Immunological investigations into the properties, and function of the phosphatidylinositol transfer protein

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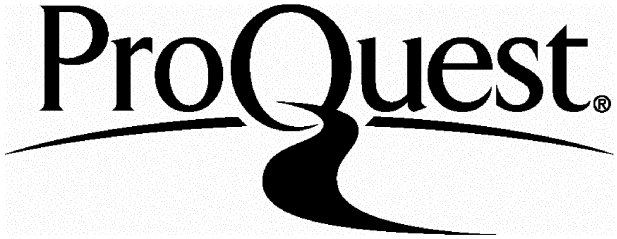

Abstract

It is becoming increasingly apparent that the inositol lipids are involved in a wide range of cellular processes, ranging from transmembrane signal transduction, to modulation of the behaviour of the cytoskeleton. To date one of their best characterised functions is in the operation of the Phospholipase C signalling pathway, whereby inositol lipid components of the cell are degraded in response to ligand binding at membrane receptors to produce the second messenger molecules diacylglycerol, and inositol-1,4,5-trisphosphate. Studies conducted on this process have implicated the mammalian phosphatidylinositol transfer protein (PI-TP) as being essential to the functioning of this pathway.

This thesis outlines studies on the nature and function of PI-TP in the leukaemic cell line HL60, and the neutrophil, for which the HL60 cell is often regarded as a model. In studies utilising HL60 cells permeabilised, and cytosol depleted using the bacterial toxin Streptolysin O (SLO), PI-TP was identified as being essential to the maintenance of the PLC signalling response. On the basis of these data, earlier work was extended by the development of immunochemicals against the two identified isoforms of mammalian PI-TP (α and β) and their use to study various characteristics of this protein. These reagents include polyclonal antibodies raised against a fusion protein of PI-TPα/GST, and a panel of monoclonal antibodies against native bovine brain PI-TPα, and bacterially expressed rat brain PI-TP β.

Among the questions addressed are the kinetics with which the proteins involved in PLC signalling leak from SLO permeabilised cells, the quantities of PI-TPα and β present in the intact cell, and the phosphorylation state of the protein in response to ligand stimulation. In the course of this work it was discovered that the β isoform is by far the predominant of the two proteins in neutrophils, and HL60 cells, and that the two protein display distinct kinetics of leakage, with the α isoform leaking very rapidly from the cell while the β isoform is significantly retained. PI-TPα appears to undergo phosphorylation in response to treatment of the cell with both PMA, and the chemotactic peptide fMLP.

Abbreviations, and symbols

ATP	Adenosine-5' triphosphate
βME	2-Mercaptoethanol
BSA	Bovine serum albumin
°C	Degrees Celcius
cAMP	Adenosine 3'5 - cyclic monophosphate
DAG	2,3-Diacylglycerol
DFP	DI-isopropylfluorophosphate
cDNA	complementary Deoxyribonucleic acid
DTT	Dithiothreitol (Clelands reagent)
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethyleneglycol-bis-(βaminoethylether) N,N,N,N tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
fMLP	Formyl-methionyl-leucyl-phenylalanine
FPLC	Fast protein liquid chromatography (TM of Pharmacia Ltd)
GDP	Guanosine-5' diphosphate
GTP	Guanosine-5' triphosphate
GTPγS	Guanosine-5'-O-(3-thiophosphate)
HAT	Hypoxanthine, aminopterin, thymidine supplement
HEPES	N-[2-hydroxyethyl]piperazine N'-[2-ethanesulphonic acid]
HRPO	Horseradish peroxidase
IEF	Isoelectric focusing
IgG	Immunoglobulin G

IgM	Immunoglobulin M
mAb	Monoclonal antibody
NMS	Normal mouse serum (Pre-immune)
OPD	ortho-Phenylenediamine dihydrochloride
PLC	Phospholipase C
PBS	Phosphate buffered saline
pCa	-Lg Ca^{2+} (M)
PA	Phosphatidic acid
PI	Phosphatidylinositol
PIP	Phosphatidylinositol-4-phosphate
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PIPES	Piperazine-N,N'bis[2-ethylsuphonic acid]
PKA	cAMP-dependent protein kinase
PKC	Ca^{2+}-associated, phospholipase dependent protein kinase
PMA	Phorbol myristyl acetate
PMSF	Phenyl-methyl-sulphonyl-fluoride
PVDF	Polyvinyldifluoride
(r)PI-TP	(recombinant) Phosphatidylinositol transfer protein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-Tetramethylethylene diamine
TLC	Thin layer chromatography
TLCK	N α–p-Tosyl-I-lysine chloromethyl ketone
Tris	Tris(hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate

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Chapter 1

Introduction

The complex biochemical activities of the cell are subject to regulation, under the influence of external conditions beyond the cell membrane. How the flux of information across the cell membrane, to modulate the activity of the intracellular effectors and ultimately the function of the cell is one of the most challenging aspects of modern biology. Until quite recently the whole process of signal transduction remained something of a 'black box'. The field as it now stands can be considered as having its birth in the work of Paul Ehrlich approximately 100 years ago. He suggested that the specificity with which heavy metal poisons effect the cells of the central nervous system probably reflects the existence of receptors on the surface of these cells which were specifically responsible for interaction with these molecules.

Cell signalling has become one of the main foci of scientific effort and as a result the field has achieved almost dazzling progress in a comparatively short space of time: today a number of transmembrane signalling processes are known and are being dissected at the molecular level. The signalling pathway with which the bulk of this work deals is the phospholipase C signalling pathway, in which the inositol lipid phosphatidylinositol-4,5-biisphosphate (PIP_2) is broken down to liberate inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), both of which are known to have a role in signalling.

As long ago as twenty years, the existence of phospholipid transfer proteins had been identified (1). Recent work in this laboratory identified one such protein, phosphatidylinositol transfer protein (PI-TP) as being involved in the efficient and sustained function of the phospholipase C (PLC) signalling pathway. The majority of the work described in this thesis relates specifically to the two isoforms of phosphatidylinositol transfer protein currently identified, PI-TP α and β.

The phospholipase C signalling system is currently one of the best understood of the transmembrane signalling mechanisms. Central to the function of this system is the constant supply of the substrate PIP_2, derived by the sequential phosphorylation of

the minor membrane lipid component phosphatidylinositol. It has been demonstrated that PI-TP plays a role in the provision of substrate for this process (2,3).

The importance of PI-TP to PLC signalling suggests that this protein may conceivably have an equal impact upon other systems which have been demonstrated as being effected by concentrations of the lipid PIP_2. Hence much effort has been directed by this lab into the further characterisation of these fascinating proteins. The multiple significance of PI-TP has been tantalisingly hinted at by the demonstration that PI-TP constitutes a component necessary for regulated exocytosis in adrenal chromaffin cells (4,5), HL60 cells (6), as well as in the formation of vesicles (7).

Before going on to consider the function of PI-TPs in detail, the phospholipase C signalling system and other systems upon which it, or the lipid PIP_2 has an impact will be considered.

Transmembrane signal transduction via the hydrolysis of inositol lipids is a widespread and well documented phenomenon, which has been extensively reviewed in the literature (8-12). In addition to the important role that PIP_2 plays in this process as substrate for the phospholipase C enzymes, PLCs, this lipid is also increasingly appreciated to play a role in a number of other processes including organisation of the cytoskeleton and regulation of some branches of the phospholipase D signalling system which operates by the hydrolysis of phosphatidylcholine to choline and phosphatidic acid. Each of these functions of PIP_2 will be discussed in more detail under the appropriate headings later on in this introduction. The first of the signalling systems to be considered will be the PLCs, which to date represent some of the best characterised of the transmembrane lipid signalling systems. Some of the key enzymes involved in inositol lipid metabolism and signalling are illustrated in Fig. 1.1.

1.1 Phospholipases C:

The enzymes responsible for the hydrolysis of PIP_2 to yield the two second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3), are the phospholipase C superfamily (11). This group comprises three subfamilies (PLCβ, PLCγ and PLCδ) related primarily as a result of sequence homology in the regions

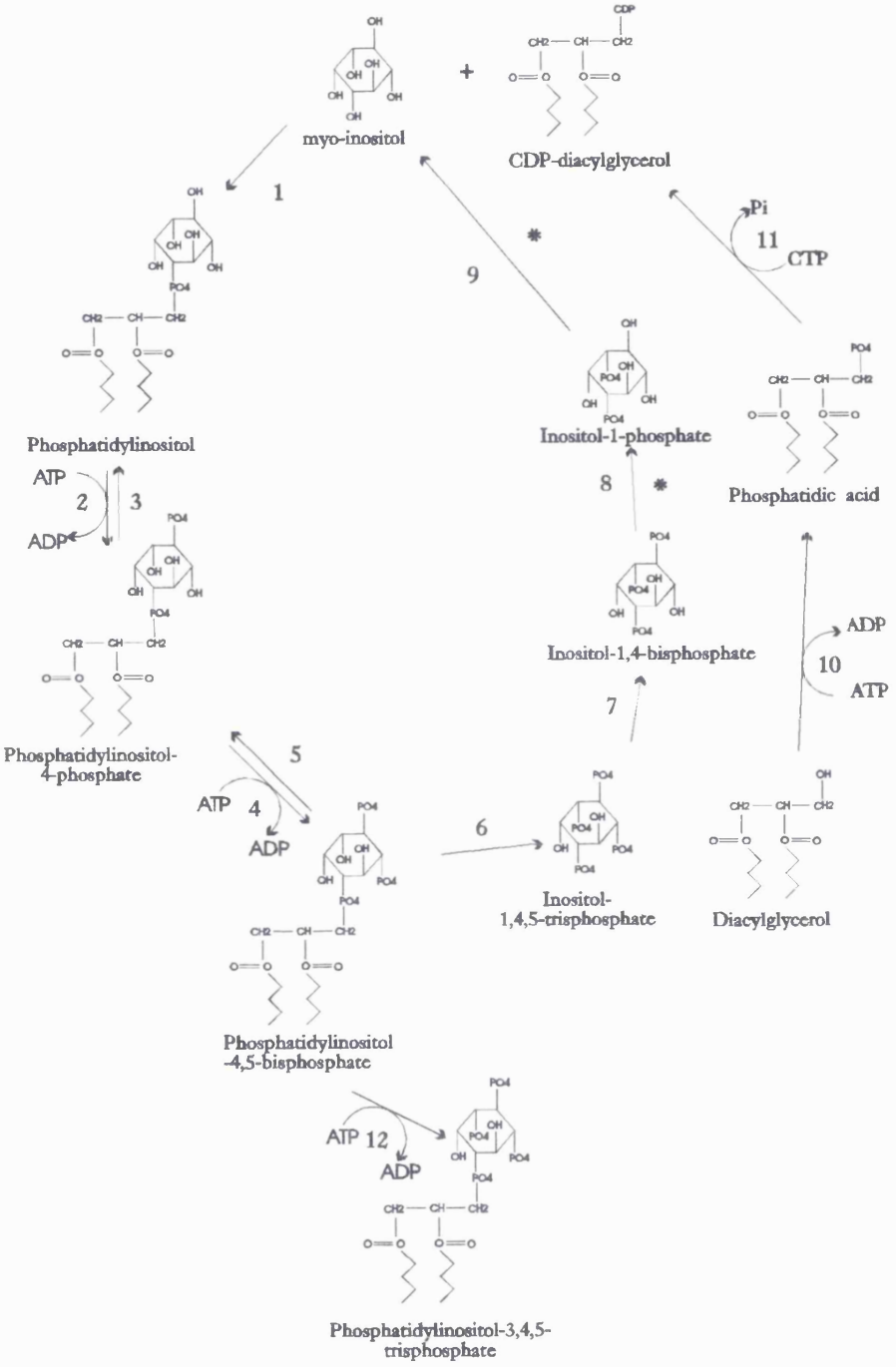

myo-inositol
CDP-diacylglycerol
1
Phosphatidylinositol
9
Pi
11
CTP
Inositol-1-phosphate
Phosphatidic acid
ATP
2
3
ADP
8
Phosphatidylinositol-
4-phosphate
Inositol-1,4-bisphosphate
ADP
10
ATP
7
5
ATP
4
ADP
6
Inositol-
1,4,5-trisphosphate
Diacylglycerol
Phosphatidylinositol
-4,5-bisphosphate
ATP
12
ADP
Phosphatidylinositol-3,4,5-
trisphosphate

Fig. 1.1

Fig 1.1 Schematic representation of the inositol cycle

The accompanying figure illustrates the steps in the cyclic metabolism of the inositol phospholipids involved in cell signalling via the phospholipase C, and PI-3-Kinase pathways. The enzymes involved in the individual steps are as follows:-

1. PI-synthase
2. PI 4 kinase
3. PI-4-phosphate phosphatase
4. PI-4-P 5-kinase
5. PI-(4,5)-P_2 5-monophosphatase
6. Phospholipase C

7-9. Various phosphatases responsible for the conversion of IP_3 to Inositol.

10. Diacylglycerol kinase
11. CTP-phosphatidate cytidyl transferase
12. PI-3 kinase

The points in the cycle which are believed to be susceptible to influence by the Li^+ ion are asterisked. This phenomenon is taken advantage in the PLC reconstitution assay, where the accumulation of inositol polyphosphates is measured.

which represent the active site. These are designated as the X and Y domains. The accompanying diagram (Fig 1.2) represents the proteins in linear form, with important regions of primary structure highlighted.

The regulation of the γ and β families of PLC is better understood than that of the δ family and it is only relatively recently that a real handle on the way that the latter family behave has been obtained, by the discovery that the pleckstrin homology (PH) domain of PLC δ1 is required for recruitment of this enzyme to the cell membrane (13). The physiological relevance of this and the details of *in vivo* regulation remain to be determined. A number of other facts suggest that calcium ions may play a role in the function of PLCδ once recruited to the membrane.

