did not hydrolyse $PA_{(18:0/20:4)}$ or $PA_{(16:0/16:0)}$. Furthermore, LPP1, LPP1a and LPP2 also appeared to dephosphorylate monounsaturated and polyunsaturated species of PA more effectively compared to the saturated $PA_{(16:0/16:0)}$. These results are in contrast to previous findings for LPP activity from native sources such as that of Tolan (1997) who reported that monounsaturated $PA_{(18:1/18:1)}$ was a more preferred substrate than the polyunsaturated $PA_{(18:0/20:4)}$ for the LPP activity from GPASM cells. In addition, Fleming and Yeaman (1995b) reported that $PA_{(18:1/18:1)}$ and $PA_{(16:0/16:0)}$ were better substrates for rat liver LPP than $PA_{(18:0/20:4)}$. Furthermore, English and colleagues reported that saturated PA species

were the best substrates for the neutrophil 'ecto'-LPP activity (English et al., 1997). However, as detailed, these studies utilised total LPP activity from native sources and so most probably contained several different LPP isoforms. The specificity constants also showed an apparent preference for $LPA_{(18:1/18:1)}$ and C8-C1P as substrates for LPP1, LPP1a and LPP2 compared to all other substrates tested suggesting that in common with the LPP purified from rat liver (Waggoner et al., 1996), the individual LPP isoforms dephosphorylate LPA with a higher catalytic efficiency than PA as a substrate. However, this is the first demonstration of an apparent high catalytic efficiency for C1P and represents an interesting observation in terms of the potential functions of the LPPs towards this relatively unstudied lipid substrate.

In addition, it is possible that the use of TX-100 micelles is not the most efficient method of presentation of S1P and LPA to the LPP enzymes. S1P and indeed LPA, are found bound to serum albumin following secretion *in vivo* (Goetzl, 2001). Therefore, it is possible that experiments investigating the kinetics of the LPPs towards S1P and LPA as well as possibly the other LPP substrates presented as complexes with albumin may result in more appropriate, physiologically relevant kinetics. In relation to this, a recent publication by our group detailed that the 'ecto'-LPP activity towards S1P presented as a complex with serum albumin was greatest in the LPP3 transfected HEK293 cells used in this study (Alderton et al., 2001). This is therefore, in contrast to the present study which used crude membranes derived from the same cells where LPP3 was found to have apparently the lowest catalytic efficiency towards S1P. Additionally, LPP3 overexpressing cells used in this study have reduced basal levels of cellular S1P compared to blank vector and LPP1, LPP1a and LPP2 overexpressing cells (Kong, 2002).

Another apparent interaction which occurred between $PA_{(18:1/18:1)}$ and other lipid phosphate substrates was that of excess substrate inhibition in the assays involving LPP1 and LPP1a. Whether this effect which is of limited physiological relevance due to the high substrate concentrations required to create it, is an effect of simple competition or due to an interaction between the substrates reulting in lower

solubility can not be determined. The effect does however, suggest possible differences in the catalytic mechanisms of LPP1 and LPP1a compared to LPP2 and LPP3 towards these substrates.

4.3.4. Evidence for substrate cooperativity

In addition to obtaining the apparent kinetic constants (Vmax and $K_{0.5}$), the Hill equation as detailed, provided values for the Hill coefficient (h). As mentioned in section 4.3.2., it is incorrect to use the value of h as an estimate of the number of substrate binding sites. The reason for this is illustrated by the case of oxygen binding to haemoglobin where the h values are typically 2.7 even though the number of binding sites is four (Cornish-Bowden, 1995). However, the Hill coefficient is still widely used as an index of cooperativity. Despite this, the conclusions on cooperativity in this study were based on the Hill coefficient in combination with secondary kinetic plots (Hanes plots). The h values obtained in this study suggested the presence of differing levels of cooperativity which ranged from apparently highly positively cooperative (LPP2 against $PA_{(18:1/18:1)}$) where h = 2.1 (Table 4.1), to slightly negatively cooperative (LPP1a against S1P) where h = 0.71 (Table 4.7). Negative cooperativity is less common and the physiological relevance of this property is less clear (Cornish-Bowden, 1995). Furthermore, it is possible that this is a result of the assay system used and if a different combination of potential LPP substrates was used or the substrates were presented in a different manner, the results may change. Nonetheless, it does show distinct variation in the kinetics of the individual LPP isoforms. The suggestion of cooperativity was in agreement with the sigmoidal nature of certain velocity versus substrate curves (e.g. Figure 4.13A) and is indicative of cooperativity. However, a more appropriate indication of cooperativity is gained through the use of secondary kinetic plots such as the Hanes plot which is linear for a typical Michaelis-Menten enzyme (Cornish-Bowden, 1995). The Hanes plots which were constructed using the data obtained in this study were found to be non-linear for all LPP isoforms against all substrates with the exceptions of LPP1a against S1P, LPP2 against $PA_{(16:0/16:0)}$ and C8-C1P, and LPP3 against $PA_{(18:1/18:1)}$, $LPA_{(18:1)}$, and S1P. This was an interesting finding as the only other report of

cooperativity displayed by LPP activity, either partially purified from a native source or in a recombinant system was by Tolan who showed an element of cooperativity for LPP activity from GPASM cells (Tolan, 1997). However, as with the present study and others investigating the kinetics of the LPPs, the author reported that the purification of LPP is required in order to determine whether the enzymes are genuinely cooperative or whether the phenomenon is due to another component of the membrane fraction (Tolan, 1997). In addition, the assay system employed in the present study is multi-substrate and therefore, differed from that used by Tolan. Therefore, instances of cooperativity in this study may also represent differing interactions between the two alternative substrates and the LPP enzymes.

4.3.5. Summary and future work

The results presented in this study show a great variation in the kinetics of the different LPP isoforms towards different combinations of $PA_{(18:1/18:1)}$ and alternative second substrates. Therefore, there are clearly interactions between the different LPP substrates and $PA_{(18:1/18:1)}$ which affect the ability of the LPPs to access and hydrolyse each substrate. However, the mechanisms resulting in these effects can not be determined from the results obtained in this study. It is possible to speculate that, as evidenced by the apparent substrate cooperativity observed for the individual LPPs in this study, the LPPs exist as multimeric complexes within cells. This idea is supported by the apparent discrepancies reported in the molecular weights of purified LPP activity from native sources (Kanoh et al., 1992, Fleming & Yeaman, 1995a, 1995b, Waggoner et al., 1995, Kai et al., 1996, Siess & Hofstetter, 1996). Indeed, Kanoh and colleagues postulated that the LPPs were channel like proteins and that they may exist as homooligomers (Kanoh et al., 1997).

To further elucidate the kinetics of the LPPs, the activities are required to be measured in a full combination of multi-substrate systems, initially, for example, by replacing $PA_{(18:1/18:1)}$ with $LPA_{(18:1)}$. Repeating this for all LPP substrates would allow all potential interactions to be investigated and would potentially reveal patterns which could be applied to physiological situations.

Information on the properties of the LPP isoforms in single substrate assay systems is still important in determining what effects are due to substrate interactions and so there is still a need for more detailed, simple kinetic studies. However, the multi-substrate kinetic studies could be directed towards determining the kinetic mechanisms. For example, in this study, the effects of varying the concentration of one substrate (A) on the dephosphorylation of a fixed concentration of a second substrate (B) was investigated. However, if the fixed concentration of substrate B was increased and the same studies repeated, the velocity measurements would rise and so the kinetic parameters would change accordingly to reflect this change in velocity. If this was repeated for several different concentrations of fixed substrate, several secondary plots would be obtained (Lineweaver-Burke or Hanes plots). The pattern of lines within these plots would change depending on the way in which the enzymes interact with each substrate and this data can be used to discriminate between potential kinetic mechanisms (Dr P. Birch – personal communication). In addition, inhibition studies using a multi-substrate system could be conducted in order to study the relative abilities of each substrate to inhibit the activity towards other substrates when several substrates are present. Another interesting study would be to conduct kinetic experiments towards LPP substrates presented as complexes with serum albumin in order to determine the importance of substrate presentation on the ability of the LPPs to recognise and hydrolyse each of the potential substrates.

CHAPTER 5

CHARACTERISATION OF THE ROLE OF THE LPP ENZYME FAMILY ON S1P SIGNALLING VIA $S1P_1$/EDG1

5. Characterisation of the role of the LPP enzyme family on S1P signalling via $S1P_1$/EDG1

5.1. Introduction

The discovery of the precise physiological role of the LPPs has been the driving force behind much recent research, but the answer remains unknown. The LPPs have been shown to localise to the plasma membrane and evidence has accumulated predicting that the catalytic site of the enzymes is extracellularly orientated (Zhang et al., 2000). Accordingly, as detailed in section 1.13., studies have demonstrated the presence of 'ecto'-LPP activities in neutrophils and keratinocytes (Perry et al., 1993, Xie & Low, 1994, English et al., 1997). Furthermore, subsequent studies have demonstrated similar 'ecto'-LPP activities for the cloned LPP isoforms when overexpressed (for review, see Pilquil et al., 2001). These findings have raised the possibility that an 'ecto'-LPP activity may function to limit the bioavailability of phosphorylated lipid agonists at their respective cell surface receptors. Indeed, several reports have recently reported data which supports this model.