While all of the members of the PLC superfamily are responsible for the conversion of the minor lipid component phoshatidylinositol-4,5-bisphosphate to the second messenger molecules IP_3 and DAG there are a number of differences in the way in which the individual families are regulated, their *in vitro* substrate specificity with respect to the other phospoinositides, PI and PIP and the products. While IP_3 is the main inositol phosphate product of the activity of these enzymes, cyclic inositol phosphates are also liberated (14). There is a distinct hierarchy with which the enzymes release these products. In order of decreasing cyclic\non-cyclic products, the following pattern is observed:-

$$PLC\text{-}\beta_1 > PLC\text{-}\delta_1 > PLC\text{-}\gamma_1$$

It has been suggested that the cyclic products may play a role in their own right as signaling molecules and have an influence on proliferation of cells. Cells with relatively low levels of cyclic inositol phosphates grow to lower densities than those with high levels (15,16). It has recently been proposed on the basis of the reaction mechanism by which PLCs cleave PIP_2 that the cyclic inositol phosphates represent reaction intermediates. The extent to which these products are liberated are also related to the substrate utilised by the enzyme i.e. PI, PIP, or PIP_2, for which the PLCs have different affinities - hence the likelihood of the cyclic intermediate being released during hydrolysis is influenced by the substrate under consideration (17). In the next section of this introduction the individual families of PLCs will be considered in more

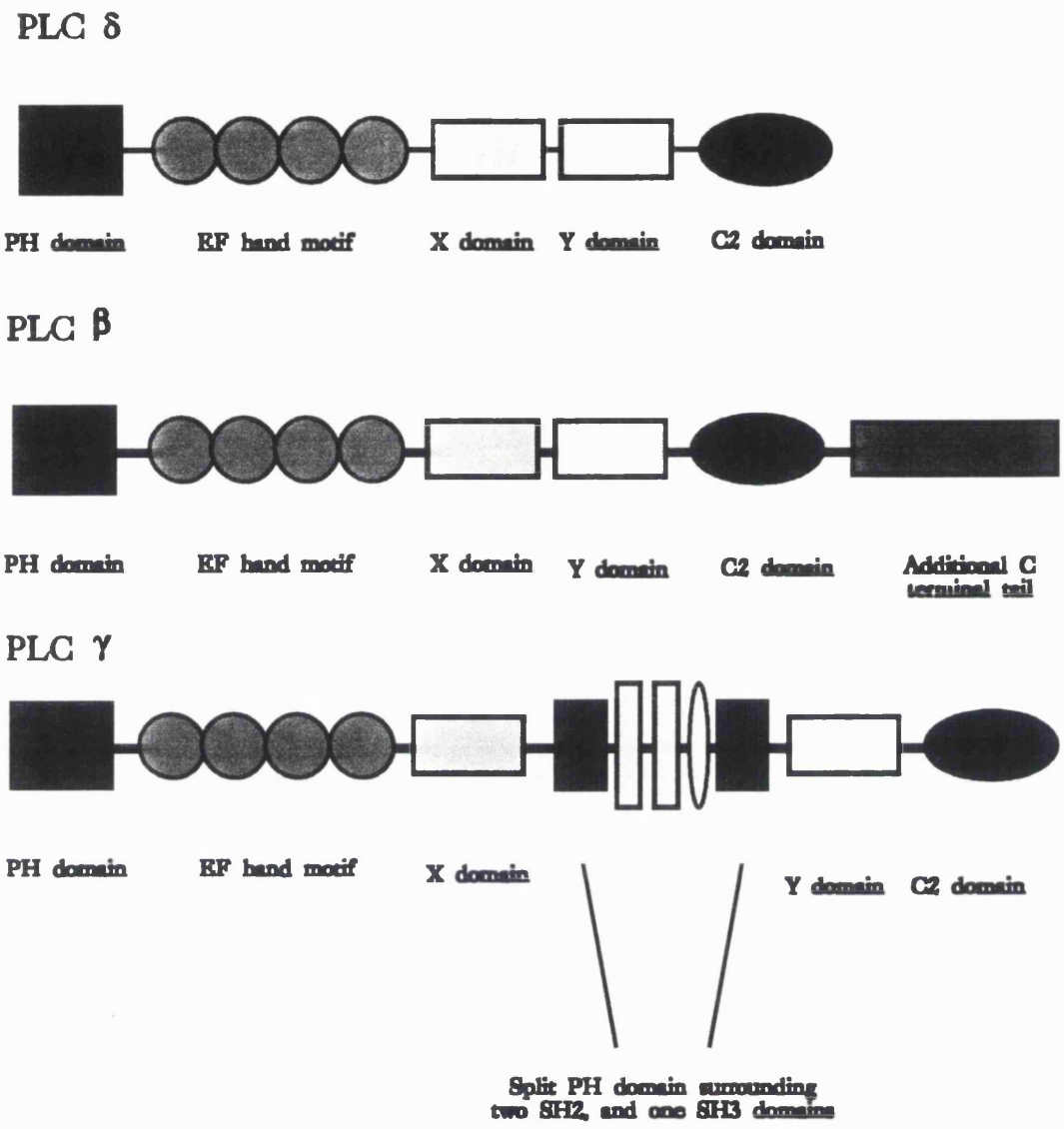


Fig. 1.2 Linear representation of the three identified mammalian phospholipase C families with their major structural motifs.

The enzymes share certain domains, while others are restricted to particular families, and reflect the manner in which the enzymes are regulated. All three families possess PH domains which are believed to be important to the binding of the enzymes to their membranes 'targets' - it has also been reported that these structures may be involved in interaction with G-protein βγ subnunits in the context of other proteins. The importance of Ca^{2+} in the activity of these enzymes is reflected in the presence of four EF hand domains, the C2 domain which binds phospholipid and Ca^{2+}. X and Y domains constitute the active site of these enzymes. The additional C-terminal sequence of PLC β is believed to interact with Gα subunits, while the SH2, and SH3 domains of the PLCs γ reflect their regulation on the basis of tyrosine phosphorylation.

In addition to those already described, a further functional Ca^{2+} binding domain is the highly conserved catalytic site which requires this ion for activity.

detail. A brief description of the various identified mammalian phospholipase C isozymes is provided in Table 1.1.

Phospholipases C ß: This subfamily of phospholipases C are activated through the superfamily of seven membrane spanning heterotrimeric G-protein coupled receptors (18-21). The family comprises four currently identified and characterised mammalian proteins designated as PLC ß 1-4 and a number of homologous proteins such as Norp-A in Drosophilla (22) and turkey erythrocyte PLC, which has recently been cloned. These proteins show variable distribution throughout various tissues, with some being ubiquitous, while other members of the family are restricted in a tissue specific manner (23).

Differential regulation of PLC ß isoforms by heterotrimeric G-proteins:

To date four isozymes of the mammalian PLC-ß family have been isolated. They are characterised as having molecular weights of around 150 kDa and are activated primarily via the superfamily of seven membrane spanning receptors, which are now known to couple to their effectors through heterotrimeric G-proteins (24, 25). These enzymes have been the focus of intense study and their properties and behaviour has been extensively reviewed in the literature (26-29).

The involvement of G-Proteins in the function of this signalling system was first hinted at by three lines of evidence:-

1. Activation by receptor directed agonists demonstrated a requirement for guanine nucleotides (30-33).

2. Poorly hydrolysable analogues of GTP including GTPγS stimulated the activity of these enzymes. Aluminium fluoride is similarly capable of activating signalling by interacting with GDP bound to the inactive form of the G-protein and mimicking the presence of a γ phosphate moiety (34-36).

3. The activity of this pathway is subject to a variable degree of inhibition by pertussis-toxin, a protein toxin from B. pertussis which is known to modulate the function of G-proteins in the $G_{i/o}$ family (37).

Name	Size	Source	References
PLC ß1	150-154kDa	Bovine, and rat brain	1. Katan M, Parker P.J. (87) Eur. J. Biochem 168:413-418 2. Katan M., Kriz, R.W., Totty, N., Meldrum, E., Aldape, R.A., Knopf, J.L., Parker, P.J. (88) Cell 54:171-177
PLC ß2	134kDa	Human leukocyte cell line HL60.	Park,D., Jhon,D.Y., Kriz,R., Knopf,J. (92) J.Biol. Chem, 267:16048-55.
PLC ß3	152kDa	Rat thyroid cell line FRTL	Jhon, D.Y., Lee, H.H., Park, D., Lee,C.W., Lee,K.H., Rhee,S.G. (93) J.Biol.Chem. 268:6654-61.
PLC ß4	134.5kDa	Bovine retina	Lee, C., Park, D.J., Lee, K-H., Kim, C.G., Rhee, S.G. (93) J. Biol. Chem. 268:21318-27
norp A	125 kDa	Drosophilla genome	Bloomquist, B.T., Shortridge, R.D., Schneuwly, S., Perdew, M., Montell, C., Steller, H., Rubin, G., Pak, W.L(88) Cell 54:723-733.
PLC γ1	145 kDa	Bovine, and rat brain	Suh, P.G., Ryu, S.H., Moon, K.H., Suh, H.W., Rhee, S.G. (88) Proc. Nat. Acad. Science USA 85: 5419-5423
PLC γ2	146 kDa	HL 60, spleen, lung	1. Banno, Y., Yu, A., Nakashima, T., Homma, Y., Takenawa, T., Nozawa, Y., (90) Biochem. Biophys. Res. Comm. 167: 396-401 2. Ohata, S., Matsui, A., Nazawa, Y., Kagawa, T. (88) FEBS Lett. 242:31-35 3. Emori, Y., Homma, Y., Sorimachi, H., Kawasaki, H., Nakanishi, O., Suzuki, K., Takenawa, T., (89) J. Biol. Chem. 264:21885-21890
PLC δ1	85 kDa	Rat, and bovine brain	1. Homma, Y., Imaki, J., Nakanishi, O., Takenawa, T., (88) J. Biol. Chem 263:6592-6598 2. Suh, P.-G., Ryu, S.H., Mon, H.H, Suh, H.W., Rhee, S,G., (88) Cell 54:161-169
PLC δ2	85 kDa	Bovine brain	Meldrum, E., Katan, M., Parker, P. (89) Eur. J. Biochem 182:673-677
PLC δ3	84kDa	Human fibroblast c-DNA	Kriz, R., Lin, L-L., Sultzman, L., Ellis, C., Heldin, C-K, Pawson, T., Knopf, J., (90) In Proto-oncogenes in cell development pp 112-127 Ciba Foundation Symposium 150
PLC δ4	84 kDa	Rat liver	Liu, N., Fukami, K., Yu, H., Takenawa, T. (96) J. Biol. Chem. 271:355-60

Table 1.1 List of currently identified Phospholipase C isozymes.

The pertussis toxin-sensitive component of the stimulation suggested the involvement of a G-protein similar to those already characterised since the activity of the toxin on members of the $G_{i/o}$ family of G-proteins was already documented. The toxin-insensitive component suggested either the involvement of some other mechanism, or of a previously uncharacterised G-protein lacking the modification site for the toxin. The toxin acts by ADP ribosylating a cysteine residue four amino acids from the C-terminal of the $G_{i/o}$ α subunit

Work being carried out simultaneously in a number of laboratories elucidated the nature of this toxin-insensitive component and identified a novel family of G-proteins as central players in the function of the PLC-ß signalling system. *Sternweis et al* (38-39) used an affinity column of purified $\beta\gamma$ subunits bound to an agarose matrix to purify two 42 kDa polypeptides from rat brain material. They could stimulate PLC-ß in the presence of AlF_4, but only poorly in the presence of GTPγS, as a result of the poor kinetics with which GTPγS displaces GDP from the nucleotide binding site. *Pang and Sternweis* (40, 41) demonstrated that this mixture of proteins, on the basis of sequencing tryptic peptides derived from them, were identical to novel α subunits identified from a mouse brain cDNA library identified earlier by *Strathmann and Simon* (42, 43). They were designated α_q and α_{11}.

Exton (44) demonstrated that $G\alpha_{q/11}$ purified from rat liver could be preincubated with GTPγS to facilitate exchange of GDP for the GTP analogue and would then stimulate the function of PLC-$ß_1$. Transfection of Cos-7 cells with $G\alpha_{q/11}$ was also demonstrated to increase the levels of IP_3 as a result of PLC-$ß_1$ activation, an effect which could be considerably increased by cotransfection with PLC-$ß_1$ (45).

To date, four members of the G_q family have been identified and these are designated as $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{16}$. Of these four proteins, $G\alpha_q$ and $G\alpha_{11}$ are the most closely related, having an 88% identity. The remaining members of the family share approximately 55-60% identity. It was demonstrated that the reason for these proteins failure to be inactivated by pertussis toxin is the absence of a crucial cysteine residue at a position four residues from the C-terminus in the portion of the protein (46). The specificity of the G_q family in the stimulation of PLC-ß was demonstrated using α-subunits from other G-proteins including $G\alpha_{i2}$, $G\alpha_{OA}$, $G\alpha_{OB}$, transducin and

constitutively active mutants of $G\alpha_Z$ and $G\alpha_{OA}$. (47). These were found to be inactive in terms of stimulation of PLC-β_1.

The ability of the various members of the G_q family to regulate different isozymes of the PLC-ß family has been investigated and a correspondence between the tissue distribution of both families can be observed. $G\alpha_{q/11}$ are ubiquitous in their tissue distribution, as is their favoured target, PLC-ß1. PLC-β_2 on the other hand is found to reside primarily in cells of an haematopoietic origin, as is the protein $G\alpha_{16}$, which has been demonstrated to activate PLC-β_2 (48).