The first report of an LPP effect on the signalling mediated by a lipid phosphate agonist was by Jasinska and colleagues (1999). In this study, overexpression of LPP1 in rat2 fibroblasts led to a decrease in the activation of ERK-1/2 and DNA synthesis induced by LPA (Jasinska et al., 1999). This study was extended by Xu and colleagues who showed that several signalling events associated with activation of LPA_1/EDG2 by LPA were shown to be attenuated by LPP1 overexpression in Rat2 fibroblasts, such as the activation of ERK-1/2 and PLD, inhibition of adenylyl cyclase, mobilisation of intracellular calcium and cell division (Xu et al., 2000). In addition, these authors reported that there was no attenuation of PDGF or EGF signalling events following overexpression of LPP1, thereby demonstrating the specificity of the LPP1 effect for LPA. Interestingly, these effects of LPP1 overexpression on signalling by LPA were observed under conditions where less than 10% of the exogenous LPA added to the cells was degraded (Xu et al., 2000). This therefore, implied the existence of a mechanism of LPP action which was more

complex than simple degradation of exogenous LPA (for review, see Pilquil et al., 2001). The authors suggested the presence of a saturable pool of LPA which is associated with the plasma membrane and is accessible to both LPP1 and the LPA receptor LPA_1/EDG2 (Xu et al., 2000). Therefore, it was proposed that exogenous LPA would first associate with cells before interacting with LPA_1/EDG2 and that the net specific association of LPA with cells could be decreased by LPP1 catalysed dephosphorylation, thus, attenuating LPA signalling events (Xu et al., 2000). In contrast, Hooks and colleagues suggested an alternative mechanism to explain the inhibitory effect of LPP1 overexpression on LPA mediated mitogenesis in HEK293 cells (Hooks et al., 2001). Thus, even though overexpression of LPP1 resulted in the degradation of around 90% of the added LPA in the medium over the period of the mitogenic assay, this could not account for the decrease in potency of LPA (Hooks et al., 2001). Furthermore, using LPP degradation resistant phosphonate analogues of LPA as tools, Hooks and colleagues reported that the mitogenic response to LPA was independent of the known LPA/EDG receptors. These authors proposed the existence of a low affinity LPA-signalling pathway, independent of LPA_1/EDG2, LPA_2/EDG4 and LPA_3/EDG7 which mediated the LPA-induced mitogenesis and platelet aggregation and was regulated by LPP1 (Hooks et al., 2001). For example, the authors proposed that the GPCR, PSP24, which was reported as an LPA receptor (Guo et al., 1996) may be involved but did not rule out the possibility of a non-receptor mediated effect (Hooks et al., 2001).

The studies described above have demonstrated a potential role for LPP1 in attenuating the signalling events mediated by LPA. However, they did not address the role of LPP1 on the signalling events mediated by other potential LPP substrates such as S1P which is an agonist for several S1P/EDG receptors (see Section 1.11.3.). Furthermore, neither of these studies addressed the potential roles of other LPP isoforms on LPA-stimulated signalling. Work by our laboratory recently demonstrated the attenuation of S1P- and LPA-mediated signalling events in HEK293 cells stably transfected with gpLPP1, gpLPP1a, hLPP2 but not with hLPP3 (Alderton et al., 2001a). This therefore, extended the potential role(s) of LPPs on signalling via exogenous phosphorylated lipids to the other members of the LPP

enzyme family and also to S1P and LPA. Significantly, the results from the latter study also demonstrated that there was no correlation between 'ecto'-LPP activity against LPA, S1P and PA and the attenuation of ERK-1/2 activation. For example, it was found that in the LPP3 overexpressing cells, 'ecto'-LPP activity against S1P was significantly elevated, even though LPP3 overexpression had no effect on S1P-mediated ERK-1/2 activation. Furthermore, LPP1, LPP1a and LPP2, but not LPP3 were found to attenuate the stimulation of ERK-1/2 by thrombin, a peptide agonist whose bioavailablity would not be regulated by the LPP enzymes (Alderton et al., 2001a). These results were therefore, in direct contrast to those reported by Xu and colleagues (2000) but agreed, in part, with those of Hooks and colleagues (2001). The study of Alderton and colleagues (2001a) also demonstrated a reduction in the basal intracellular PA levels in the LPP1, LPP1a, and LPP2, but not LPP3 transfected HEK293 cells (Alderton et al., 2001a). This led to the suggestion that the LPPs may act on a pool of basal intracellular PA to regulate the membrane dynamic for signalling via GPCRs.

The aim of the present study was to extend the investigation of the LPP-mediated attenuation of S1P-mediated signalling. Therefore, the aims of this section were:

(i) To investigate the effects of stable overexpression of LPP1, LPP1a, LPP2 and LPP3 in HEK293 cells on the basal growth rates of the cells.

(ii) To investigate the effects of stable overexpression of LPP1, LPP1a, LPP2 and LPP3 in HEK293 cells on $S1P_1$/EDG1 mRNA levels.

(iii) To investigate the effects of transient overexpression of LPP1, LPP1a, LPP2 and LPP3 in HEK293 cells on S1P signalling to ERK-1/2.

(iv) To investigate the effects of transient overexpression of LPP1, LPP1a, LPP2, LPP3 and antisense LPP1 in airway smooth muscle cells, on S1P signalling to ERK-1/2.

5.2. Results

5.2.1. The effect of stable overexpression of LPP1, LPP1a, LPP2 or LPP3 on basal cell growth rates of HEK293 cells

S1P and LPA are two potent signalling molecules which have been shown to be involved in the survival, growth, differentiation but also death of cells (for reviews, see Pyne & Pyne, 2000a, Tigyi, 2001). Overexpression of the LPPs in HEK293 cells has been shown to attenuate ERK-1/2 activation in response to these lipid agonists (Alderton et al., 2001a). Therefore, the effects of stable overexpression of LPP1, LPP1a, LPP2 and LPP3 on the basal growth rates of HEK293 was investigated. HEK293 cells, stably transfected with either blank vector (pcDNA3.1 zeo(-)), or plasmid constructs encoding LPP1, LPP1a, LPP2 or LPP3 were plated separately at a density of approximately 33000 cells/well and basal growth rates measured daily for a period of 8 days (Figure 5.1). Figure 5.1 shows that the five HEK293 stable cell lines were observed to grow continuously and at similar rates throughout the 8 days of study. Therefore, overexpression of each of the LPP isoforms did not significantly affect the basal growth rates of the HEK293 cells.

5.2.2. The effect of stable overexpression of LPP1, LPP1a, LPP2 or LPP3 on $S1P_1$/EDG1 receptor mRNA transcript levels in HEK293 cells

One possible mechanism that might account for the attenuation of S1P- and LPA-signalling in HEK293 cells that stably overexpress LPP1, LPP1a or LPP2 is a reduction in the expression levels of S1P- and LPA-specific receptors. For example, a recent report by Sato and colleagues demonstrated the downregulation of $S1P_3$/EDG3 mRNA expression by inducers of differentiation such as retinoic acid in HL-60 leukaemia cells (Sato et al., 1998). The authors also reported this effect to correlate with an attenuation of S1P-induced calcium mobilisation (Sato et al., 1998). Therefore, the $S1P_1$/EDG1 receptor mRNA levels were assessed through semi-quantitative PCR (Section 2.9.4.) in each of the HEK293 cell lines stably transfected

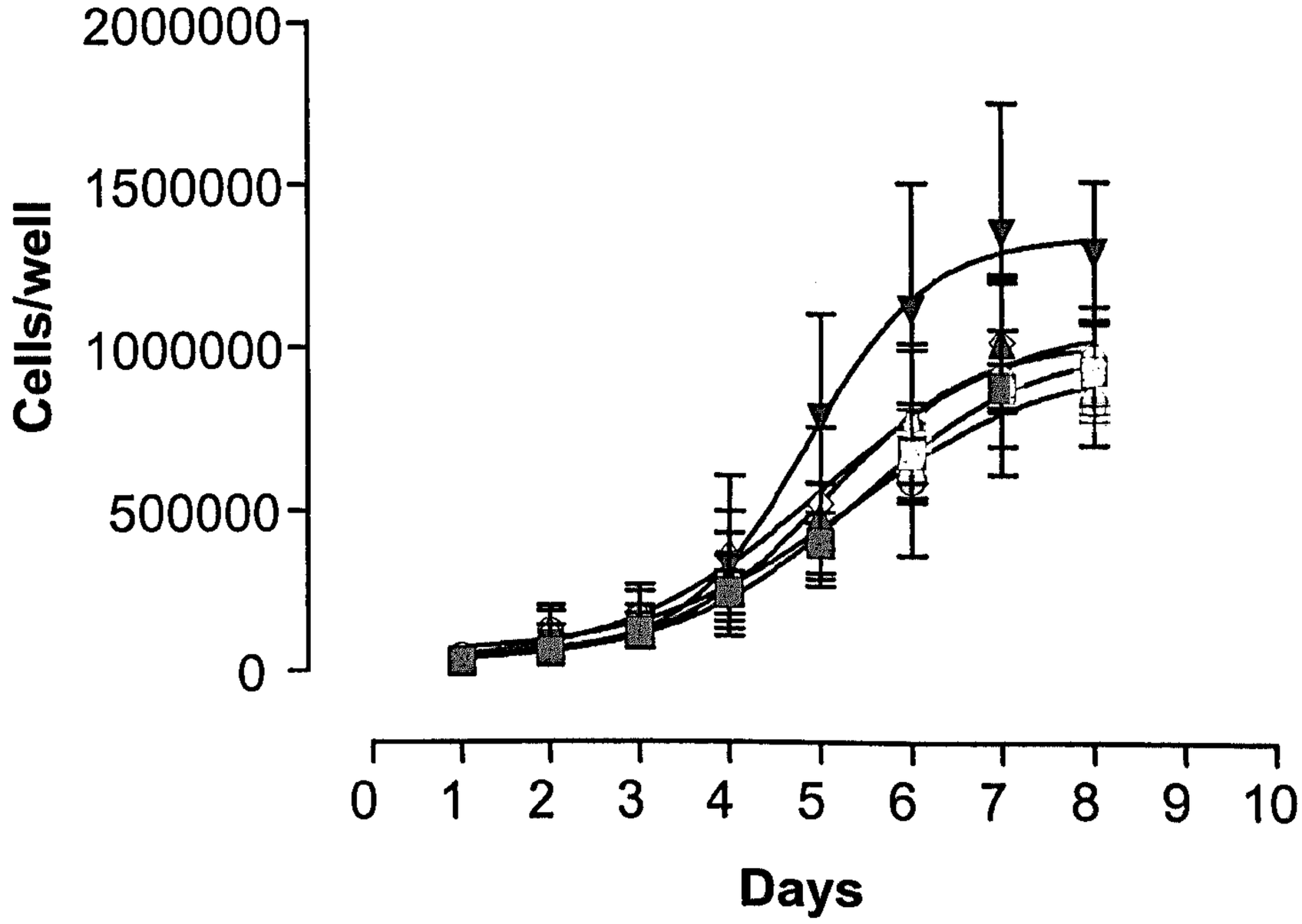

Figure 5.1: Basal rates of proliferation of HEK293 cells stably overexpresing LPP1, LPP1a, LPP2 or LPP3

HEK293 cells, stably transfected with either blank vector, LPP1, LPP1a, LPP2 or LPP3 were seeded onto 12 well plates at a density of approximately 33000 cells/well. Basal rates of proliferation were monitored through daily cell counting for a period of 8 days as described in 2.8.7. . Results are expressed as cells/well (mean +/- SEM of data pooled from three separate experiments performed in triplicate).

■ = Blank Vector

▲ = LPP1

▼ = LPP1a

◇ = LPP2

○ = LPP3

with blank vector, or overexpressing LPP1, LPP1a, LPP2 or LPP3. Human G3PDH mRNA levels were analysed in parallel to confirm equal addition of cDNA in the reaction mixtures for each of the cells types (Section 2.9.4.).

5.2.2.1. Linear amplification of $S1P_1$/EDG1 and G3PDH mRNA by RT-PCR

Semi-quantitative RT-PCR requires the linear amplification of each gene transcript of interest to sub-maximal levels. This allows any relative changes in transcript levels to be assessed. Therefore, each gene transcript of interest was amplified for various cycle lengths (conditions as in 2.9.4.) in order to characterise the amplification of each (Figures 5.2A and 5.2B). Firstly, Figure 5.2B confirms the presence of $S1P_1$/EDG1 transcript in HEK293 cells. In addition, Figures 5.2A and 5.2B show that, in the absence of reverse transcriptase (-RT), no products were obtained following PCR in any samples. Furthermore, Figures 5.2A and Figure 5.2B clearly show that at 25 cycles, $S1P_1$/EDG1 (347bp product) did not show maximal amplification whereas G3PDH (983bp product) showed high levels of transcript. However, no transcript was observed for either $S1P_1$/EDG1 or G3PDH at 20 cycles. Therefore, 25 cycles were used in all subsequent RT-PCR reactions in order to assess $S1P_1$/EDG1 and G3PDH gene transcript levels in each of the LPP overexpressing cell lines.

5.2.2.2. Assessment of $S1P_1$/EDG1 transcript levels in HEK293 cells stably overexpressing LPP1, LPP1a, LPP2 or LPP3

Figures 5.2A and 5.2B show the RT-PCR amplification (25 cycles) of $S1P_1$/EDG1 (347bp product) and G3PDH (983bp product) from HEK293 cells stably transfected with blank vector or a plasmid construct encoding LPP1, LPP1a, LPP2 or LPP3. Figure 5.2A demonstrates that G3PDH transcript levels are similar across the five cell lines. However, as shown in Figure 5.2B, $S1P_1$/EDG1 transcript levels appear to be lower in the LPP1, LPP1a, LPP2 and LPP3 overexpressing cell lines compared to the control cells. The percentage decrease in $S1P_1$/EDG1 transcripts (estimated by

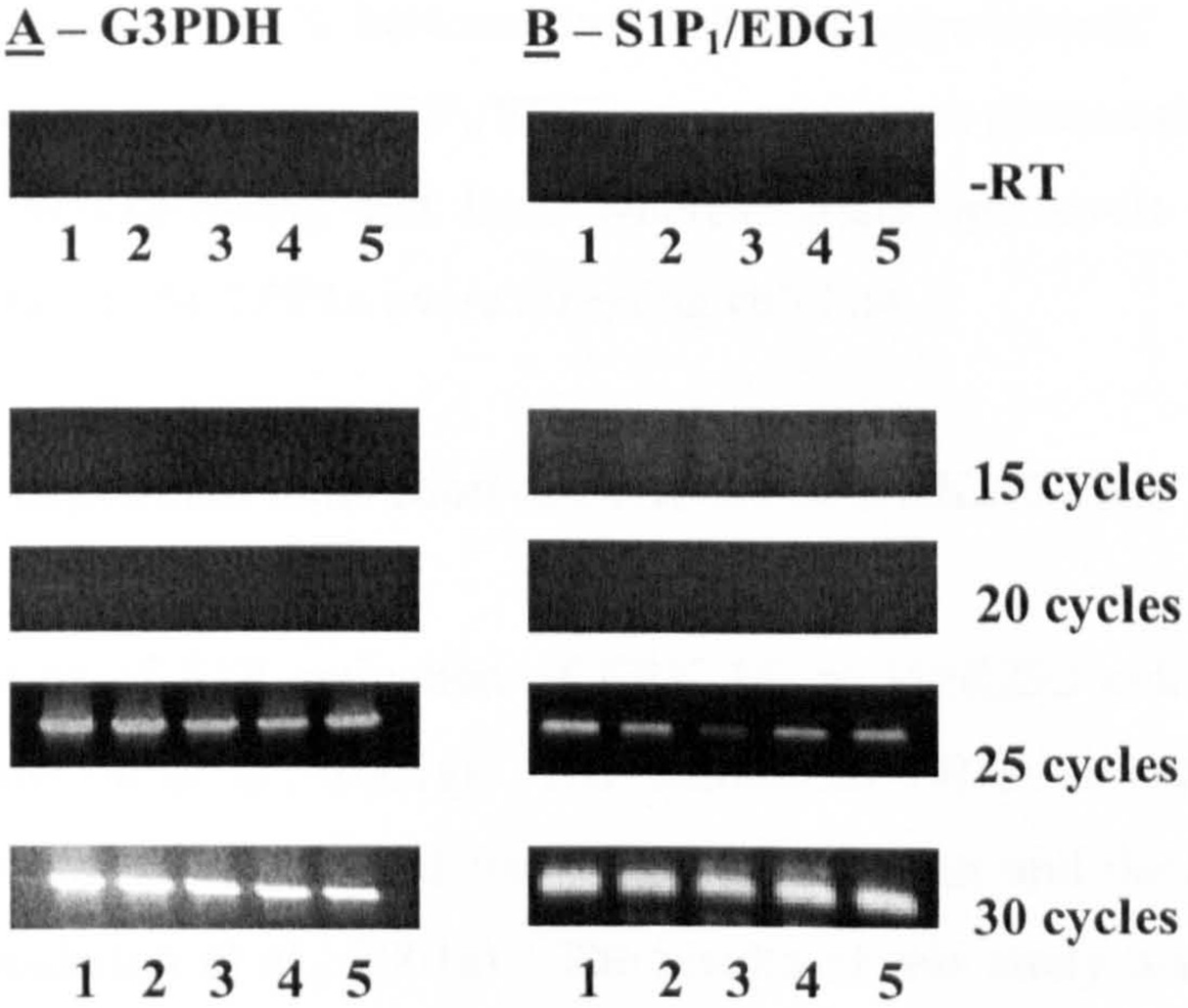

Figure 5.2: Semi-quantitative RT-PCR of G3PDH and $S1P_1$/EDG1 mRNA from HEK293 cells stably transfected with blank vector or plasmid constructs encoding LPP1, LPP1a, LPP2 or LPP3 at various cycle lengths

Total RNA was isolated from HEK293 cells stably transfected with blank vector or plasmid constructs encoding LPP1, LPP1a, LPP2 and LPP3 (2.9.1.). 2μg total RNA/sample was used as a template for cDNA synthesis (2.9.3.) of which one fifth was used for each PCR. PCR was conducted using gene specific primers designed towards **A**- G3PDH (983bp product) and **B**- $S1P_1$/EDG1 (347bp product) for –RT (25 cycles), 15, 20, 25 and 30 cycles (2.9.4.). **1** – Blank vector, **2** – LPP1, **3** – LPP1a, **4** – LPP2, **5** – LPP3 transfected HEK293 cells. Samples were analysed by agarose gel electrophoresis (2.9.5.) and quantified by densitometry using Scion Image© - Version 3b. These are representative results from an experiment conducted twice.

densitometry) were typically: LPP1 overexpressing cells, 52%; LPP1a overexpressing cells, 96%; LPP2 overexpressing cells, 68% and LPP3 overexpressing cells, 66% between two separate experiments. Therefore, similar levels of down-regulation in $S1P_1$/EDG1 transcript were observed in the LPP1, LPP2 and LPP3 overexpressing cell lines whereas transcript levels were substantially reduced further in the LPP1a overexpressing cell line.

5.2.3. S1P-dependent activation of ERK-1/2 in HEK293 cells

The timecourse of S1P activation of ERK-1/2 in HEK293 cells has already been defined (Alderton et al., 2001a). S1P-stimulated ERK-1/2 activation is initiated within 2-5 minutes, maintained for at least 30 minutes and declines towards basal thereafter (Alderton et al., 2001a). The results of this study also suggests that the S1P signal to ERK-1/2 occurs via a Gαi GPCR dependent mechanism in HEK293 cells (Alderton et al., 2001a).