The demonstration of the activity of the G_q family in stimulating PLC-ß isozymes accounts well for the pertussis toxin-insensitive component of their stimulation. The toxin-sensitive component of the stimulation may well be explained by the more recent discovery that at least some of the PLC-ß isozymes may be regulated via the βγ subunits of heterotrimeric G-proteins (38, 49-51). There is an observable hierarchy with which the different members of the $G\alpha_q$ and Gβγ subunits stimulate the different members of the PLC-ß family:-

$G\alpha_q$	PLC-$\beta_1 \geq$ PLC-β_3 > PLC-β_4 > PLC-β_2	
Gβγ	PLC-β_3 > PLC-β_2 > PLC-β_1	PLC-β_4 is insensitive

It has been observed that the relative amounts of each of the subunits required for activation of the PLC-ß show disparity i.e. micromolar quantities of βγ subunits are required for a level of stimulation comparable with that obtained using nanomolar concentrations of the α subunit. This fact has been interpreted in a number of ways. Either the Gα subunit represents the main stimulus for the PLC-ß, which is then more subtly modulated by Gβγ subunits released via other receptor/G-protein combinations as a form of fine tuning of the response, or the conditions of the stimulation have not yet been optimised in terms of the appropriate Gβγ combination. Alternatively it has been suggested that the differential stimulation achieved by α, or βγ subunits reflects the degree to which they are represented in the cell - the concentration of $G_{i/o}$ in most cells is higher that of G_q family members and stimulation of the cell via receptor directed agonist, or GTPγS will consequently liberate considerably more βγ subunits from $G_{o/i}$ than α subunits from G_q.

To date, diversity within the Gα subunit has been the benchmark for classifying heterotrimeric G-proteins. It is now known that there is considerable variation within the Gβγ subunits, with the γ subunit showing particular diversity. Thus specificity of Gβγ stimulation may well reside within these subunits. Differential sensitivity of PLC-ß isozymes to the heterotrimeric G-protein subunits would not be without parallel - the important group of signalling enzymes, the adenylate cyclases exhibit an array of sensitivities to Gβγ subunits, while all are stimulated by $G\alpha_S$ (52).

Sternweis and Smrcka demonstrated that in the presence of saturating quantities of βγ subunits, addition of a $G\alpha_q/G\alpha_{11}$ mixture produced an additive stimulation of PLC-β1 (38). This demonstrates that the sites of interaction are distinct. It was determined that the site of interaction of Gα subunits with PLCβ was at the carboxy terminus and comprised two distinct domains within the region 903-1142. The domain represented by residues 903-1030 was demonstrated as being responsible for particulate fraction association as well as Gα activation. Residues 1030 to 1142 were found to be necessary for Gα activation only (53, 54).

Activation of PLCβ isozymes by βγ subunits has been attributed to a portion of the protein at the N-terminus (55) and specifically incorporating the N-terminal PH (56) domain. The βγ binding site has been specifically located as being at the carboxy terminal of the PH domain and to include residues immediately distal (57). It has been proposed that these motifs represent βγ binding sites in a number of proteins involved in signalling. This potential regulation via the PH domain raises the anomalous behaviour of the PLC δ and γ isozymes, as neither of these families appear to be regulated in this fashion, with one exception. An EGF stimulation of PLC γ in hepatocytes has been demonstrated to be pertussis-toxin-sensitive (58).

An examination of the stimuli which lead to the activation of the PLC-ß family of enzymes reveals a consistent pattern. The range of ligands which trigger the enzymes include hormones and chemotactic factors for which a rapid and fairly transient response is required. A list of receptors, the binding of ligand to which stimulate the activity of PLC-ß isozymes would include thromboxane B2, bradykinin, bombesin, angiotensin II, histamine, vasopressin, acetylcholine m1, m2, and m3 and α1-adrenergic agonist. An homologue of the mammalian PLC-ß enzymes, Norp-A

also plays a role in the phototransduction system of Drosophilla (59). A schematic diagram represents the activation of PLCβ isozymes via the Gα subunit of heterotrimeric G-proteins in Fig. 1.3.

Regulation of Phospholipases C γ:

This family of PLCs so far encompasses two isozymes, designated as PLC-γ_1 and PLC-γ_2. PLC-γ_1 is ubiquitous in distribution, while PLC-γ_2 is restricted to cells of an haematopoietic origin. The molecular weight of these proteins is slightly lower than that of the PLC-ß isozymes, with the prototype of the family, PLC-γ_1 of approximately 145kDa. Neither of these enzymes are coupled to heterotrimeric G-proteins.

An examination of the linear sequence of this family of PLCs and a consideration of the significant areas of primary structure thus revealed suggests that phosphorylation plays a significant role in the regulation of PLCs-γ. This has indeed proved to be the case. (See the accompanying schematic diagram, Fig. 1.4).

Both of the PLC-γ isozymes so far identified are coupled to receptors which mediate fairly long term changes in the behaviour of cells, including various growth factors, where the requirement for stringent and rapid on/off regulation of activity is not so great. Receptors coupling to PLC-γ include Platelet Derived Growth Factor (PDGF) (60, 61), Epidermal Growth Factor (EGF) (60, 62), and Fibroblast Growth Factor (63).

A common feature of all of the receptors acting through these enzymes is that they either possess tyrosine kinase domains, or are associated with non-receptor tyrosine kinases. The sequence of events leading to the activation of PLC-γ isozymes runs as follows:-

1. Binding of ligand to the receptor triggers the dimerisation of the receptor within the cell membrane. This dimerisation brings the tyrosine kinase portions of the receptors into close juxtaposition and allows the mutual phosphorylation of the receptors. The dimerisation of the receptor is facilitated by the fact that the majority

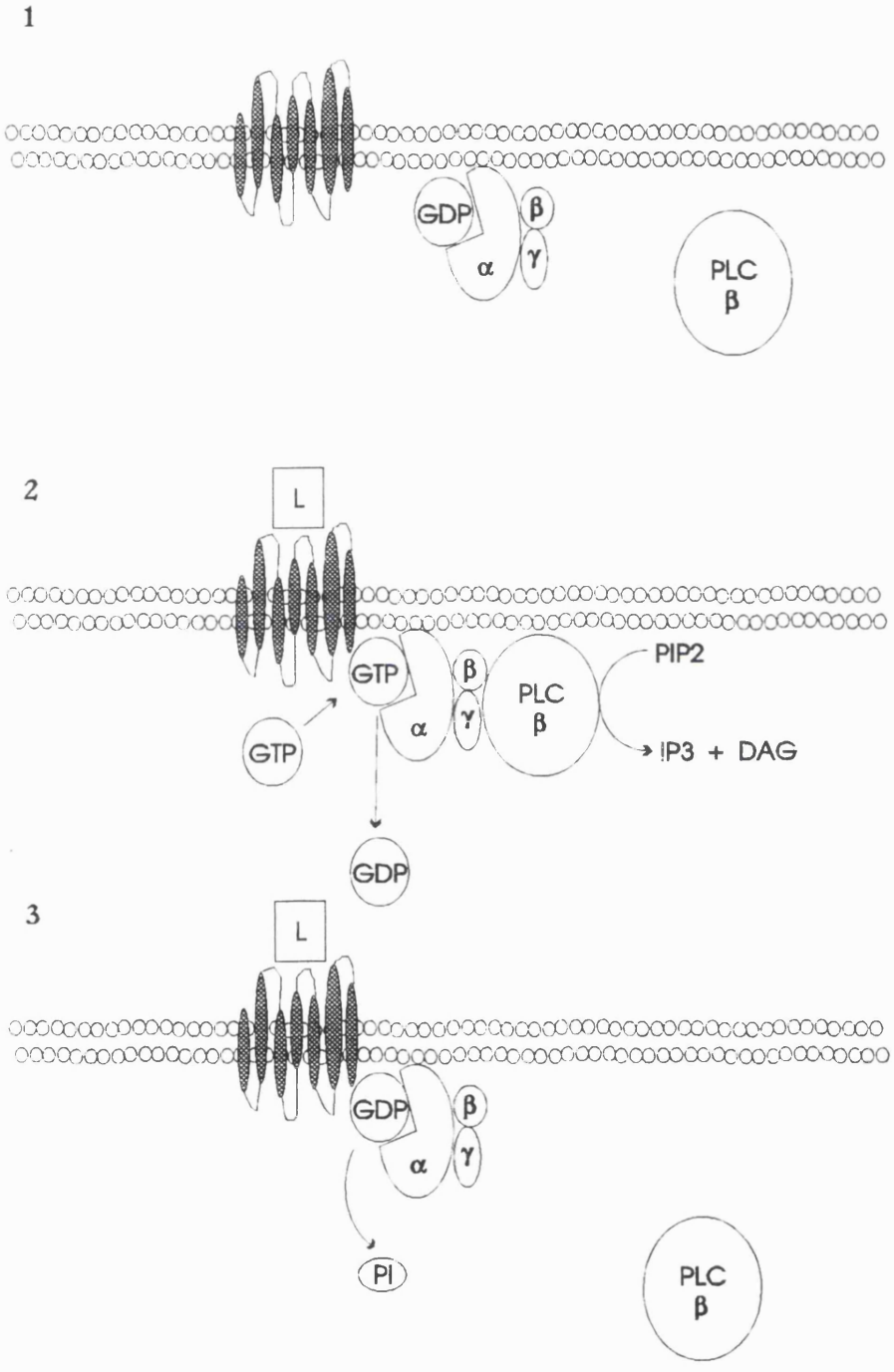
Fig. 1.3 Schematic representation of the activation of PLC β via heterotrimeric G-protein-coupled receptors.

The accompanying figure illustrates the steps in the activation of a G-protein regulated PLC β enzyme:-

1. Ligand (L) binds at the extracellular portion of a seven membrane spanning receptor, leading to conformational shifts within the receptor.

2. Conformational change within the receptor lead to the recruitment of an heterotrimeric G-protein into the complex. Initially in the inactive GDP bound state, interaction with the ligand bound receptor causes release of GDP and binding of GTP in which state the G-protein α and in some cases βγ subunits are capable of stimulating the activity of PLC β, which is recruited from the cytosol or bound at the membrane.

3. The inherent GTPase activity of the G-protein leads to hydrolysis of the bound GTP to form GDP and return the G-protein to the inactive state, also switching off the effector, in this case PLC β. The GTPase function of the G-protein may be enhanced by interaction with the PLC which behaves as a GTPase activating Protein, or GAP.


1
GDP
β
γ
α
PLC
β
2
L
GTP
β
γ
α
PLC
β
PIP2
IP3 + DAG
GTP
GDP
3
L
GDP
β
γ
α
PI
PLC
β

of these receptors are single membrane spanning proteins, which consequently have significantly more freedom of movement within the plane of the membrane than the G-protein coupled receptors for instance. Another consequence of the relatively simple structure of these proteins is that there is considerably less scope for shifts in secondary and tertiary structure such are as proposed for the function of the seven membrane spanning receptors. Covalent modification, such as the observed phosphorylation represents the most efficient mechanism by which the activation of these proteins could be determined.

2. As illustrated in the schematic representation (Fig 1.4), PLCs-γ possess two src homology-2, (SH-2) domains. SH2 domains are regions of conserved primary structure shared with the tyrosine kinase src. They are now known to mediate physical binding of proteins with this motif to phosphorylated tyrosine residues situated within a consensus SH2 domain binding site (64) - as a consequence of possessing SH2 domains, PLC-γ is recruited to the appropriate phosphorylated site on the receptor (65).

3. Activation of the PLCs-γ is then completed by phosphorylation of the enzyme with a concomitant conversion of PIP_2 to IP_3 and DAG.