5.2.4. The effect of transient overexpression of LPP1, LPP1a, LPP2 and LPP3 in HEK293 cells on S1P- and PMA-mediated activation of ERK-1/2

It has already been established by our group that stable overexpression of LPP1, LPP1a, LPP2 but not LPP3, attenuates S1P-mediated ERK-1/2 activation (Alderton et al., 2001a). However, the levels of overexpression obtained from stable transfection are generally substantially higher than those obtained with transient transfection. Therefore, to confirm that the effects observed were not peculiar to the stably transfected cell lines and an artefact of high levels of expression of the LPPs, the LPPs were individually, transiently overexpressed HEK293 cells to assess their effects on the S1P-induced ERK-1/2 activation. Figure 5.3A shows that S1P-mediated activation of ERK-1/2 was substantially reduced in HEK293 cells transiently overexpressing LPP1, LPP1a and LPP2, but not LPP3. Furthermore, Figure 5.3B shows that transient overexpression of LPP1, LPP1a, LPP2 and LPP3

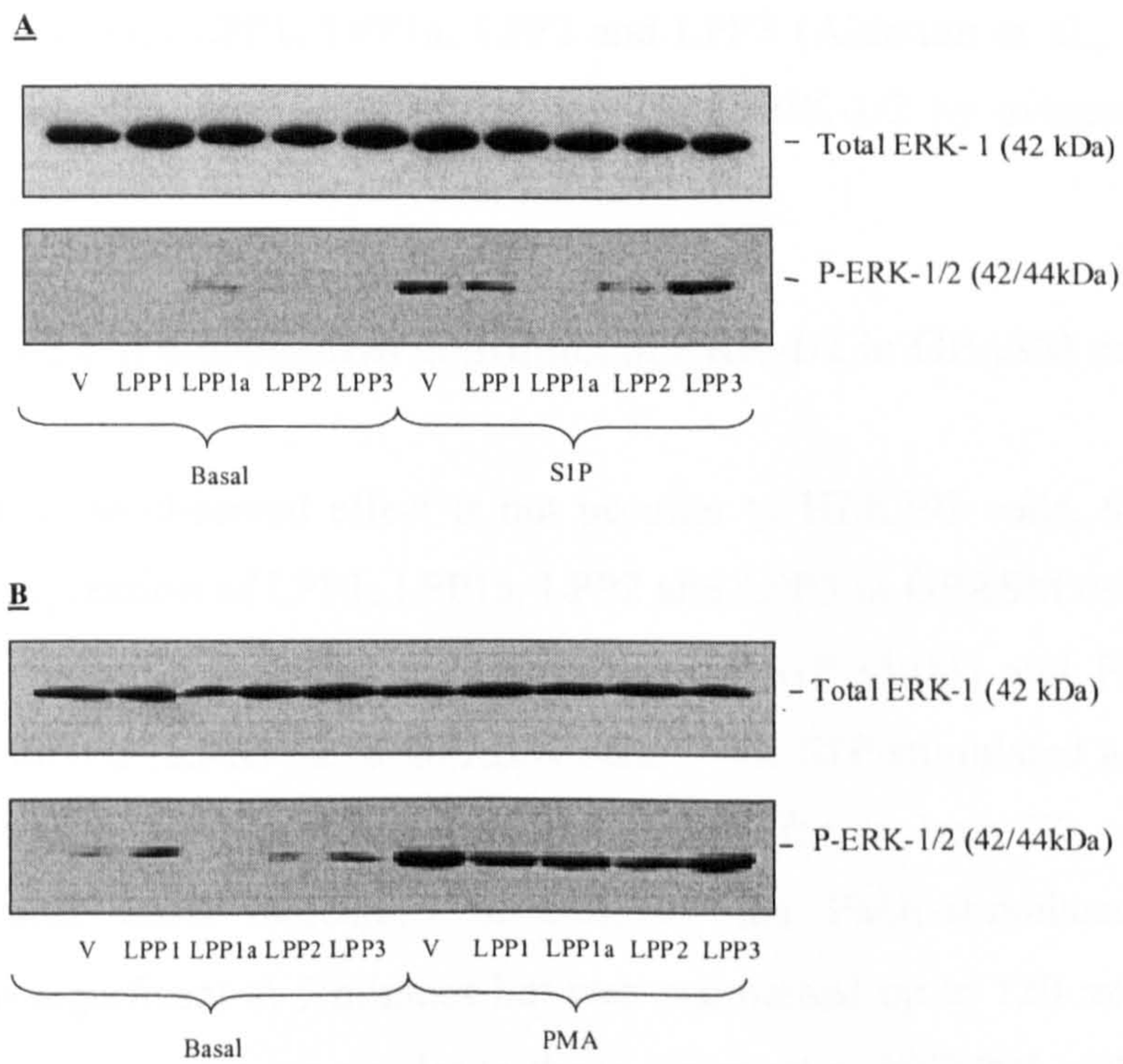

Figure 5.3: The effect of transient overexpression of LPPs on the S1P-induced activation of ERK-1/2 in HEK293 cells

HEK293 cells were transiently transfected with either blank vector (V), or plasmid constructs encoding LPP1, LPP1a, LPP2 or LPP3 (2.8.6) prior to stimulation for 10 minutes with S1P (5μM) or PMA (1μM). Cell lysates were prepared (2.4.1) and analysed by Western blot using anti-phospho ERK-1/2 antibody (2.4.9). Blots were stripped (2.4.10.) and reprobed with an anti- ERK-1 antibody (2.4.9) to ensure equal protein loading. This is a representative result of an experiment conducted twice.

had no effect on the PMA-mediated activation of ERK-1/2. PMA was used as a control as it bypasses GPCRs and growth factor receptors to activate ERK-1/2. These results are in direct agreement with the results obtained in the HEK293 cells stably transfected with LPP1, LPP1a, LPP2 and LPP3 (Alderton et al., 2001a) and further establishes the attenuation of S1P-mediated ERK-1/2 by overexpression of LPP1, LPP1a and LPP2, but not LPP3 in HEK293 cells.

5.2.5. S1P- and PMA-dependent activation of ERK-1/2 in GPASM cells

To confirm that the observed effect is not peculiar to HEK293 cells, the effect of transient overexpression of LPP1, LPP1a, LPP2 and LPP3 in GPASM cells was also investigated. Figure 5.4, shows the timecourse of S1P (5μM) and PMA (1μM) induced activation of ERK-1/2 in GPASM cells. The S1P-stimulated activation of ERK-1/2 was significant at 5 minutes, maintained for at least 20 minutes and declined towards basal thereafter. In contrast, the PMA-stimulated ERK-1/2 activation was significant at 5 minutes but was maintained up to 120 minutes. The kinetics of these responses are similar to those observed in HEK293 cells (Alderton et al., 2001a) and agree with previously published observations in GPASM (Pyne & Pyne, 1996). In addition, pre-treatment of GPASM cells with the bacterial toxin, pertussis toxin (PTX, 0.1μg/ml) (which ADP-ribosylates the G-proteins, Gαi/o, and uncouples the G-proteins from their receptors) substantially reduced the S1P-dependent activation of ERK-1/2 but had little effect on PMA-stimulated ERK-1/2 activation (Figure 5.5). This confirms that S1P appears to exert its effect on ERK-1/2 via a Gαi/o dependent mechanism in GPASM cells (Pyne & Pyne 1996), as is also the case in HEK293 cells (Alderton et al., 2001a).

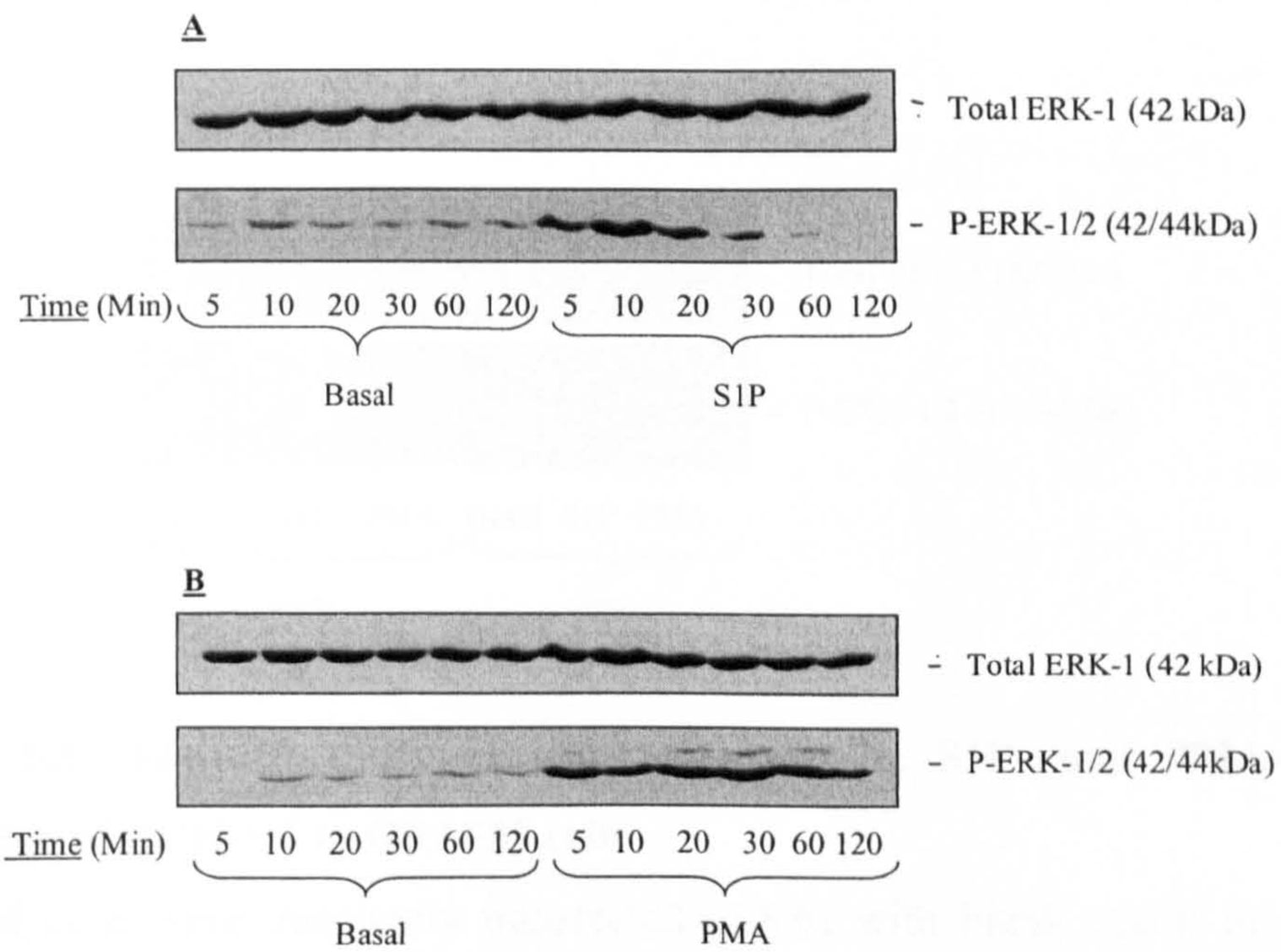

Figure 5.4: S1P and PMA dependent activation of ERK-1/2 in GPASM cells

GPASM cells were transiently transfected with blank vector (2.8.6) prior to stimulation with **A-** S1P (5μM) or **B-** PMA (1μM) for the indicated times. Cell lysates were prepared (2.4.1) and analysed by Western blot using anti-phospho ERK-1/2 antibody (2.4.9). Blots were stripped (2.4.10) and reprobed with an anti- ERK-1 antibody (2.4.9) to ensure equal protein loading. This is a representative result of an experiment conducted three times.