This model is supported by a number of lines of evidence and has been the subject of considerable research effort from a number of laboratories. Treatment of a number of different cell types with growth factors including EGF, PDGF and FGF, led to an increase in the phosphorylation of PLC-γ_1 on both tyrosine and serine residues (11). (It should be noted that despite similarities in the structure of the receptors to most polypeptide growth factors, signalling through the PI system is not universal, as reflected by the fact that insulin and CSF-1 appear not to effect PLCγ activity.) This association of PLC-γ with receptors is demonstrated by the fact that antibodies raised against either the PLC-γ or the appropriate receptor will immunoprecipitate the receptor/effector complex from cell membranes in a number of cases (60, 66, 67). There is some apparent degree of variability in the specificity with which specific phosphorylation sites are used for the docking of PLC-γ. The EGF receptor has five phosphorylation sites - of these Tyr-992 appears to be critical for the recruitment of

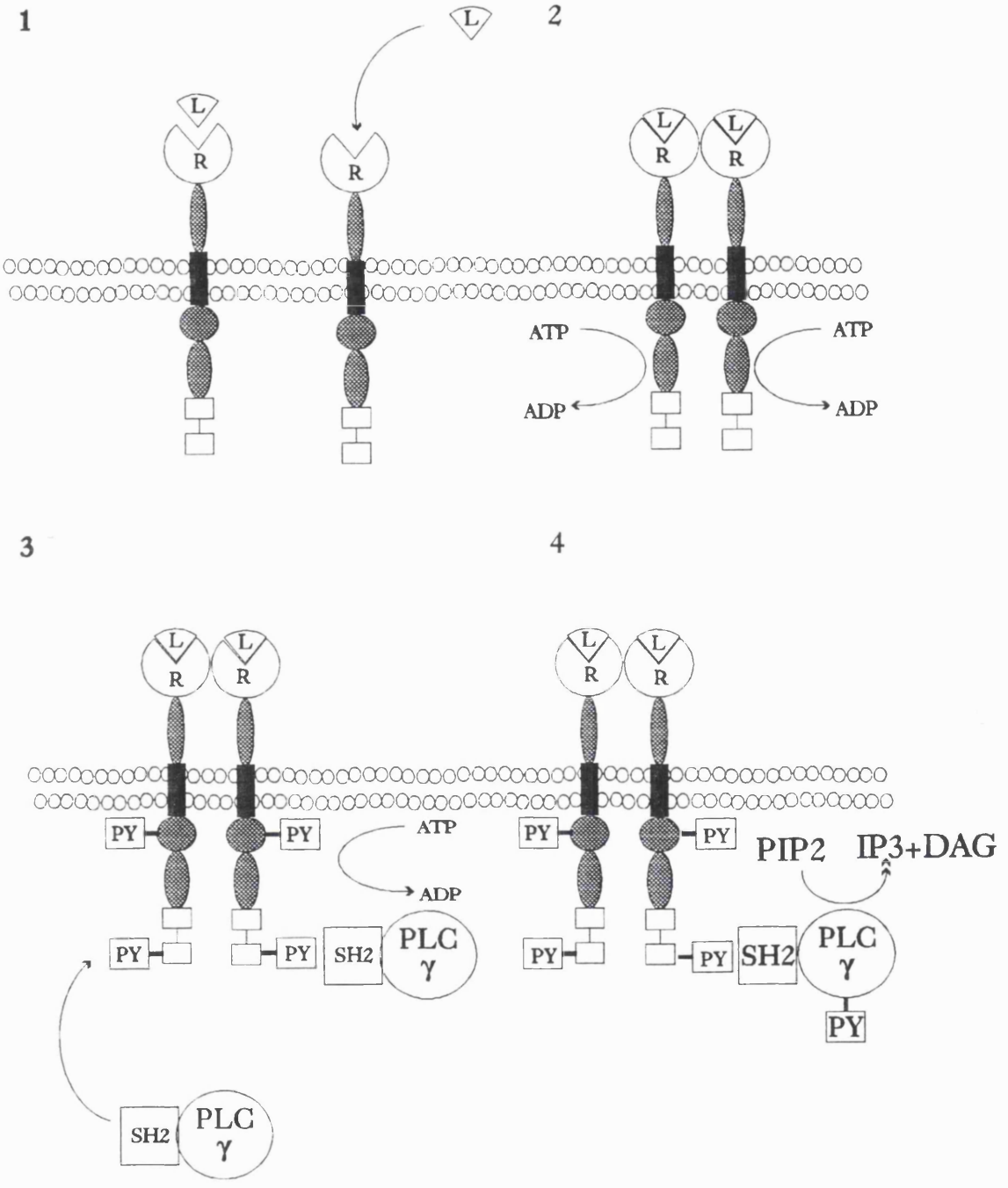
Fig. 1.4 Schematic representation of the mechanism by which PLCγ isozymes are activated by binding of ligand to tyrosine kinase receptors.

The accompanying figure illustrates the steps in the activation of PLC γ isozymes by receptor tyrosine kinases:-

1 and 2. Binding of ligand to the cell surface receptor leads to dimerisation of the receptors. This causes the tyrosine kinase domains of the receptors to be brought into close juxtaposition, and enables the mutual phosphorylation of the monomers in the complex.

3. PLC γ molecules are recruited into the complex via SH2 domains which interact with the phosphotyrosine moieties on the phosphorylated receptor. Further kinase activity phosphorylates the bound PLC.

4. The PLC is now fully activated, and capable of processive catalysis, releasing IP_3, and DAG using PIP_2 substrate.


1
2
L
R
ATP
ADP
3
4
PY
SH2
PLC
γ
PIP2
IP3+DAG

PLC-γ (68). Similarly the FGF receptor utilises Tyr-766 as the main binding site for PLC-γ (69).

The autophosphorylation of the receptors for PDGF and FGF was studied by altering Tyr-1021 and Tyr-766 respectively to a phenylalanine residue. It was observed that while autophosphorylation of the receptor was reduced, the PLC-γ_1 showed good levels of phosphorylation but failed to show a concomitant increase in activity. This demonstrates that both phosphorylation and association with the receptor are required for activation of PLC-γ (70-72).

Tyrosine phosphorylation recruits PLC-γ to the activated receptor via the SH2 domains of the enzyme. The SH3 domains therein serve to target the activated enzyme towards the proline rich portions of the actin cytoskeleton (73). It is suggested that this interaction with the actin microfilament system is facilitated by a conformational change arising as a result of interaction between phosphotyrosine groups on the PLC and internal SH2 domains such that the active site of the enzyme is also brought into close juxtaposition with substrate in the membrane (74).

A number of receptors activating PLC-γ are not in themselves intrinsic tyrosine kinases. These include the T-cell receptor of lymphocytes and the IgE receptor in mast cells . This is a complex receptor comprising a number of peptides. It is believed that this receptor activates PLC-γ via the src tyrosine kinases lck, or fyn. Examination of the phosphorylation of PLC-γ by activation of these receptors using two-dimensional phosphopeptide analysis show that the modified sites correspond to those produced by the inherent tyrosine kinase activity of the PDGF and EGF receptors (75).

That the state of the cytoskeleton, the substrate of the PLCs, PIP_2 and the activity of the PLC signalling pathway via tyrosine phosphorylation are closely interconnected is suggested by the following observation. *In vitro* examination of the kinetics with which PLC-γ consumes PIP_2 reveals that both phosphorylated and unphosphorylated forms of PLC-γ show the same activity. The unphosphorylated form of the enzyme is however selectively inhibited by micellar concentrations of Triton X-100 and also by the small actin binding protein profilin (76), which tightly binds PIP_2. Once phosphorylated the enzyme is capable of hydrolysing PIP_2 under

both sets of conditions. The ability of PLC-γ to hydrolyse PIP_2-bound profilin, releasing profilin to alter the state of the cytoskeleton suggests a connection between activation of PLCs and reorganisation of the actin microfilament system.

The Phospholipases-C_δ

This family of enzymes is structurally the most simple of the three, but has remained the most mysterious in terms of regulation of function. To date it remains unclear what factors actually turn the enzyme on, compared with the plethora of information available for the β and γ isozymes. Studies of PLCs from simpler eukaryotes such as slime moulds and the relative structural simplicity of these enzymes suggest that they are the most primitive and represent the evolutionary prototype for all of the mammalian PLCs. With this in view, recent work from *Paterson et al* (13) has provided valuable information concerning the regulation of this family of enzymes, as well as the crystal structure and potential catalytic mechanism (17, 77) - in consideration of the sequence homologies apparent throughout the X and Y catalytic regions of all three families of PLC, this suggests that the mechanism is similar in all cases. It has been demonstrated that PLC-δ1 requires the presence of a pleckstrin homology domain to facilitate recruitment of the enzyme to the plasma membrane. Using deletion mutants of PLCδ1 in CHO cells and immunofluorescent microscopy it was demonstrated that deletions within the PH domain of this protein completely inhibited binding to the cell membrane.

X-ray diffraction studies using crystallised protein have now revealed the three dimensional structure of an N-terminally truncated PLCδ1 deletion mutant. This has revealed a number of interesting facts, including striking similarities between PLCδ1, (and by extension, potentially the other PLCs) and a number of other signalling molecules.

As can be observed from the linear representation of the PLCs in Fig. 1.2, PLCδ can be considered as comprising four distinct domains. These are :-

1. An N-terminal PH domain, a structural motif that has already been suggested as playing an important role in the interaction with PIP_2, as well as potentially the βγ subunits of heterotrimeric G proteins.

2. A domain composed of four EF hand structures, that have been revealed by diffraction studies as being arranged in two lobes, each composed of two EF motifs. This arrangement has been compared with that observed in Troponin-C and calmodulin. The similarity between calmodulin and PLCδ is further enhanced by the nature of the interactions between the EF hand domain and the C terminal. The EF hand is known to be a calcium binding motif, common to a number of proteins which bind this ion, or have activities which are modulated by it.

3. A catalytic domain created by the spatial juxtaposition of the X and Y regions which are the areas of highest sequence homology within the PLC superfamily (78). This has been designated the Triosephosphate Isomerase-like domain (TIM domain). In the case of the PLCδ isozymes, the X and Y regions are separated by a relatively short sequence of charged residues which appear to be afunctional in terms of activity. The PLCγ isozymes are noteworthy for the presence of a split PH domain encompassing two SH2 and a single SH3 domain in this region. The active site includes two catalytic histidine residues close to the C terminal of the structure.

4. The C terminal C2 domain is a structure shared with in excess of 40 other proteins involved with membrane interactions, or signalling. At the time of writing, only the C2 domain of synaptotagmin has been fully characterised and it has been proposed that this represents a Ca^{2+} sensitive phospholipid binding site. It is of note that the PLCβ isozymes have a further C terminal domain believed to be responsible for interactions with the Gα subunits of heterotrimeric G proteins (44).

On the basis of these observations *Paterson et al* et al have gone on to propose that the PLCδ is initially bound at the membrane via the N terminal PH domain and then, possibly under the influence of calcium, the C2 domain causes the active site to be brought into juxtaposition with the membrane and the substrate for the enzyme. Despite these revelations it remains to be demonstrated whether other factors play a role in the stimulation of the PLCδ isozymes, or whether the activity of these proteins

is governed solely by Ca^{2+} through the calcium binding motifs already described and the active site.

Effects of PLC activation

As described in the accompanying diagram, Fig. 1.1, PLC hydrolyses PIP_2 into the second messenger molecules inositol-1,4,5-trisphosphate and diacylglycerol. Both of these are potent influences on cellular activities and IP_3 stimulates Ca^{2+} release from intracellular stores, while DAG stimulates protein kinase C (PKC).

IP_3 generates a biphasic elevation of intracellular calcium. The initial binding of IP_3 to the receptor for this molecule on the surface of Ca^{2+} storage organelles results in an initial pulse, or transient of Ca^{2+} release. Subsequent to this there is a more sustained elevation of intracellular concentrations as a positive feedback loop increases the sensitivity of the IP_3 receptor to the ligand (79). This second, more sustained release of Ca^{2+} is believed to be mediated by IP_3 in concert with inositol(1,3,4,5)tetrakisphosphate, a phosphorylated derivative of IP_3 (80). This latter phase of Ca^{2+} increase occurs as a result of 'capacitative influx' from the extracellular medium and appears to be dependent on the state of intracellular stores. The exact mechanism by which the second wave of calcium is induced has been investigated and three main potential mechanisms have been proposed:-

1. The IP_3 receptor in some way is capable of transducing a signal concerning the state of the intracellular organelles to the inositol tetrakisphosphate receptor at the cell membrane (80).

2. A chemical factor designated as CIF, or Calcium Influx Factor has been suggested (81).

3. Depletion of the calcium stores may lead to conformational change of the organelles involved and be communicated to the cell membrane, possibly via the cytoskeleton (82).

The diacylglycerol moiety that is released by the action of PLCs remains resident in the cell membrane where it is able to diffuse in the plane of the lipid bilayer. Here it acts as a stimulus for the activity of protein kinases C, or PKCs. The importance of this group of enzymes in regulating the activity of the cell is reflected by the transforming effects produced on cell growth by exposure to phorbol esters which specifically stimulate PKCs. It is believed that the mode of action of this agent lies in the fact that it mimics diacylglycerol (83).

1.2 Phosphatidylinositol-3 kinases

As suggested by the name this group of enzymes is responsible for the phosphorylation of the inositol ring of PI, PIP, or PIP_2 at the 3' position. The activity of this group of enzymes is illustrated in Fig. 1.1. Again, as in the case of the PLCs and as will be seen in the case of the kinases responsible for the synthesis of PIP_2, the study of the PI-3 kinases is complicated by the fact that multiple isozymes exist, some of which are specific for different phosphorylated substrates and under differential regulation - some appear to function as basic "house-keeping" activities, while others have a role in signal transduction. For the purpose of brevity, only those isozymes believed to be involved in signal transduction will be considered here.

fMetLeuPhe has the ability to stimulate the accumulation of phosphatidylinositol-(3,4,5)-trisphosphate in neutrophils (84) and similar effects have been observed in response to various growth factors and activated oncogenes in a number of other cell types (85).

The first mammalian PI-3-kinase identified is an heterodimer composed of an 85kDa regulatory unit and a 110kDa catalytic subunit, designated as p85 and p110 respectively. The p85 occurs in two forms, α and β and possesses two SH2 domains and a single SH3 domain. The catalytic subunit shares homology throughout the C-terminal half with the yeast protein VPS34P and enzyme of similar catalytic nature involved in vacuolar protein sorting.

The presence of two SH2 domains on the regulatory unit suggests an interaction with phosphotyrosine residues and this has been observed to be the case, with PI-(3,4,5)-P_3 accumulating in response to the stimulation of growth factor

receptors e.g. PDGF (86) with intrinsic tyrosine kinase activity, as well as some requiring the activity of a non receptor tyrosine kinase (87). Phosphorylation, auto, or otherwise, of tyrosine residues on the receptor recruit the regulatory subunit into a complex. The 110kDa subunit is then recruited, becomes phosphorylated and in the process activated.

The prototype PI-3-kinase depends on the activity of tyrosine kinases. However it has been observed that activation of a PI-3-kinase activity in neutrophils and U937 cells could be abrogated by treatment with pertussis-toxin. As discussed in the sections relating to regulation of the PLCβ isozymes, this implies the involvement of an heterotrimeric G-protein of the Gi family (88). This effect was assumed to be mediated through G-protein regulated tyrosine kinase activity. This supposition was found to be incorrect by the observation that in excess of 90% of the PI-3-kinase activity observed in neutrophils was directly regulated by G-proteins and was insensitive to an extensive panel of tyrosine kinase inhibitors (89) . Stephens et al subsequently partially purified a PI-3-kinase activity which showed distinct pharmacological, chromatographic and immunological properties to the tyrosine kinase regulated activity. They further demonstrated that the activity was not stimulated by $G\alpha i_1$, $G\alpha i_2$, $G\alpha i_3$, or $G\alpha_q$, but that it was sensitive to $G\beta\gamma$ subunits. This closely parallels the situation in the PLCs and adenylate cyclases, where different isozymes are differentially regulate in a manner dependent upon receptor coupling. This G-protein coupled activity was not recognised by antibodies directed against the tyrosine kinase regulated activity and the kinetics with which wortmannin inhibits the activity are significantly different, with this activity being much less sensitive to inhibition. On the basis of chromatographic data, the mass of the protein has been put at 210-220kDa. This protein has now been cloned by Stoyanov et al and is designated as PI 3-kinase-γ (90).

The function of the PI-3-kinases in regulation of cellular activities has recently been subject to intense scrutiny. So far the accumulation of PI-(3,4,5)-P3 has been connected with generation of the oxidative burst in neutrophils, as well as modulation of cytoskeletal change via actin polymerisation (88). These observations have been supported by the inhibitory effects of wortmannin on these phenomena (91). There is however some degree of ambiguity connected with experiments conducted with

wortmannin, as it has now been demonstrated as having a broader inhibitory activity than originally appreciated (92).

PI-(3,4,5)P3 produced by the activation of PI-3-kinase is rapidly dephosphorylated by the activity of a 5-phosphatase that is membrane associated. Further dephosphorylation leads to the accumulation of PI-3-P in a process that obviously continues on from signal induced activation of PI-3-kinase. An observed anomaly was the accumulation of PI-3-P in unstimulated cells, independent of receptor activation (84). It is now known that there is a PI specific PI-3-kinase which is immunologically and pharmacologically distinct from the better characterised, agonist stimulated PI-4,5-bisphosphate specific form and is not regulated by tyrosine phosphorylation. This protein is more closely related to VPS34p from yeast and plays a house keeping role (93, 94).

1.3 Phospholipase D

Another important group of phospholipases involved in intracellular signalling are the phospholipases D. Until relatively recently, despite the PLD response in intact and fractionated cells being partially characterised, little was known about this group of enzymes in animal cells. Using molecular biological methods, a number of members of this family have now been cloned (95, 96) and expressed in a baculovirus system, identified on the basis of similarities between the active site of the well characterised plant enzyme and sequences in the animal enzymes. The enzyme has however proved singularly recalcitrant in terms of purification to homogeneity.

It was observed that treatment of neutrophils with fMLP led to a transient increase in levels of phosphatidate (PA). This was not accounted for by the phosphorylation of DAG. After a transient elevation in levels it then declined as a result of the activity of phosphatidate phosphohydrolase. The concomitant headgroup release led to an increase in free choline - hence the enzyme in this case was found to be PC specific. This activity could also be stimulated by GTP analogues in permeabilised cells, demonstration that it is G-protein coupled.

The situation in the PLDs almost certainly mirrors that in the PLCs, in that there are multiple isozymes, with different substrate specificities and differential

regulation - for instance two PI specific PLD activities have been identified in neutrophils which exhibit different sensitivities to Ca^{2+}.

Attempts to unravel the regulation of PLD have revealed that small G-proteins, PKC and Ca^{2+} can each independently and co-operatively stimulate the activity of this enzyme. Of particular interest has been the discovery of the role of the small G-protein Arf-1 in the regulation of PLD (97). This small G-protein is believed to represent a prototype for both the other small G-proteins such as the Ras family and the α-subunits of heterotrimeric G-proteins.

The importance of PLD appears to lie in a correlation between the time course with which PA accumulates and that of the secretory process in neutrophils. Inhibition of the activity of PLD, by the addition of small quantities of ethanol to reaction mixtures and the subsequent formation of phosphatidylethanol is also found to have a profound effect on exocytosis in neutrophils. In addition to a role in exocytosis, the product of PLD activation, PA, has also been found to have a regulatory function on type I PI-4-phosphate 5-kinase, part of the synthetic machinery of PIP_2 (98).

Recent investigations carried out in the laboratory of *P. Sternweis* have revealed a role for PIP_2 in the regulation of PLD activity (99). Use of the antibiotic neomycin which selectively binds to PIP_2 in the membrane, inhibits PLD function. This effect can be abrogated by the addition of extraneous PIP_2. This has been demonstrated in the lab of *M. Liskovitch* (100, 101).

1.4 Regulation of the cytoskeleton by calcium and polyphosphoinositides

1.4.1 Calcium effects: As can be appreciated from the earlier discussion of the function of the phospholipase C family, the metabolism of the polyphosphoinositides in intimately connected with the regulation of intracellular calcium and hence in part to the function of many other effectors which are in turn regulated by this. One cell component which is of particular importance to motile cells such as neutrophils and other leukocytes is the cytoskeleton and the behaviour of this appears to be influenced by both Ca^{2+} concentrations and the levels of PIP and PIP_2.

Motility and structural change in cells occurs in response to various stimuli including the binding of ligands to cell surface receptors. It is events such as this that lead to activation of PLCs and the elevation in intracellular calcium levels. The morphology of the cell is governed by the physical state of the cytoskeleton, which undergoes transitions between gel and liquid states (102,103). Since the original statement of the concept that the organisation of the cytoskeleton may be governed by intracellular concentrations of calcium, the area remains one of uncertainty, but a considerable body of evidence now exists which points to the fact that many of the proteins involved in regulation of the cytoskeleton bind calcium and interact with polyphosphoinositides.

In 1992 *Marks and Mansfield* demonstrated that the transient increase of intracellular calcium was necessary for changes in the cytoskeleton of neutrophils and their migration and produced an increase in the concentration of polymerised actin (102). By contrast it has been demonstrated that increased intracellular Ca^{2+} decreases actin polymerisation in adrenal chromaffin cells (104). Similar apparently contradictory data have been obtained from a number of systems and it is becoming increasingly clear that the effect of calcium on the cytoskeleton is cell type specific and depends on the presence of other activating factors. In vitro studies with various actin binding proteins have shown a requirement for Ca^{2+} in excess of that measurable in the cytosol - this, coupled to the fact that the range of diffusion of Ca^{2+} within the cytosol is limited to 100nm (105) suggest that these effects are mediated by localised transients which may well exceed the gross cytoplasmic levels. It is noteworthy also that cells can perform some of these change under extremely well buffered conditions, suggesting that Ca^{2+} changes may not be essential under all circumstances. This has been demonstrated in neutrophils (106).

From studying the effects of calcium on a number of actin binding proteins a consensus has been arrived at whereby it is generally the case that increased Ca^{2+} leads to the activation of proteins which disrupt the actin filament system, while those proteins responsible for cross linking and stabilisation are inhibited (107).

1.4.2 Effects of polyphosphoinositides: Only relatively recently, in the '80s was the potential role of lipid/protein interactions appreciated in the context of the

cytoskeleton. Certainly, a connection between the PLCs and hence PIP_2 levels and the state of the cytoskeleton is suggested by the observation that the actin binding protein profilin is capable of sequestering PIP_2. The unphosphorylated form of PLCγ is unable to hydrolyse its lipid substrate in this circumstance, but once phosphorylated, the enzyme has access to the substrate and is able to breakdown profilin bound PIP_2 (108). Observations in neutrophils and platelets have demonstrated that when these cells are stimulated, the amount of F-actin increases and the amount of actin complexed with profilin drops. This was demonstrated to be independent of calcium levels and the search for another regulatory component led to the observation that PIP and PIP_2 were capable of specifically dissociating the actin/profilin complex (109, 110). The accompanying table (Table 1.2), which is taken from a recent review by P.A. Jamney (107) lists some of the actin binding proteins now known to interact with PIP_2.

Studies on a number of actin severing proteins have revealed that they are without exception so far regulated by both Ca^{2+} and PIP/PIP_2 concentrations (see table for references), and that the effects of the two different regulators are in opposition to one another - i.e. PIP/PIP_2 inhibits severing, nucleation and actin monomer binding. In the case of gelsolin, a filament severing protein, in vitro studies have shown that 100μM PIP_2 is capable of producing 90% inhibition of function - since the concentration of PIP_2 within the cell is around 300μM, this suggests that the in vitro effect may well be of physiological relevance (111). Inhibition of the nucleating activity of gelsolin however requires approximately ten times more PIP/PIP_2 than the severing activity. Data relating to the physical presentation of PIP/PIP_2 to these proteins suggest that clustered polyphosphoinositides are required for these interactions, since in mixed micelles with PC, a critical ratio is required between PIP_2 and PC for the effect to be observed, independent of the gross amount of inositol lipid present (112).

α-actinin, a cross linking protein, responsible for the stabilisation of the actin cytoskeleton has been demonstrated to be enhanced by the presence of PIP/PIP_2 (113). α-actinin is particularly striking in that the interaction with PIP_2 appears to be so stable as to permit it to survive the harsh denaturing conditions of SDS-PAGE. Studies of other actin cross-linking proteins, including gizzard filamin (114) have revealed

Protein	Effect of phosphoinositides	Reference
Profilin	Sequestered actin released from profilin/actin complexes	Nature 314:472-72
Gelsolin Villin	Severing activity inhibited more strongly than nucleating activity. Actin/Gelsolin complexes dissociated Barbed ends uncapped. Multiple PPI binding sites	Nature 325:362-64 J. Biol. Chem. 263:16738-43
Severin		Biochemistry 31:4779-87
Adseverin		J. Biol. Chem. 266:4581-85
Cofilin Destrin	Binding to actin inhibited, PPI binding site identifed	J. Biol. Chem. 265:8382-86
gCap 39	Dissociated from actin monomers, and barbed filament ends.	Science 250:1413-15
Alpha actinin	F-actin cross-linking activity increased	Nature 359:150-52
Filamin	F actin cross-linking activity decreased	B.B.R.C. 184:1261-65
MAP-2C	F-actin bundling activity decreased	B.B.R.C. 190:710-15
Myosin I	Links F-actin to acidic phospholipid bilayer	Nature 340:565-68
Talin		B.B.A. 106:121-31
CapZ	Released from barbed filament ends	B.B.R.C. 181:833-39
Cap 32/34		Biochemistry 30:8753-58
Cap 100		Cell Motil. Cytoskel 23:133-44

Table 1.2 This table is taken from the recent review by P.A. Jamney.

interactions at variance with those observed for α-actinin. One interpretation of these variations is that the observed effects arise as a result of interaction between the actin cross-linkers and PIP/PIP_2 rather than a direct effect of PIP/PIP_2 on the actin itself.

In summary of the effects of the polyphosphoinositides (PPIs) on the actin cytoskeleton, PPIs tend to produce effects in opposition to those induced by calcium, i.e. promotion of actin polymerisation and strengthening of the cortical actin gel. These data have led to the proposition of the following model for crawling cell motility (103, 115):-

1. Stimulation of the cell via surface receptors etc. causes elevation of intracellular calcium and a drop in local PPI concentrations. This favours Ca^{2+} sensitive actin binding proteins release of bound actin filaments from their associations at the membrane and the disruption of the cytoskeleton by the filament severing proteins. The cytoskeleton loses rigidity as a result and facilitates distortion of the cell membrane.

2. Stimulation of the synthesis of PPIs would favour polymerisation of the actin cytoskeleton at the cell membrane and enhance some of the actin cross-linking activities. Profilin/actin complexes would be disrupted, liberating actin monomers for the formation of fresh cytoskeleton.

1.5 Biosynthesis of phosphatidylinositol

De novo synthesis of PI occurs by the reaction of *myo*-inositol with cytidyl diphosphate-diacylglycerol (CDP-DAG). This reaction is catalysed by the enzyme CDP-DAG:inositol phosphatidyltransferase. For convenience this enzyme is usually referred to as PI-synthase. In addition to the de novo synthesis catalysed by this enzyme, another headgroup exchange activity is observed which is responsible for the rapid incorporation of labelled inositol into cellular lipids. This activity does not produce an overall synthesis of PI, but is of interest, having been characterised as having a similar requirement for divalent cations to the PI synthase and also requiring

the presence of CMP, one of the products of the activity of the phosphatidyltransferase - taken together these pieces of evidence suggest that the headgroup exchange is a consequence of the reversible nature of the reaction catalysed by PI-synthase.

It has been demonstrated that the enzymes responsible for the generation of the PI precursor CDP-DAG and the PI-synthase itself are membrane bound enzymes (116). A number of studies have been conducted to determine the intracellular distribution of PI-synthase. To date the majority of this body of evidence suggests that upwards of 90% of the activity observed in the cell resides in the endoplasmic reticulum. By utilising the sensitivity of the PI-synthase to proteolysis and mercury dextran it has been demonstrated that the majority of this activity may further be localised to the cytosolic, rather than the luminal surface of the ER (117). A relatively small amount of data suggests that some activity may also be present at the cytoplasmic membrane. Imai and Gerschengorn demonstrated PI-synthase activity in the cell membranes of cultured GH_3 rat pituitary tumour cells (118). Whether the detected activity represented an headgroup exchange , or de novo synthesis remains to be answered. A number of studies with different cell types have suggested that activity detected in the cell membrane is possibly attributable to ER contamination on the basis of ER markers being detected at levels comparable with PI-synthase activity in the prepared material (119). The steps in the synthesis of PI and phosphorylated derivatives are illustrated in Fig. 1.1.