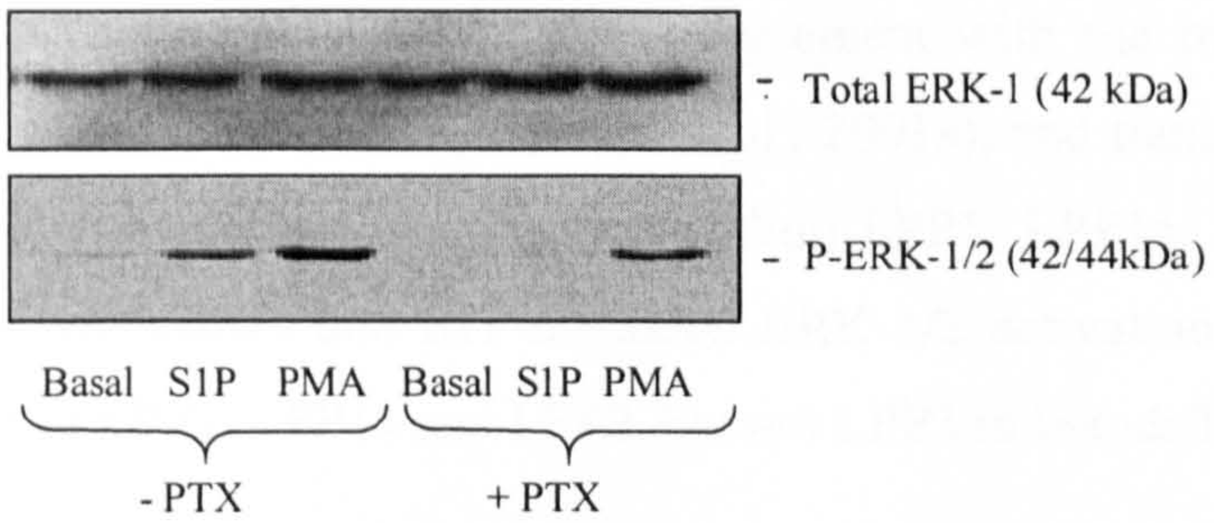

Figure 5.5: The effect of pertussis toxin on the S1P- and PMA-mediated activation of ERK-1/2 in GPASM cells

GPASM cells were transiently transfected (2.8.6) with blank vector prior to pre-treatment with and without pertussis toxin (0.1μg/ml, 18 hours) before stimulation with S1P (5μM) or PMA (1μM) for 10 minutes. Cell lysates were prepared (2.4.1) and analysed by Western blot using anti-phospho ERK-1/2 antibody. Blots were stripped (2.4.10) and reprobed with an anti- ERK-1 antibody (2.4.9) to ensure equal protein loading. This is a representative result of an experiment conducted three times.

5.2.6. The effect of transient expression of LPP1, LPP1a, LPP2 and LPP3 in GPASM cells on S1P- and PMA-mediated activation of ERK-1/2

Figure 5.6A shows that S1P-mediated activation of ERK-1/2 was substantially reduced in GPASM cells transiently overexpressing LPP1, LPP1a and LPP2, but not LPP3. Furthermore, Figure 5.6B shows that transient overexpression of LPP1, LPP1a, LPP2 and LPP3 had no effect on the PMA-mediated activation of ERK-1/2 in these cells. These results are in direct agreement with the results obtained in HEK293 cells stably transfected, (Alderton et al., 2001a), and transiently transfected (present study) with plasmid constructs encoding LPP1, LPP1a, LPP2 and LPP3. This therefore, establishes that S1P-mediated ERK-1/2 activation is attenuated by overexpression of LPP1, LPP1a and LPP2, but not LPP3 in two different cell types.

5.2.7. The effect of transient transfection of a plasmid construct encoding the antisense sequence for LPP1 in GPASM cells on S1P- and PMA-mediated activation of ERK-1/2

Whilst overexpression studies are useful to investigate the role(s) of specific proteins in signalling systems, it is possible that the results obtained may not be relevant to the physiological function of the protein. To substantiate a proposed function, it is useful to investigate the effects of reducing the levels of a protein as well, the prediction being that the opposite effect may be apparent. Therefore, as a further extension of the investigation into the role of the LPPs in S1P-mediated ERK-1/2 activation, the effect of transient transfection of a plasmid construct encoding the antisense sequence of LPP1 on S1P- and PMA-mediated ERK-1/2 activation was investigated. The antisense plasmid construct for LPP1 was generated by the insertion of the LPP1 nucleotide sequence into the mammalian expression vector (pcDNA3.1zeo(+)) where the multiple cloning site was in the reverse orientation (R.J.Tate – Molecular Biology facility, University of Strathclyde). Figure 5.7 demonstrates the attenuation of the S1P-induced but not PMA-induced ERK-1/2 activation in GPASM cells that had been transiently transfected with the plasmid construct encoding antisense-LPP1.

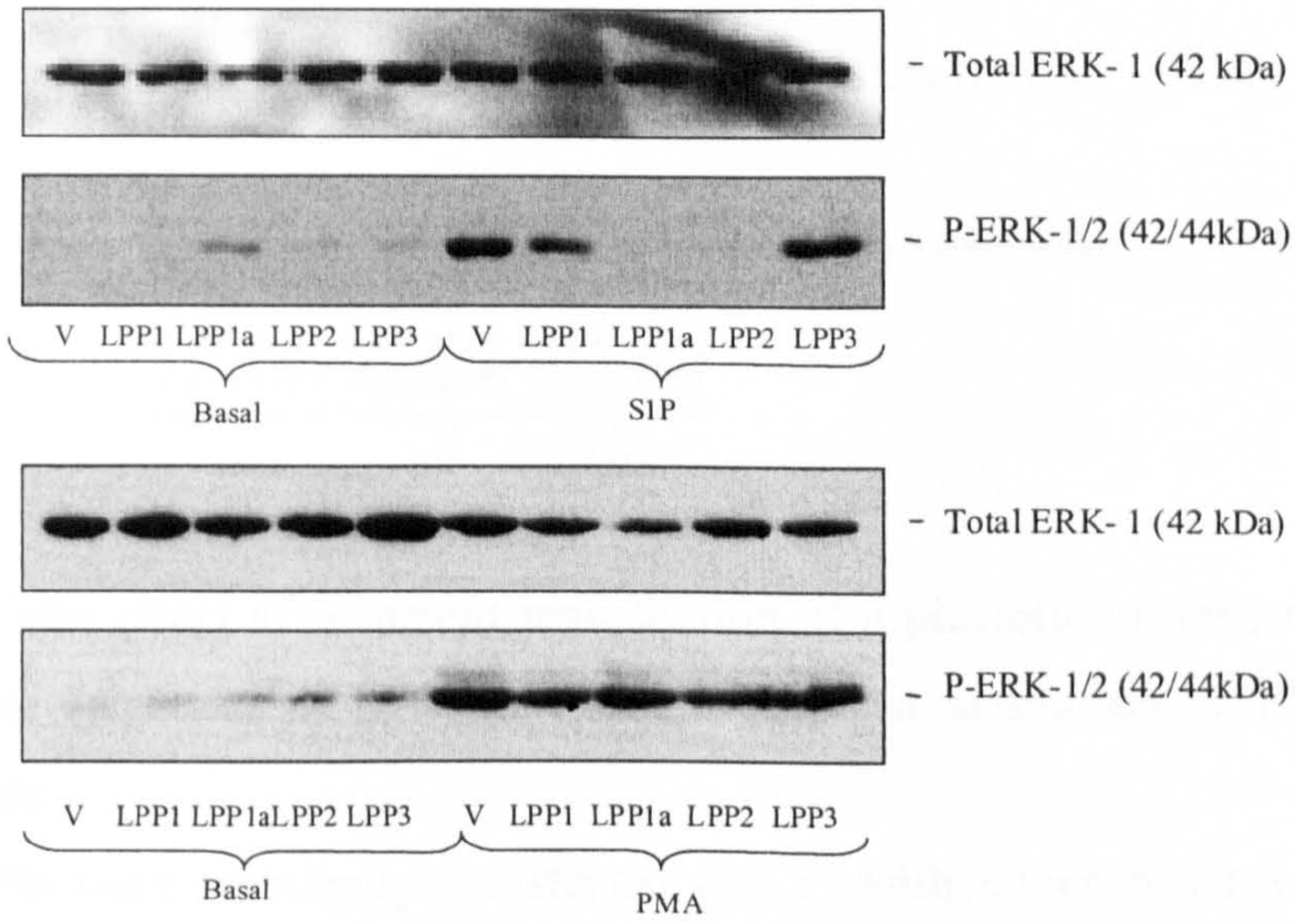

Figure 5.6: The effect of transient overexpression of LPPs on the S1P-induced activation of ERK-1/2 in GPASM cells

GPASM cells were transiently transfected (2.8.6) with blank vector (V), or plasmid constructs encoding LPP1, LPP1a, LPP2 or LPP3 prior to stimulation for 10 minutes with **A**- S1P (5μM) or **B**- PMA (1μM). Cell lysates were prepared (2.4.1) and analysed by Western blot using anti-phospho ERK-1/2 antibody (2.4.9). Blots were stripped (2.4.10) and reprobed with an anti- ERK-1 antibody (2.4.9) to ensure equal protein loading. This is a representative result of an experiment conducted twice.