1.6 Synthesis of polyphosphoinositides

It has been demonstrated that the preferred in vivo substrate for the PLC enzymes is phosphatidylinositol-4,5-bisphosphate despite the preponderance of PI and PIP and the fact that the PLC enzymes can be driven to hydrolyse PI in vitro by use of higher, non-physiological concentrations of calcium. This compound is synthesised by the further phosphorylation of PI by the two kinases phosphatidylinositol-4-kinase and phosphatidylinositol-4-phosphate 5 kinase. As is the case with the phospholipase C enzymes however, the study of the kinases is complicated by the fact that the catalytic activities described appear to be carried out by families of enzymes rather than individual proteins and multiple isozymes are found within the same cell. The steps

and their corresponding enzymes involved in the synthesis of PIP_2 is illustrated in the accompanying diagram (Fig. 1.1).

There are at least two isozymes of PI-4-kinase responsible for the phosphorylation of PI to PI-4-P. These have been designated as phosphatidylinositol kinase Type II and Type III and have been shown to be distinct by immunological methods (120) as well on the basis of their biochemical properties (The type I kinase phosphorylates the inositol moiety on the D3 position.). In addition a PI-3-P 4 kinase activity has also been identified and characterised as an enzyme distinct from those above.

The type II PI-4-kinase has been characterised as a polypeptide of approximately 56 kDa and is distinguished from the type III kinase studied in bovine brain which has a molecular mass in the region of 230 kDa , an higher Km for ATP and a lower sensitivity to inhibition by adenosine. Studies using a monoclonal antibody against the type II enzyme have demonstrated that it is ubiquitous throughout a range of cell types and species. It has been suggested that it represents a distinct gene product, as the larger type III enzyme is not recognised by the 4C5G antibody.

The type III PI-4-kinases has recently been cloned from a rat brain library and subject to localisation studies in cells using epitope tagging methods (121). The cloned protein has been found to comprise 2041 amino acids and has a calculated molecular weight of 231.3 kDa. The protein exhibits 52.3% and 34.4% homology with the yeast kinases STT4 and PIK1 and 31.7% homology with the 110 kDa subunit of PI-3-Kinase from rat brain. The 3' coding half of the DNA was found to be 87.6% identical in nucleotide sequence and 98.2% identical in terms of coded amino acids with the type II kinase PI-4-Kinase α, a recently cloned human protein (122) suggesting that this protein may be an alternative splicing product rather than a distinct gene product. The identity of the protein as a type III kinase is confirmed by the facts that its activity is enhanced by Triton X-100 and that it is insensitive to inhibition by adenosine. The 3' half of the protein also contains a PH domain suggesting a possible role of $\beta\gamma$ subunits in regulation of activity. Immunocytochemistry using epitope tagging has allowed the localisation of this protein to the Golgi. This is in agreement with Liscovitch et al (123).

Both of the kinases have been demonstrated as being intrinsic to the membrane, as extraction requires treatment with detergent. Examination of the retrieved activity from bovine brain has demonstrated that type II and type III enzymes account for approximately 40% and 60% of the retrieved activity.

Further phosphorylation of the resultant phosphatidylinositol-4-monophosphate at the D5 position on the inositol ring yields phosphatidylinositol-4,5,-bisphosphate, quantitatively the most important substrate for the phospholipase C enzymes (124). In the case of this activity, at least two distinct isozyme families have again been identified, designated as Type I and Type II (125). Of the two, the type II activity is the best understood and has been purified to homogeneity and characterised as being a 53 kDa polypeptide. The two activities are best identified by means of their differential activation by the polyamine spermine and by phosphatidic acid - the type I activity is stimulated by both of these agents, while the type II enzyme is relatively insensitive. The type I enzyme has recently been cloned by two groups (126-129).

Because of the importance of PIP_2 as a substrate in various signalling pathways and other cell processes, the regulation of the synthesis has been subject to much investigation. Upon ligand stimulation it has been demonstrated that the turnover of PIP_2 may be many times greater than the reserves of this lipid within the cell - hence it has been postulated that the kinases responsible for the production of PIP_2 from PI are subject to regulation, with their activity being stimulated to meet demands of the various processes consuming PIP_2. As may be expected with such a complicated, multi-step synthesis unravelling the regulation of the kinases has proved both difficult and time consuming - the concentration of PIP_2 within the cells arises from the balance of a number of dynamic processes, all of which are independently subject to regulation. Despite the knowledge that multiple isozymes exist, it remains to be determined which isozyme is responsible for the provision of substrate for PLC signalling, maintaining PIP_2 levels for interaction with cytoskeletal components and all of the other functions with which PIP_2 has been implicated - it has been suggested that the relevance of the multiple isozymes lies in the maintenance of distinct pools of PIP_2 within the cell.

PIP-5-kinase activity has been recovered from erythrocytes, brain, adrenal medulla and liver. Within cells the activity has been found associated with plasma

membranes (130), cytoskeleton (131), endoplasmic reticulum (132), and the nucleus (133). The two isozymes have been investigated immunologically and antibodies raised against the individual proteins demonstrated no cross reactivity, suggesting that they are distinct gene products. The two isozymes may further be differentiated by the fact that in vitro, the type II enzyme shows little activity towards native membranes. This may reflect one or more of the possibilities below:-

1. The enzyme may require some form of modification for activity.
2. It may require the presence of some specific targeting factor
3. PIP-5-kinase may be active towards substrate complexed with another protein - for example cytoskeletal components, or PI-TP.

Type I PIP-5-kinase has been cloned and partially characterised (98, 134). This has led to the revelation that PIP-5-kinase has no recognisable homology with the other lipid kinases, protein kinases, or sugar kinases (PI-4-Kinase, PI-3-kinase and protein kinases show some degree of homology). This establishes the PIP-5-kinases as a distinct family.

Work carried out by Hay et al has suggested a role for PIP-5-kinase in exocytosis (5) . Studies carried out using the rat cell line PC-12 has shown that resolution of cytosol on gel filtration produced three distinct peaks of activity that reconstituted exocytosis in cytosol depleted cells. Peak 1, designated as PEP1, for Priming in Exocytosis Protein was found to contain both PIP-5-kinase Ia and b. Another peak of particular interest was PEP3, which upon examination was found to contain PI-TP.

The regulation of type I PIP-5-kinase activity by both the small and heterotrimeric G-proteins has been subject to investigation. Of particular interest was the potential regulation of type I PIP-5-kinase by the Rho family of small G-proteins. It has been suggested that these proteins may be involved in the regulation of the cytoskeleton possibly through the concentration of PIP_2. It is believed that this family of proteins acts in an hierarchical cascade (135, 136). cdc42h is believed to be responsible for the formation of philopodia, rac responsible for the formation of lamellopodia and rho is responsible for the formation of focal attachments and stress

fibres. Different growth factors are able to enter the system at different points in the cascade.

Tolias et al demonstrated that a PIP-5kinase activity bound directly to a rac1-GST fusion protein attached to a solid matrix. However he found that only 0.3% of the total activity could be captured in this way and suggested that either the activity represented a minor isozyme, or that PIP-5-kinase required some form of post translational modification to become activated (137). Chong et al have identified a Rho A activated PIP-5-kinase activity (138). In addition a cholera toxin-sensitive PIP-5-kinase activity has been identified in placenta, but the nature of the G-protein involved and the directness with which the regulation is effected has yet to be determined (139).

Having now considered some of the most important systems which either directly utilise inositol lipids as substrates, or are influenced by their presence, the two proteins with which the bulk of this work is concerned will now be introduced : the phosphatidylinositol transfer proteins.

1.7 The phosphatidylinositol transfer proteins:

The existence of phospholipid transfer proteins and their activities have now been documented for almost 30 years. In 1968, Wirtz and Zilversmitt demonstrated the activity of what is now believed to be the phosphatidylcholine transfer protein (PC-TP) (140) . The involvement of cytosolic liver proteins in the exchange of membrane lipids was demonstrated by Wirtz and Zilversmitt in 1969 (141). Work in the field continued and over the course of the 1970s, the state of knowledge relating to the phospholipid transfer proteins continued to increase, with the identification of a number of proteins exhibiting specific lipid transfer activities.

In 1976 Lumb, et al identified the PC-TP and demonstrated that it shared many of the properties exhibited by the liver extract activity identified by Wirtz and Silversmitt (142). In 1977, Bloj and Zilversmitt identified the non-specific- lipid transfer protein nsL-TP, also now known to be Sterol Carrier Protein 2, or SCP2 (143).

In 1974 the existence of the phosphatidylinositol transfer protein was identified (144). Despite considerable effort to determine the function of this protein it was not

until comparatively recently that this protein was proven to have a physiologically relevant role in the functioning of the PLC signalling system (2). Workers in this laboratory were employing the bacterial cytolysin, Streptolysin O (SLO) to study the behaviour of various lipid signalling systems in cytosol-depleted HL60 cells. This toxin forms pores in the cytolplasmic membrane through which cytosolic components of the cell are able to diffuse from the interior of the cell. The result of this depletion is that the membrane asssociated components of the signalling systems remain in place, while cytosolic components are removed. As a consequence of the loss of various cytosolic factors, the function of the PLC pathway in response to the GTP analogue GTPγS was found to decay.

The permeabilised and cytosol depleted cells were utilised as the basis of an assay to detect cytosolic factors capable of restoring PLC function. HL60 cells were cultured in the presence of ^{3}H-myo-inositol so that the inositol lipids became labelled. The cells were then permeabilised using SLO and the cytosolic proteins allowed to diffuse from the cell. The cells retained a basal responsiveness to stimulation by GTPγS. Chromatographically resolved components of cytosol could then be assessed for their ability to enhance this basal responsiveness, according to a protocol that is fully described in Chapter 2.

Utilising this method in parallel with *in vitro* PLC assays it was discovered that PLC β1 present in bovine brain cytosol could reconstitute GTPγS stimulated PLC activity in permeabilised cells (145). In addition another cytosolic factor with no detectable *in vitro* PLC activity could also potently restore GTPγS responsiveness. This factor was subsequently identified as being the mammalian phosphatidylinositol transfer protein (2).

In 1994, Tanaka and Hosaka demonstrated the existence of a second isoform of this protein which demonstrated a considerable degree of identity with the original protein, now designated as PI-TPα, while the latest addition to the family is PI-TPβ (146).

The accompanying table (Table 1.3) illustrates some of the properties of the phospholipid transfer proteins so far identified. The proteins with which this thesis is predominantly concerned are the PI-TPs and these will now be considered in more depth.

1.7.1 Distribution: PI-TP is of universal distribution among the higher eukaryotes and the highly conserved nature of this protein is illustrated by the fact that antibodies raised against rat brain PI-TP have been demonstrated to cross react with PI-TPs from species as diverse as human, to the fungus Neurospora crassa (147). The accompanying diagram (Fig. 1.5) shows the amino acid sequences of the α and β isoforms of the mammalian PI-TP (146,148). As can clearly be seen, the two proteins are very highly conserved, with 77% identity and 91% amino acid similarity.

A remarkable example of convergent evolution appears to be represented by the PI-TPs of the lower fungi, including the yeast Saccharomyces cerevisae. This protein is designated sec 14 and was initially identified on the basis of gene screens to identify proteins required for the normal function of the secretory process. It was only subsequently identified as functioning as a PI-TP (149). *In vitro* this protein exhibits an ability to transfer both PI and PC from one membrane compartment to another in a manner very similar to the mammalian PI-TPs and has also demonstrated an ability to reconstitute PLC signalling in permeabilised HL60 cells.

Sec14 shares many of the physical properties of the mammalian PI-TPs and has a molecular weight of approximately 35 kDa as determined by electrophoretic mobility, similar behaviour on isoelectric focusing and almost identical properties with regards to substrate specificity. Remarkably, however, amino acid sequence analysis demonstrates no significant sequence homology between this protein and the mammalian PI-TPα, or PI-TPβ (148, 150). On the basis of the convergent natures of these two proteins, a number of models have been proposed suggesting that they may well serve similar purposes in both yeasts and higher organisms (1). This however may not be the case, as recent work has identified the sec 14 equivalent in the dimorphic fungus *Yarrowia lipolytica* as playing a role in the alternation between single cell, yeast-like and filamentous, mycelial form (151). Unlike the situation in Saccharomyces species, this sec 14 homologue does not appear to be essential to the

Protein	Source	Molecular weight	Isoelectric point	Substrate specificity	References
α PI-TP	Bovine brain	32500	5.3 & 5.6	PI>PC=PG	JBC **249**:6382-9
	Bovine heart	33500	5.3 & 5.6	PI>PC>>SPM	JBC **254**:7795-7802
	Rat liver	35000	5.1 & 5.3	PI>PC	Eur.J.Bioch **69**:15-22
	Rat brain	36000	4.9 & 5.3	PI>PC	BBA **946**:119-28
sec 14	S. cerevisiae	35000	5.1 & 5.3	PI>PC	BBA **794**:385-91
β PI-TP	Rat Brain	31500	5.1 & 5.3	PI>PC>SPM	
nsL-TP	Rat liver	13500	8.6	PC,PI,PE,PS	JBC **252**:1613-19
	Bovine liver	14000	9.5	Cholesterol	Biochem. **19**:1433-39
	Human liver	14000	n.d.	n.d.	BBA **919**:149-55
	Goat liver	12000	8.7	PC,PI,PE,SPM	BBA **959**:134-42
	Rat hepatoma	11200	5.2		Eur.J.Bioch **82**:463-71
PC-TP	Bovine liver	24680	5.8	PC	BBA **318**:313-25
	Rat liver	28000	8.4	PC	BBA **600**:376-36