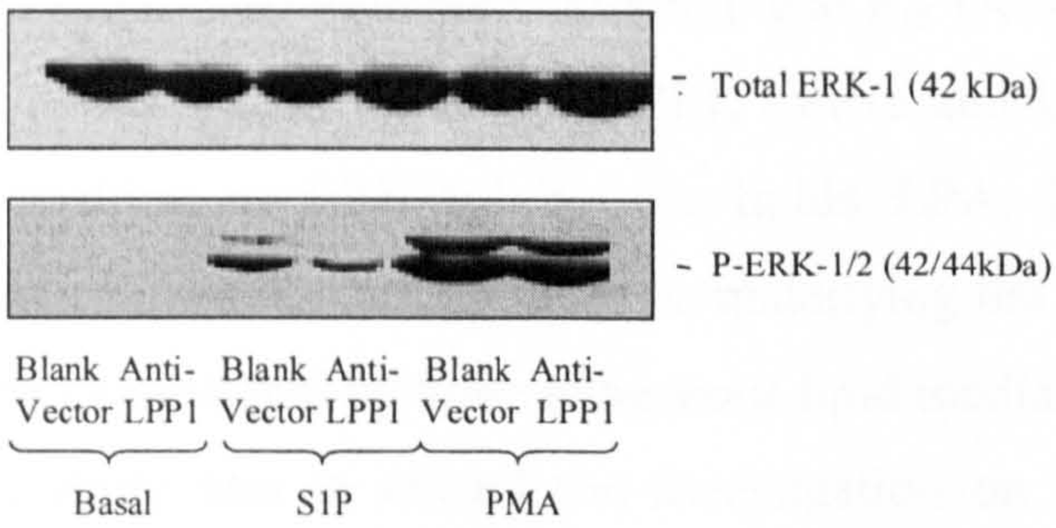

Figure 5.7: The effect of transient transfection of a plasmid construct encoding the antisense sequence of LPP on the S1P-induced activation of ERK-1/2 in GPASM cells

GPASM cells were transiently transfected (2.8.6) with either blank vector or a plasmid construct encoding the antisense sequence of LPP1 prior to stimulation for 10 minutes with **A**- S1P (5μM) or **B**- PMA (1μM). Cell lysates were prepared (2.4.1) and analysed by Western blot using anti-phospho ERK-1/2 antibody (2.4.9). Blots were stripped (2.4.10) and reprobed with an anti- ERK-1 antibody (2.4.9) to ensure equal protein loading. This is a representative result of an experiment conducted twice.

5.3. Discussion

Several studies have reported the attenuation of exogenous LPA-mediated signalling events following overexpression of LPP1 (Jasinska et al., 1999, Xu et al., 2000, Hooks et al., 2001). Furthermore, we recently extended these findings to other members of the LPP family, namely LPP1, LPP1a, LPP2 and LPP3, and also to the other exogenous phosphoryated lipid mediators S1P and PA (Alderton et al., 2001a). In the latter study, it was demonstrated that LPP1, LPP1a and LPP2, but not LPP3 could attenuate signalling to ERK-1/2 by the lipids LPA, S1P and also PA. However, controversy surrounds the mechanisms underlying the effects of the LPPs on the signalling events mediated by these exogenous lipid mediators. Therefore, the aim of the present study was to extend the investigation on the effects of LPP overexpression on exogenous S1P-mediated signalling.

S1P and LPA have well recognised proliferative properties and this led to the initial investigation to assess whether overexpression of the LPPs in HEK293 cells had an effect on basal cell proliferation. Figure 5.1 shows that no significant difference was observed in the proliferation of HEK293 cells which separately, stably overexpress the LPPs compared to vector control cells. No other study has examined the effects of LPP overexpression on basal rates of proliferation but Jasinska and colleagues demonstrated a reduction in LPA-mediated DNA synthesis in cells overexpressing LPP1 (Jasinska et al., 1999). Similar results were also obtained by Xu and colleagues (2000) in rat2 fibroblasts and by Hooks and colleagues (2001) who demonstrated the ablation of LPA-mediated mitogenesis by overexpression of LPP1 in HEK293 cells. The difference between the results of the former studies compared to the present study is not surprising in that, here, basal proliferation was measured in the initial presence of serum. Therefore, numerous growth factors would be present in the medium. Notably, serum-stimulated and EGF-stimulated activation of ERK-1/2 was not affected by stable overexpression of LPP1, LPP1a, LPP2 or LPP3 in HEK293 cells (Alderton et al., 2001a). Furthermore, LPA and S1P were unable to induce DNA synthesis in blank vector transfected HEK293 cells. Only incubation with serum was found to increase DNA synthesis and this was unaffected by

overexpression of each of the LPPs (S. Pyne, unpublished data). This therefore, suggests that the LPA and S1P-induced activation of ERK-1/2 may not be a growth signal in the HEK293 cells used in the present study and that the proliferation of the HEK293 cells is governed more by the growth factors in the serum.

It is notable that, large errors are observed in the data sets obtained in the present study, possibly due to the low number of repeats (three separate experiments conducted in triplicate). Figure 5.1 demonstrates a possible trend whereby, the HEK293 cells stably transfected with LPP1a may have had an increased rate of growth compared to all other cells types. It is therefore, possible that increasing the number of observations would reduce the errors in the data and this effect would be significant. The reasons for this effect, if present, would not be clear and would require further investigation.

Previous studies have provided possible mechanisms to explain the effects of the LPPs on signalling by phosphorylated lipids based on the ability of the LPPs to degrade their lipid substrates. However, as detailed previously, conflicting results have been reported. We extended this type of study to assess whether the levels of $S1P_1$/EDG1 mRNA transcript were altered in HEK293 cells stably overexpressing LPP1, LPP1a, LPP2 or LPP3. A reduction in $S1P_1$/EDG1 expression in HEK293 cells overexpressing LPP1, LPP1a and LPP2, but not LPP3 is a possible molecular mechanism for the effects observed on S1P signalling events. However, Figure 5.3 shows a decrease in the mRNA transcript levels for $S1P_1$/EDG1 in all LPP transfected cell lines compared to vector transfected cells. These results suggest that overexpression of LPP1, LPP1a, LPP2 and LPP3 may alter the *de novo* synthesis of $S1P_1$/EDG1. Despite this, drawing conclusions from the present data is difficult for several reasons. Firstly, only two independent experiments were performed and so the finding requires further confirmation. In addition, it is well recognised that mRNA transcript levels do not necessarily correlate with levels of protein expression. Therefore, experiments investigating the levels of $S1P_1$/EDG protein are also required to support any conclusions. Notably, there is no correlation between the reduced mRNA transcript levels and the loss of S1P-stimulated ERK-1/2

activation. For example, using these stable cell lines, Alderton and colleagues (2001a) demonstrated that overexpression of LPP3 had no effect on the S1P-induced activation of ERK-1/2. However, the results of the present study suggest that LPP3 overexpression decreases $S1P_1$/EDG1 mRNA transcript to a level comparable to that in HEK293 cells that stably overexpress LPP1 or LPP2, which were shown to exhibit reduced S1P-mediated ERK-1/2 activation. The reason for this discrepancy is unclear. However, it suggests that a complex mechanism exists in the control of S1P-mediated signalling by the LPPs and that although a reduction in the $S1P_1$/EDG1 mRNA transcript levels may contribute to this regulation, other factors/mechanisms may also be involved.

The only other study of this type was conducted by Xu and colleagues who investigated the levels of LPA_1/EDG2 mRNA transcript in cells overexpressing LPP1 (Xu et al., 2000). In this study, the authors reported no alteration in the levels of LPA_1/EDG2 mRNA transcript. However, Xu and colleagues only conducted RT-PCR at 30 amplification cycles and as demonstrated in this study, they may not have obtained non-maximal, linear amplification in their study which could mask any differences present.

Figure 5.3 shows the results of transient transfection of LPP1, LPP1a, LPP2 and LPP3 on exogenous S1P- and PMA-induced ERK-1/2 activation in HEK293 cells. The results obtained in this study are identical to those obtained in the HEK293 cells stably overexpressing the LPPs where LPP1, LPP1a and LPP2, but not LPP3 are seen to attenuate S1P-mediated ERK-1/2 activation but that the PMA response is unaffected in all cases. Similar results were also obtained using transiently transfected GPASM cells (Figure 5.6). The use of transient transfection is expected to provide lower expression levels compared to stable transfection where, in the latter case, the gene of interest is incorporated into the genome of the whole population of cells. In contrast, with transient tranfection, expression levels vary with transfection efficiency such that a mixed population of cells exists with some cells expressing the gene of interest and others that are not. However, transient overexpression of the LPPs in either HEK293 cells or GPASM cells confirmed that the effects were not

peculiar to the apparently highly overexpressing stably transfected HEK293 cell lines or to the HEK293 cell type alone. This further strengthens the possibility that the LPPs function as negative regulators of ERK-1/2 activation mediated by exogenous lipid phosphate agonists.

The final section of this study investigated the effects of reducing the amount of LPP1 in GPASM by transient transfection of a plasmid construct encoding the antisense sequence for LPP1. Unexpectedly, the expression of antisense LPP1 decreased S1P-induced ERK-1/2 activation, rather than increasing it or having no effect whereas, the PMA-mediated activation of ERK-1/2 was unaffected. The result with S1P-stimulated ERK-1/2 activation was therefore, opposite to what was predicted. It was also contrary to the results reported by Xu et al., (2000) where the transfection of rat2 fibroblasts with antisense oligodeoxynucleotides for LPP1, increased LPA-stimulated ERK-1/2 activation. This also correlated with an increase in [^{3}H]-thymidine incorporation in response to LPA, reduced LPP activity by up to 90%, decreased dephosphorylation of exogenous LPA and an increase the net association of LPA with the cells (Xu et al., 2000). This was therefore, in agreement with their theory that the LPPs function to reduce the association of LPA with cells which they suggest is a pre-requisite for signalling by LPA via the LPA/EDG receptors (Xu et al., 2000).

There are two possible explanations for the results obtained in the present study. Firstly, high basal levels of LPP activity are present in GPASM cells (results not shown) and this may have been unaffected by transient transfection of the antisense LPP1 plasmid construct. Notably, the relative levels of each LPP isoform in GPASM cells is unknown and the effects of reducing the levels of LPP1 may have been masked by the actions of the other LPPs or may even have resulted in up-regulation of another LPP. Alternatively, reducing the levels of LPP1 in GPASM cells may have resulted in up-regulation of another S1P-specific enzyme such as the sphingosine 1-phosphate phosphatase reported by Mandala and colleagues (2000) and also Le Stunff and colleagues (2002) which then masked the effects of reducing the levels of LPP1.

5.3.1. Summary and future work

The results of this study have further confirmed the recent reports suggesting that the LPPs may function as negative regulators of signalling events mediated by exogenous phosphorylated lipid mediators. The S1P-mediated activation of ERK-1/2 has been shown to be attenuated in both HEK293 cells and GPASM cells, transiently overexpressing LPP1, LPP1a or LPP2, but not LPP3, which is in agreement with previous studies in stably transfected HEK293 cells (Alderton et al., 2001a). Furthermore, this study has shown that there is a reduction of $S1P_1$/EDG1 mRNA transcript levels in HEK293 cells stably overexpressing LPP1, LPP1a, LPP2 and LPP3. However, the relevance of this finding is unclear and needs to be further investigated. The use of an antisense LPP1 plasmid construct was found to have the opposite effect to that expected in that S1P-induced ERK-1/2 activation in GPASM cell was also reduced.

There are several experiments, which need to be conducted to support the findings of the present study. For example, the assessment of $S1P_1$/EDG1 mRNA transcript levels needs to be repeated and could also be extended to similar investigations on the mRNA transcript levels of the other S1P/EDG and also LPA/EDG which are expressed in HEK293 cells and known to signal to ERK-1/2. $S1P_1$/EDG1, $S1P_2$/EDG5 and $S1P_3$/EDG3 are expressed in HEK293 cells (B. Singh – unpublished data) and have all been demonstrated to signal to the ERK-1/2 pathway (Windh et al., 1999, An et al., 2000) (Section 1.11.4.). In addition, Alderton and colleagues (2001b) demonstrated the expression of LPA_1/EDG2 but not LPA_2/EDG4 and LPA_3/EDG7 in HEK293 cells and these receptors have also been demonstrated to couple to the ERK-1/2 signalling pathway (Ishii et al., 2000) (Section 1.8.2.). It is currently not known if the HEK293 cells express $S1P_4$/EDG6 or $S1P_5$/EDG8 and little is known at present with regards to which effector pathways these receptors couple to (Section 1.11.4.). Furthermore, with the recent advent of commercially available anti-S1P/EDG and LPA/EDG antibodies, Western blot analysis would also provide evidence on the levels of protein expression to support any findings obtained from the investigations on mRNA levels.

LPP activity measurements and Western blot analysis using anti-LPP isoform specific antibodies on the transiently transfected cells would confirm the overexpression of the specific LPP isoform and allow the estimation of transfection efficiency in each cell line investigated in the present study. In relation to the former, as detailed, GPASM cells displayed high basal levels of LPP activity and transient overexpression of LPP1 could not be detected as an increase in total LPP activity in membranes derived from these cells (results not shown). However, as a functional alteration in ERK-1/2 activation was observed compared to vector transfected cells, it is possible to suggest that the transfections were successful. It is possible to speculate that a small local change in LPP1 activity could result in the loss of the ERK-1/2 response to S1P through the reduction of S1P levels in small, localised signalling domains such as caveole or detergent resistant membrane domains. Indeed, LPP1, LPP1a and LPP3 have recently been demonstrated to localise to caveolin-enriched domains and also detergent resistant membrane domains (Sciorra et al., 1999, Nanjundan & Possmayer, 2001). Activity measurements could also be conducted on the transiently transfected HEK293 cells which showed low basal levels of LPP activity (Alderton et al., 2001a) to confirm expression of the LPPs and so support the conclusions of this study.

The use of specific antibodies to detect expression of the LPPs is another method by which the results reported in this study could be supported. However, the endogenous, and overexpressed LPP1 and LPP1a can not be detected by the anti-LPP1/LPP1a, or the anti-LPP catalytic domain antisera available. Affinity purification or refinement of the procedure is therefore required to improve reactivity. LPP2 and LPP3 have been demonstrated through Western blot and confocal microscopy, to be expressed in the stably transfected HEK293 cell lines (Alderton et al., 2001a). This could therefore, be extended to the transiently transfected HEK293 cells. However, it should be noted that LPP2 is resistant to SDS-PAGE analysis without prior detergent solubilisation, highlighting the difficulty of studying this enzyme family.

Further experiments of interest include investigating the effects of overexpressing the LPP isoforms on other exogenous S1P-mediated signalling events such as the inhibition of adenylyl cyclase. In addition, a more detailed investigation of the effects of reducing the levels of LPP isoforms in systems through the use of antisense technology, on signalling events mediated by exogenous phosphorylated lipids is also required. Furthermore, an extension of the investigation into basal cell proliferation is required to increase the number of repeats but could also be extended to assess the effects of stable overexpression of the LPPs on DNA synthesis and proliferation induced by the individual phosphorylated lipids. For example, even though S1P and LPA failed to induce DNA synthesis in HEK293 cells (S. Pyne – unpublished data), Rakhit and colleagues (1999) reported a similar finding for S1P in ASM cells but demonstrated S1P to function as a co-mitogen with PDGF. The present study could therefore, be extended to see if similar co-mitogenicity exists in HEK293 cells and whether overexpression of the LPPs affects this co-mitogenicity in both GPASM and HEK293 cells. There is therefore, still much work to be conducted to further assess the roles of the LPP enzymes in signalling events mediated by exogenous phosphorylated lipids and determine the mechanisms involved.

CHAPTER 6

GENERAL DISCUSSION

6. General Discussion

Recent advances in LPP research have revealed the potential of this diverse enzyme family to regulate the signalling events mediated by bioactive lipid phosphates, including PA, LPA, C1P and S1P. One proposed function of the LPPs is to regulate the balance between these lipid phosphates and their dephosphorylated products of which DG, ceramide and Sph also have potent signalling roles (for review, see Waggoner et al., 1999). Furthermore, evidence is accumulating showing a complex ordering of diverse lipid signalling pathways where 'cross-talk' exists between sphingolipid and glycerolipid signalling (section 1.14). In addition, recent advances in receptor signalling has revealed the presence of cross-regulation of GPCRs (such as those for bioactive lipid agonists such as LPA and S1P) and classical growth factor receptor tyrosine kinases (section 1.12.3.). Therefore, regulation of signalling events mediated by lipid phosphates may have far reaching implications to the physiology and pathophysiology of cells.

Currently, four mammalian LPP isoforms, LPP1, LPP1a, LPP2 and LPP3 have been identified. However, very little is known about their respective physiological roles, biochemical regulation, or contribution to signal transduction. There is therefore, still a great amount of information regarding the basic properties of the LPPs to be discovered. This has prompted a great amount of research into this diverse enzyme family.

6.1. Purification of LPP isoforms

Molecular biological techniques and the ability to express recombinant proteins in a variety of systems has revolutionised the production, purification and subsequent characterisation of proteins. Advances in DNA technology, combined with projects such as the Human Genome Project have allowed the identification, cloning, subsequent expression and characterisation of proteins which have never been purified from their native source. Furthermore, these advances have enabled proteins

to be engineered in forms which aid their purification and so characterisation following expression.

The purification of LPP activity has been limited to the partial purification of, most probably, mixtures of LPP isoforms from native sources (Kanoh et al., 1992, Kai et al., 1996, Fleming & Yeaman, 1995b, Waggoner et al., 1995) (See Chapter 3). In the present study, gpLPP1 and gpLPP1a have been engineered with a hexa- and deca-histidine tag. This allowed the partial purification of LPP1 and LPP1a using IMAC but resulted in the loss of all catalytic activity (Chapter 3). The refinement of this purification protocol or the development of an alternative protocol such as the pMal-c2x system reported in this study also, would allow the extensive characterisation of the LPP isoforms. For example, pure protein would allow the precise investigation of the kinetics and substrate preference of the individual LPPs. Furthermore, studies on the regulation of the LPPs through processes such as phosphorylation/dephosphorylation could be conducted using the purified enzymes as substrates for various protein kinases. In addition, the purified enzymes could be attached to resins to create affinity columns to investigate potential protein-protein interactions and so reveal potentially crucial clues to the physiological roles of the LPPs.

Another important development which would be provided by the successful purification of individual LPPs would be the ability to perform detailed structural analysis of the LPPs through techniques such as X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy. The discovery of the precise 3-dimensional structure of the LPPs is vital to discovering and understanding the function of the LPPs. The importance of this information has led to the structure of the LPPs being predicted using techniques such as hydrophobicity plots. Furthermore, the structure of rLPP1 was recently predicted by Waggoner and colleagues (1999) using the coordinates from the X-ray structure of chloroperoxidase which is a related member of the phosphatase superfamily to which the LPPs belong. Even though this model is entirely hypothetical it does provide clues as to the possible orientation of LPP1 in the membrane of cells. Accordingly, the models of

the structure of the LPPs and subsequent studies using techniques such as site directed mutagenesis have predicted the LPPs to possess six transmembrane domains and an active site which is expressed on the outer leaflet of the plasma membrane or the lumen of the golgi or endoplasmic retculum (Pilquil et al., 2001). This model structure has been used as evidence to explain the reason for the ability of the LPPs to dephosphorylate exogenous lipid phosphates and so illustrates the fundamental information that structural analysis can provide.