Table 1.3
Properties of identified phospholipid transfer proteins

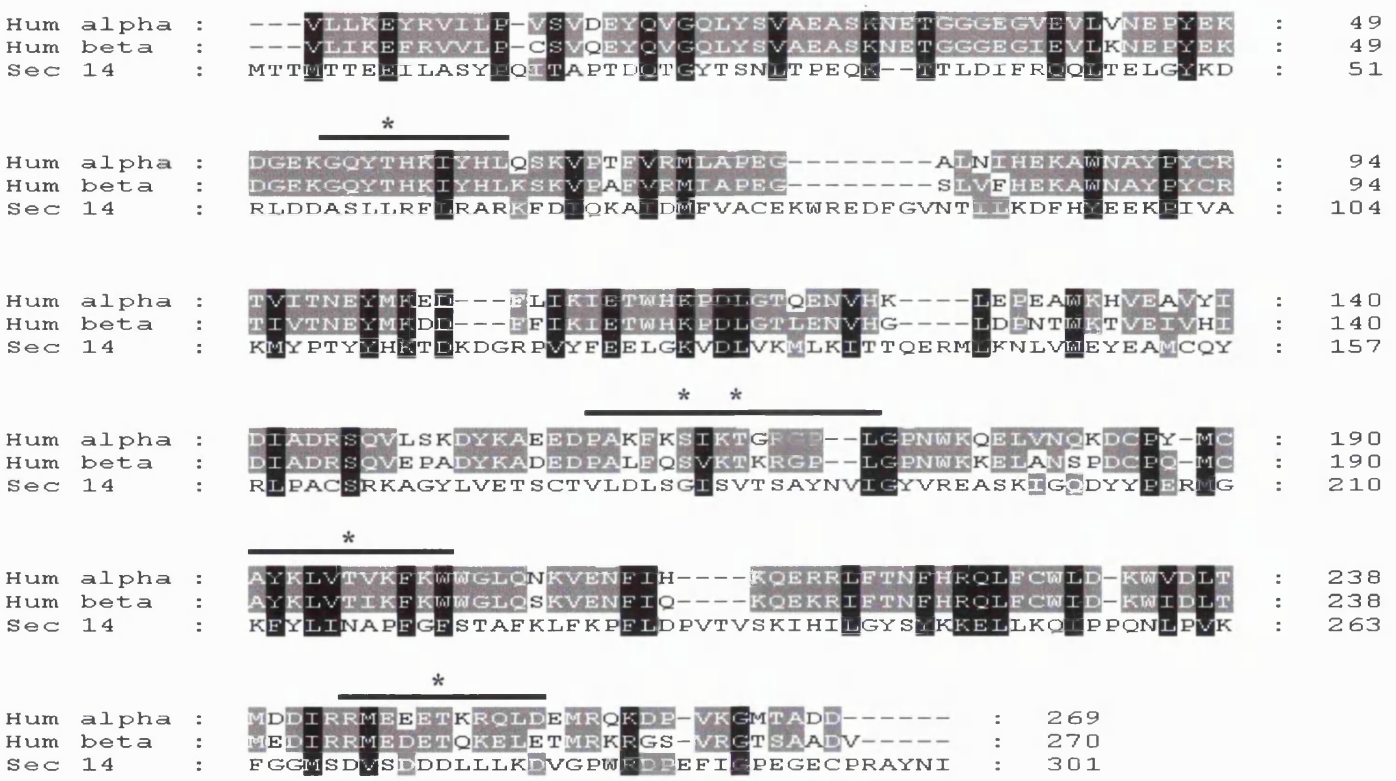


Fig 1.5 Sequence alignment of human PI-TP α, and β, and the yeast homologue sec 14.

The above figure shows an alignment of the three proteins. Areas of identity or 75% conservative substitutions between the proteins are shown in black, and amino acids common to two of the proteins, or conservative amino acid differences are shaded grey. While the human PI-TP isoforms show a considerable degree of homology over their entire length there is only slight but tantalising homology between these and the yeast PI-transfer protein. Potential PKC phosphorylation sites in the human proteins are marked with lines above the text, with the specific residues asterisked.

survival of the cell. The non-essential nature of this protein is not a result of another PC/PI-TP activity in these cells, as cytosols from deletion mutants were found to have no significant PC/PI-transfer activities.

Studies conducted on the ability of sec 14, PI-TPα and PI-TPβ to reconstitute PLC signalling in permeabilised HL60 cells has demonstrated that all of these proteins are capable of restoring the function of this pathway (152). In the absence of significant sequence homology between the yeast protein and the mammalian proteins, the ability of the proteins to restore PLC signalling clearly demonstrates that their respective abilities to bind and transfer PI lie at the heart of their function in this system. Furthermore, if ,as has been invoked in a number of models, PI-TP interacts with proteins involved in PLC signalling, some of these interactions may be mediated through the protein bound inositol head group, rather than through direct protein/protein interactions.

The involvement of PI-TP in the function of the PLC signalling system is further supported by the identification of the rdg-B mutant in Drosophilla. This mutation results in the degeneration of the retina upon light stimulation. The protein which is effected by this mutation has been identified as being an integral membrane protein, the amino terminal 30% of which shows marked homology to the mammalian PI-TP. The protein is found in the sub rhabdomeric cisternae of the eye and also present in large amounts in the olfactory organs of Drosophilla. Unlike the mammalian PI-TPs which are significantly cytosolic in distribution, this protein is anchored within a membrane by a hydrophobic transmembrane portion (153).

1.7.2 Properties: On the basis of the encoding DNA, the molecular weight of PI-TPα is 31.91 kDa and the molecular weight of the β isoform is calculated as 31.45 kDa These values are at variance from those determined by a number of methods. Because of "non-ideal" interactions of the proteins with chromatography support media, gel filtration of mammalian PI-TP yields a molecular size in the region of 17 kDa as a consequence of the protein being retained by the column. SDS PAGE analysis of the protein gives it a molecular weight in the region of 35kDa (2, 4, 144). The reason for this latter anomaly is less clearly understood.

The purified proteins, as resolved on SDS PAGE gels yield single bands, as determined by staining and immunological probing of Western blots. Analysis of the protein using isoelectric focusing yield two bands, the more prominent of the two being at pH 5.3 and the minor band being at pH 5.6. This effect was originally documented by *van Parridon* et al (154) and the different isoelectric points exhibited by the protein were attributed to the fact that the PI-loaded form of the protein carries an extra negatively charged moiety in the form of the PI headgroup, when compared to PC. This demonstrates that the protein exists in two predominant states within the cell - PI bound, or PC bound and that occupancy of the protein by one of these lipids precludes the presence of the other. This phenomenon was used as a partial proof of the identity of the PLC reconstituting activity described by Thomas et al (2).

The two forms of PI-TP are readily interconverted by incubation with vesicles of the appropriate lipid composition. The sn1 and sn2 acyl groups of both PI and PC share common binding sites which are believed to occupy 'troughs' on the surface of the protein at right angles to each other, as revealed by studies on the behaviour of bound, fluorescent parinaroyl lipids. This accounts for the exclusive nature of lipid binding to these proteins - occupancy of the acyl binding sites by the fatty acid groups of one of the lipids prevents binding of the other lipid to the protein (155, 156). PI represents only a relatively minor lipid component within the cell, with the phosphorylated forms, PIP and PIP_2 being represented to an even lesser extent. PI represents 5 to 8% of cellular lipids and PC 40%, but the mammalian PI-TP has been demonstrated to have 16 times greater affinity for PI than PC. These factors mean that purified PI-TP is divided approximately 65:35 PI:PC bound forms. The fact that the acyl chains of the two lipids occupy the same site suggest that the specificity for lipid binding is attributable to the headgroup binding site (2, 157).

PI-TP displays an absolute requirement for the presence of an intact inositol ring in order for PI-transfer to occur. If the inositol ring is reduced by borohydride treatment, the ability of PI-TP to bind and transfer the resultant lipid is reduced ten fold with respect to the unmodified lipid (156). The ability of PI-TP to transfer the phosphorylated derivatives of PI have been investigated in both bovine brain and human PI-TP. In both cases specific transfer of these lipids was not observed (147, 158). It is possible that this failure to mediate transfer of these lipids between vesicles may reflect avid binding of these lipids to the protein and a consequent lack of release

into an acceptor membrane. It is also worth noting that the relatively high charge density of PIP_2 means that this lipid displays an unusually high degree of 'stickiness', thus making the case for specific binding of this lipid to PI-TP more difficult to prove.

The efficiency with which PI-TP is able to interact with a particular membrane is influenced by a number of factors. These include:-

1. Headgroup composition of the membranes with which the protein is presented. Studies conducted on acceptor membranes with elevated amounts of PI present demonstrated that the rate of transfer was actually reduced in this case. This effect was initially attributed to the specific interaction of PI-TP with the PI in the vesicle. Incorporation of other acidic lipids into the membrane however was capable of replicating this effect. This was observed using PA, PIP and PIP_2. The effect could be reversed by inclusion of elevated quantities of Mg^{2+} in the assay, or, in the case of PIP_2 inhibition, the presence of the antibiotic neomycin, which is capable of specifically binding to PIP_2 and screening the negative charge of this lipid. This effect probably reflects a positively charged membrane binding site on the protein (156, 159). This demonstrates that interaction of the protein with the membrane and the specific transfer of PI-TP are separate activities. The incorporation of PIP and PIP_2 into the acceptor membranes reduces the efficiency of transfer as a consequence of lowering the dissociation constant for the protein and the membrane in question with respect to a more physiological norm.

2. The pH of the environment in which the PI-transfer protein finds itself effects the efficiency of transfer. If the pH of the environment rises above the isoelectric point of the protein i.e. approximately pH 5.5, the protein becomes negatively charged and as a result is subject to electrostatic repulsion by the negatively charged lipids in the membrane (Both acceptor and donor.). The converse is also true and lowering of the pH below the isoelectric point of the protein induces an overall positive charge to the protein resulting in an elevation of the association constant of the protein for the negatively charged lipid moieties in the membranes (159).

3. The fatty acyl moieties in the phospholipids also effect the efficiency with which the protein effects lipid transfer. Because of the fact that the fatty acid groups of the phospholipids share a common binding site, as demonstrated on the basis of time resolved fluorescence quenching studies (154). It is presumably possible to extend observations made using PC to PI and vice versa. Studies utilising PC have demonstrated that PI-TP has a distinct preference for the transport of lipids containing cis and trans mono-unsaturated fatty acids. phosphatidylcholine carrying saturated acyl groups was found to inhibit the efficiency of lipid transfer (160). This observation is of interest in light of the finding that the activation of PLC signalling has been demonstrated to stimulate the turnover of a limited range of PIP_2 in terms of the acyl composition of the diacylglycerol generated (161).

4. The degree of curvature of the donor membrane has been observed to have an effect on the efficiency of transfer - highly curved donor vesicles have been demonstrated to be better sources of phospholipid for transfer (162). This can easily be visualised as being a result of the greater degree to which the head group of the lipid in question will be exposed in the more highly curved membrane model. It was demonstrated that the dissociation constant for multilamellar liposomes is 100 times lower than that for monolamellar vesicles.

1.7.3 Behaviour of PI-TP in the cell - net transfer, or shuttle:

The ability of PC-TP to mediate the net transfer of phosphatidylcholine about the cell has been documented i.e. this protein displays bulk transfer of material from one membrane to the other. This function of the protein is entirely dependent on the ability of the protein to exist free in the cytosol in a non-lipid bound form. PI-TP purified from a variety of sources exists in either of two readily resolved forms, either PI, or PC bound, separated on the basis of their respective isoelectric points (2). Thus, in the mammalian cell, the PI-TP appears to be always bound to either of the lipids which it transfers. This would appear to preclude the function of the protein in the net transfer of phospholipids within the cell.

On the basis of the above facts and the observation that the yeast homologue of this protein, sec14, appears to play a crucial role in the regulation of lipid composition

of secretory Golgi vesicles (149, 163), a model has been suggested whereby PI-TP serves either to monitor the lipid composition of the intracellular membranes, possibly to ensure function of the exocytotic pathway, or actually regulates directly the composition of the intracellular membranes on the basis of lipid transfer - it has also been suggested that PI-TP may regulate the composition of lipid vesicle to ensure an appropriate lipid composition for transfer of lipids about the cell by a vesicular trafficking mechanism. The latter of the above models reflects the situation thought to exist in yeast cells with the protein product of sec-14. Again it should be pointed out however that the striking evolutionary convergence between the mammalian PI-TP and sec 14 in terms of their physical properties may not reflect a functional homology. This is demonstrated by the apparent difference of function of this protein in different species of fungi - in one the protein regulates the operation of the secretory pathway and is essential to the viability of the cell, while in another the sec14 homologue appears to regulate the alternation between yeast-like and mycelial morphology (151).

It has been demonstrated that PI-TP can mediate the "net" transfer of PI between donor membranes composed of purely this lipid to membranes composed purely of PC (164, 165). The effect of PI-TP as a net transporter of PI could also be visualised as occurring in a situation in which PI is being consumed. This situation is evoked by state of affairs extant during stimulation of PLC signalling, when PI is presumably converted to PIP and PIP_2 to act as substrate for the PLC. A recent paper by *Alb et al* (166) invoked a possible mechanism whereby a vectorial activity for PI-TP could be possible, regulated on the basis of phosphorylation. By generating a cohort of mutant PI-TPs and examining the ability of this protein to rescue sec 14 defective yeast strains, a number of mutant PI-TPs have been identified which have amino acid substitutions in the inositol headgroup binding site and were consequently defective in terms of PI transfer, but still capable of PC transfer. One of the sites in the inositol head group binding site was a threonine residing within a PKC consensus sequence.