6.2. Substrate specificity and kinetics of the LPP isoforms

Substrate preference is also of critical importance in defining the physiological roles of the LPPs with respect to signalling by their lipid phosphate substrates. All the proposed LPP substrates have been shown to be potent signalling molecules within cells. Furthermore, several of the products of the LPP catalysed reaction (DG, ceramide and Sph) have also been demonstrated to mediate a plethora of signalling events. Therefore, defining the substrate preference and kinetics of action of the individual LPP isoforms may provide crucial information on the potentially different functions of the individual LPP isoforms. The only reports of substrate preference investigations on individual, cloned LPPs are those by Kai and colleagues (1997), Roberts and colleagues (1998) and Hooks and colleagues (1998). These studies were limited in that they utilised single substrate assay systems, varying assay conditions, investigated only certain LPP isoforms or substrates and, in some cases, reported contrasting results (see Chapter 4). For example, Kai and colleagues (1997) reported that when LPP1 and LPP3 were overexpressed, only LPP3 possessed the ability to dephosphoryate S1P. Hooks and colleagues (1998) only tested PA and LPA as substrates and Roberts and colleagues (1998) demonstrated the differing ability of LPP1, LPP2 and LPP3 to dephosphorylate PA, LPA, C1P and S1P in an assay which varied from that used by Hooks and colleagues and also the present study. Furthermore, none of these studies investigated the effects of acyl chain length and degree of saturation on the ability of the LPPs to dephosphorylate PA.

In the present study, the different LPP isoforms were shown to exhibit broad substrate specificity's similar to those reported by Roberts and colleagues (1998). In addition, the individual LPPs were shown to display variable kinetics and differing abilities to dephosphorylate various molecular species of PA as well as LPA, C8-C1P and S1P. Furthermore, the present study represents the first demonstration of potential substrate cooperativity for an individual LPP isoform. Tolan (1997) reported the only other demonstration of cooperativity for total LPP activity from GPASM cells. The substrate cooperativity observed for the individual LPPs was found to vary considerably depending on the LPP being investigated and also on the substrates being presented to the enzymes. This illustrates the potential presence of complex and variable interactions between the lipid phosphate substrates and the individual LPP enzymes. Furthermore, this represents a significant advance in the understanding of the catalytic mechanisms of the LPPs since it suggests the possibility that the LPPs may function as multimeric complexes within cells.

The complex interactions which may exist between the LPPs and the many lipid phosphate substrates may be critical in determining the direction and overall ability of an LPP to dephosphorylate a particular substrate. The lipid phosphate substrates may determine the signalling pathways affected by the LPPs and thereby serve as regulators of LPP activity in order to allow the LPPs to act as tightly controlled, 'molecular integrators' of signalling events mediated by the many lipid phosphate substrates. A more detailed kinetic analysis of the individual LPP isoforms investigating the many possible combinations of substrates which may be presented to the LPPs *in vivo* is required in order to fully appreciate the interactions which exist and the kinetic mechanisms of LPP action. This process would be aided greatly by the successful purification of the LPP isoforms.

6.3. 'Ecto'-LPP or an alternative intracellular action?

Transient transfection of LPP1, LPP1a or LPP2, but not LPP3 in HEK293 and GPASM cells was found to attenuate S1P-induced ERK-1/2 activation. This was similar to previous results obtained in our laboratory with HEK293 cells stably

overexpressing LPP1, LPP1a, LPP2 or LPP3 (Alderton et al., 2001a). Furthermore, several reports have also detailed the attenuation of LPA-mediated ERK-1/2 activation following overexpression of LPP1 (Jasinska et al., 1999, Xu et al., 2000, Hooks et al., 2001, for review, see Pilquil et al., 2001) and also LPP1a, LPP2 and LPP3 (Alderton et al., 2001a). The mechanisms surrounding these effects remain to be defined and several theories have been proposed (Chapter 5). The theories originally proposed by Xu and colleagues (2000) and Hooks and colleagues (2001) were based on the ability of the LPPs to dephosphorylate exogenous lipid phosphates either in the surrounding medium or following association with the plasma membrane. However, Alderton and colleagues suggested an alternative interpretation since 'ecto'-LPP activity did not correlate with the attenuation of lipid phosphate-induced ERK-1/2 activation (Alderton et al., 2001a). Furthermore, Alderton and colleagues (2001) demonstrated the ability of the LPPs to attenuate ERK-1/2 activation in response to thrombin (a peptide agonist which would therefore, not be degraded by the LPPs) in a manner almost identical to the effects observed for LPA and S1P. This suggested the involvement of more complex and diverse mechanisms apart from the 'ecto'-LPP activity.

In relation to the potential intracellular action of the LPP isoforms, Alderton and colleagues (2001) observed that basal PA levels were reduced in LPP1, LPP1a and LPP2, but not LPP3 transfected HEK293 cells. Therefore, this provided a better correlation with the observed attenuation of ERK-1/2 activation. In an earlier study, Leung and colleagues (1998) also reported reduced PA levels in HEK293 cells transfected with LPP1 and LPP1a. Therefore, Alderton et al., (2001), proposed that the LPP isoforms may act on a basal pool of PA, distinct from agonist-stimulated PA, which functions to regulate the membrane dynamic required for signalling via certain GPCRs. There is a considerable amount of evidence to support a role for PA in the process of ERK-1/2 activation. Firstly, PA has been found to inhibit p21rasGAP (Tsai et al., 1989) and activate p21rasGIP (Tsai et al., 1990), thereby prolonging the lifetime of activated ras and so activated raf-1 which is essential for activation of ERK-1/2 (Section 1.5.2). Secondly, PA has been reported to directly interact with raf-1 (Ghosh et al., 1996) and be required for the translocation of raf-1

to the plasma membrane for its subsequent activation and signalling to ERK-1/2 (Rizzo et al., 1999) (Section 1.5.2). Thirdly, along with phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2), PA has been shown to stimulate lipid penetration by dynamin (Burger et al., 2000) which is required for the pinching off of endocytic vesicles and subsequent activation of ERK-1/2 by certain GPCRs (Daaka et al., 1998). Further to this, the ratio of LPA/PA at the inner leaflet of the plasma membrane has been found to be important in the formation of endocytic vesicles and vesicle budding (Schmidt et al., 1999). This involves the enzyme endophilin-1 which is a LPAAT and so converts LPA into PA which has been found to be essential for synaptic vesicle endocytosis (Schmidt et al., 1999, Huttner & Schmidt, 2000).

Therefore, LPP1, LPP1a and LPP2-mediated alteration of basal PA levels may (i) disrupt the integrity of specific lipid microdomains within which, signalling complexes are assembled, (ii) disrupt the recruitment of signalling proteins to caveolae, or (iii) disrupt the endocytosis required for stimulation of ERK-1/2 certain by certain GPCRs (Alderton et al., 2001a). Support for an LPP role in regulating the levels of PA, for example in lipid microdomains or caveolae has been provided by the findings that LPP1, LPP1a and LPP3 have been demonstrated to localise, along with several other signalling components, to caveolin-enriched domains or detergent resistant domains (Sciorra et al., 1999, Nanjundan & Possmayer, 2001). These theories are currently under investigation in our laboratory.

The present study showed a reduction in the mRNA transcript levels of $S1P_1$/EDG1 in the LPP1, LPP1a, LPP2 and LPP3 transfected HEK293 cells. Even though the levels of mRNA transcript do not necessarily correlate with the levels of protein expression, this highlights another possible mechanism of action of the LPPs where there may be a reduction in the *de novo* synthesis of $S1P_1$/EDG1 or other lipid phosphate receptors. The natural progression of research attempting to determine the role of the LPPs is to reduce the levels of LPPs and observe the functional effects. The present study utilised a plasmid construct encoding the antisense nucleotide sequence for LPP1 in order to reduce the levels of expression of LPP1 in HEK293

cells. However, as detailed previously, this was found to result in attenuation of S1P-induced ERK-1/2 activation similar to that observed with overexpression of LPP1. This may have been due to upregulation of alternative S1P-specific phosphatases. In contrast, Xu and colleagues (2000) demonstrated antisense oligodeoxynucleotides to LPP1 to decrease 'ecto'-LPP activity and remove the LPP-mediated attenuation of signalling events by LPA. These results further suggest the presence of several different mechanisms of action of the LPPs. Investigations are required to discover the significance of the different potential mechanisms and whether several of the mechanisms are required in combination and are stimulus and cell-type specific.

6.4. Summary and conclusions

The reactions catalysed by the LPP isoforms place the LPPs in a critical position in the control of the levels of bioactive lipid phosphates which have been shown to have roles in regulating processes ranging from cell growth and survival, to cell death. Recent research has revealed the important functions of the LPPs in attenuating the signalling events mediated by both intra- and extra-cellular lipid phosphates. Therefore, the differential regulation of LPP activity in cells has the potential to control the balance of several potent lipid mediators and could be critical in the decision of a cell to live or die. The current study has highlighted the presence of potentially crucial interactions between the LPPs and their lipid phosphate substrates that may control the direction of LPP activity towards different substrates and also the possible existence of the LPPs as multimers within cells. Therefore, it is possible to speculate from the results here that, depending on the combination of substrates available to each of the LPP isoforms, the activity of the enzyme may be differentially targeted towards different substrates. Therefore, it is possible that localisation of individual LPP isoforms to distinct microdomains within cells may allow the LPP isoforms to act as 'molecular integrators' of several different lipid signalling pathways, depending on the lipid composition of the microenvironment.

There is a need for further research to determine the importance of LPP action in the attenuation of signalling events mediated by lipid phosphates and other GPCR agonists with respect to 'ecto'-LPP activity and alternative intracellular effects. The purification of individual LPP isoforms would be a significant progression in LPP research and would allow the discovery of many basic properties of the LPPs. This would greatly assist the elucidation of the physiological roles of the LPPs. Questions, which remain to defined, include the exact subcellular localisation of the LPPs, the exact 3-dimensional structure of the LPPs and the precise physiological substrates. The answers to these questions will provide fundamental clues to the roles of the LPPs in health and disease and also to the reasons for the molecular diversity displayed by this enzyme family.

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