1.7.4 Subcellular localisation and phosphorylation

In studies using Swiss 3T3 cells, the localisation of PI-TP within the cell was initially studied using indirect immunofluorescence (167). The distribution of the protein was studied under a number of conditions, including exponential growth, confluence and a condition of serum starvation, under which non confluent cells were caused to enter a state of semi-quiescence. The distribution of PI-TP within these cells was demonstrated to be predominantly cytosolic. In quiescent, confluent cells which were experiencing contact inhibition, the PI-TP was observed to be almost purely cytosolic in distribution. However in exponentially growing cells, the protein was found to be localised to some clear extent to the Golgi and nucleus. Serum starved cells demonstrated an intermediate state of affairs. Stimulation of the cells with bombesin, which acts through a seven membrane spanning, G-protein coupled receptor and PMA led to a redistribution of the protein from the cytosol to the membrane compartments already described (168). PMA also resulted in an elevation of phosphorylation of PI-TP which was independent of redistribution of the protein i.e. PI-TP was apparently able to be recruited to membranes without the necessity of phosphorylation. The function of this phosphorylation remains to be determined - it has been demonstrated that PI-TP experiences a basal level of phosphorylation (168) which can be enhanced by treatment with PMA and certain receptor directed agonists. The physiological relevance of the observed phosphorylation remains to be determined. The genuine nature of the redistribution of the protein to the Golgi was demonstrate by the fact that treatment of the cell with brefeldin A destroyed the observed staining pattern - this toxin destroys the organisation of the Golgi stack. The staining pattern was observed again if the Brefeldin A was removed from the cells and the Golgi system allowed to reform.

In a recent paper (202) it has been demonstrated that there is a differential redistribution of PI-TP isoforms within the cell. Upon SLO permeabilisation of Swiss 3T3 cells, it was observed that a considerable quantity of 'a novel acidic isoform' of PI-TP remained associated with the Golgi system. This protein is now believed to be the PI-TPβ. This possibly reflects involvement of the two proteins in distinct

processes, although both isoforms have been demonstrated as having the ability to reconstitute PLC signalling (152). Attempts to validate the observation of PI-TP in the nucleus of the cell were unsuccessful - the case for this however is supported by the knowledge that there is a very active PI-cycle active within the nucleus (169).

1.7.5 Tissue distribution of PI-TP

Probing of various rat tissues using immunological methods has demonstrated that PI-TPα is virtually universal in distribution and molecular biological methods have shown that the same is true of PI-TPβ (146, 170). There is however quite considerable variation in terms of quantity. The tissue showing the highest activity of PI-TPα was found to be in the brain, with the Pons Medulla being a particularly rich source - the pituitary gland was the weakest source of the protein within the brain. Subcellular localisation of the protein on the basis of cell disruption showed that purified synaptosome fractions from this organ were particularly rich in PI-TP. Electrical stimulation of neurones has been demonstrated the stimulate the hydrolysis and turnover of inositol lipids in neurones. This is interesting observation in that it ties in with the observation of Hay et al. that PI-TP is one of the Priming in Exocytosis Proteins (PEPs) identified by this group as playing a role in regulated exocytosis (4,5).

1.7.6 Function of PI-TP within the cell

On the basis of observed evolutionary convergence between the yeast homologue of PI-TP and PI-TP itself, models have been suggested in which PI-TP plays a role in regulation of the lipid composition of intracellular membranes (163). It has also been suggested in the past that PI-TP may play a role in the function of the PLC signalling system (1). Until fairly recently this remained speculation until Thomas et al. demonstrated a function for this protein in PLC β mediated signalling in permeabilised HL60 cells (2). This was followed up later with the observation that PI-TP is recruited to become part of a complex in A431 fibroblasts including PLC γ, EGF receptor and PI-4-kinase (171). Thus the involvement of PI-TP in PLC signalling is now well documented and accepted.

The exact function of PI-TP in the PLC signalling system remains to be determined. PI itself, which is the predominant lipid bound to this protein in the cell is not the major substrate for PLC activity under physiological conditions. This role is fulfilled by PIP_2. It has been demonstrated that the rate of synthesis of PIP_2 in permeabilised cells is influenced by the presence of PI-TP (3). This suggests that PI-TP may well perform a role in the supply of PI for conversion to PIP_2 by a sequence of lipid kinases. *In vivo*, this rate limiting behaviour is unproven.

The manner in which this function is performed is not presently characterised. It is conceivable that the transfer protein simply delivers PI from the site of synthesis in the endoplasmic reticulum to the inner leaflet of the plasma membrane where it joins the pool of inositol lipids already there. An alternative model involves PI-TP actually remaining bound to the lipid and carrying it along the sequence of kinases to produce PIP_2 for hydrolysis in signalling etc.

The multiple enzymes involved in inositol signalling at the cell's membrane can for kinetic purposes be grouped together as a single mathematical entity. It has also been shown that the PLC β enzymes present in the cell in both cytosolic and membrane distributions represent a considerable excess above that level which is required to maintain PLC signalling under normal circumstances. In permeabilised HL60 cells it has been demonstrated that even after prolonged permeabilisation, sufficient PLCs remain bound at the membrane to provide for a very robust response when PI-TP is added back to the system. This state of affairs is probably similar to that demonstrated in turkey erythrocytes, where significant quantities of PLCβ have been demonstrated as being present in cell ghosts, associated with cytoskeletal components (18). The magnitude of this response is linear with respect to the quantities of PI-TP added to the system, suggesting that in the cell, the factor limiting PLC signalling is the PI-TP (3).

The proposition that PI-TP may have a role to play in the regulation of membrane composition is quite attractive. It has been demonstrated that a number of functions of the cell are dependent on the lipid compositions of the various membrane components. In particular, the function of PLD and the exocytotic machinery has been demonstrated to be sensitive to the concentration of PIP_2 (100, 172) and the reports that PI-TP and a PIP 5-kinase activity have been identified as PEPs by *Hay et*

al (4, 5) highlight the possible relevance of PI-TP to these processes (173) - and in conjunction with the demonstrated function of PI-TP in PLC signalling suggest that the PI-TPs may be truly multi-functional proteins.

PI itself is a minor lipid component of the cell and is fairly uniformly distributed throughout the various membrane compartments. PIP and PIP_2 are however very much more limited, both in terms of total amount and distribution within the cell. PIP and PIP_2 represent no more than 5% of the total inositol lipids in the cell and are predominantly distributed in the plasma membrane. It has been demonstrated that cells are capable of very rapidly turning over this lipid and it has been shown that in rat parotid gland cells, stimulation via the muscarinic receptor can turn the entire cellular pools of these lipids over one, to two times per minute and furthermore that this level of turnover can be maintained for at least 30 minutes (174). This obviously requires the rapid recycling of the DAG and IP_3 generated during signalling to replace the substrate consumed. It is possibly as a part of this process that PI-TP may be playing a pivotal role, by rapidly returning PI from the site of synthesis in the endoplasmic reticulum to the cell membrane.

It has been suggested that this process of recycling may be conducted at the plasma membrane, but to date most of the enzymic machinery necessary for this process has been found to be resident in the endoplasmic reticulum. Both PI synthase and CTP phosphatidate cytidyltransferase are required and have been consistently found in no locations other than the E.R.. The mechanisms that would mediate the return of PA to the interior of the cell are presently unclear and no specific PA transfer protein has so far been found and may well invoke the operation of a vesicular trafficking mechanism.

1.7.7 Distinct phospholipid pools within the cell

As early as 1964, *Hokin and Hokin* (175) were proposing the existence of specific lipid pools within the cell, involved in distinct functions. This was based on the observation using avian salt glands, that stimulation with acetylcholine led to the turnover of a distinct and limited set of inositol lipids. This observation was later confirmed by the work of *Fain and Berridge*, utilising a double labelling protocol in blow-fly salivary glands. The tissue was labelled to equilibrium with ^{32}P-

orthophosphate and then pulse labelled with ^{3}H-inositol (176). The more recently labelled tritiated phospholipids appeared to be hydrolysed in preference to the ^{32}P labelled material. This state of affairs was extended to mammalian cells when Monaco et al studied the turnover of inositol lipids in mouse mammary tumour cells. These cells were labelled with ^{32}P orthophosphate while undergoing stimulation with Vasopressin. Subsequent stimulation of the cells following labelling in this manner appeared to lead to the preferential hydrolysis of PIP_2 labelled during the period of stimulation (177). Michell however failed to duplicate Monaco's observations using a slightly modified labelling protocol (178). Since the cell is capable of very rapidly turning over the entire PIP_2-stock in a very short period of time, any model which involves the hydrolysis of a limited pool of these lipids in signalling events must also invoke a state of affairs in which the synthetic enzymes are either in close juxtaposition to the PLCs, or are very efficiently coupled to them, possibly by means of a PI-transfer protein. This implies that in the absence of PI-TP linking the biosynthetic machinery to the signalling enzymes, the whole of the synthetic mechanism would be found in close juxtaposition to the cell membrane, or nuclear membrane, which has been demonstrated as having a very active PI-cycle (169, 179).

Attempts to ascertain the location of the PI-synthetic enzymes within the cell have in the past proved less than clear cut and to date no single study has confirmed the presence of all the necessary enzymes for the PI-cycle to be present in the plasma membrane. PI-synthase has been demonstrated as present in the plasma membranes of GH3 pituitary tumour cells (118), rabbit proximal tubule (180), 1321 N1 astrocytoma cells (181) and turkey erythrocytes (36). It can be argued however that the latter example at least represents a special case as the erythrocyte is singularly devoid of internal membranes and has no endoplasmic reticulum, which had previously been supposed the site of the PI synthase. A number of other studies have also failed to locate PI-synthase in the plasma membrane (119, 182). Another enzyme required for the regeneration of PI in situ with the PLC is CTP-phosphatidate cytidyltransferase. So far only one study has suggested that this enzyme is present in the plasma membrane (116).

PI-TP has been demonstrated as enhancing the release of IP_3 from permeabilised cells as a result of PLC reconstitution. It is unknown whether it has an effect on the rate of *de novo* synthesis of PI. These effects may be explained as being

a result of the protein enhancing the efficiency with which a component, or components of the PI-signalling system work. The mechanism by which this enhancement is achieved may be any one , or more of those already proposed, including the proposed function of the protein in presentation of substrate to the kinases responsible for the generation of PIP_2, the main in vivo substrate for the PLCs.

Studies using rat hepatocytes have demonstrated that there is a distinct lag between stimulation of PLC activity by receptors and the synthesis of PIP_2. During this period, the levels of PIP_2 in the cell may drop to 50-70% below the level of control cells at 30s post stimulation Various studies place this lag at between 10 seconds and three minutes. This suggests that the synthesis of PIP_2 and the function of PLCs are not directly linked, in that the switch that turns on PLC activity does not simultaneously activate PIP_2 synthesis (183). The upturn in PIP_2 synthesis could result from either a direct stimulation of the enzymes responsible for this conversion, or from the input of some co-factor, possibly acting through enhanced substrate presentation. The increase of PIP_2 synthesis from PIP has been demonstrated as being stimulated by a pertussis-toxin-sensitive G-protein in the case of human neutrophils and NRK cells (184, 185). The interconversion of PIP and PIP_2 has been suggested as being subject to a very significant degree of futile cycling. Thus it is suggested that an increase of PIP synthesis could be mediated by reduced dephosphorylation of PIP.

1.8 Rationale behind the project:

As has already been stated, the phosphatidylinositol transfer proteins play a role in the operation of the PLC signalling system and also possibly in the balance of the inositol lipids within the cell. These lipids themselves play an increasingly appreciated role in the function of multiple cellular systems - any entity which plays a role in their metabolism merits further study.

To date, the exact mechanisms by which PI-TP is able to mediate its function are unknown - whether the protein is regulated in some fashion to provide a vectorial transport system, the relative amounts of the two isoforms within the cell and the differential functions performed by the two proteins are just some of the questions yet to be answered. In order to further understand these proteins it was therefore decided to raise a panel of immunological reagents through which various aspects of these

proteins behaviour can be examined, ranging from quantification, to phosphorylation studies, to an examination of other proteins with which they may interact. With this in mind, this thesis outlines the production of these reagents, their characterisation and commences to answer some of the above questions.

Chapter 2

Materials and methods

2.1 Materials:

Raw materials:

Bovine brains were obtained frozen from Advanced Protein Products.

Rat brains were obtained from the in house animal facility which regularly culled large numbers of white Wistar rats.

Tissue culture reagents and plasticware:

RPMI1640 single strength working solution, either ICN-Flow Ltd, or Sigma Ltd.

Medium 199 ICN-Flow.

Penicillin/Streptomycin solutions ICN-Flow

L-glutamine was from ICN-Flow.

Foetal calf serum either Advanced Protein Products Ltd., or Imperial Laboratories Ltd..

Tissue culture flasks and other consumables were manufactured by Falcon.

Radiolabelled compounds:

^{3}H-D-myo-inositol Amersham International

Pro-Mix^{TM} ^{35}S methionine/cysteine mixture Amersham International

^{32}P-orthophosphoric acid ICN-Flow, or Amersham International

Electrophoresis reagents:

Acrylamide\Bisacrylamide Bio-Rad, or National Diagnostics Ltd.

Materials for electrophoresis buffers Sigma Ltd.

Ampholytes BioRad

Immobilon P, PVDF blotting membrane Millipore Ltd.

Immunoreagents

Horse Radish Peroxidase conjugated goat anti-rabbit, anti-mouse and anti-rat IgGs - Sigma Ltd.
ECL™ reagents - Amersham International Ltd.
Luminol and p-Coumaric acid - Sigma Ltd.
ortho-Phenylenediamine Dihydrochloride (OPD) substrate for ELISA - Sigma Ltd.
Diaminobenzidine - Sigma Ltd.
Preimmune mouse serum - Sigma Ltd.
Anti-phosphotyrosine PY20 - ICN Flow.

Gifts:

Antisera against PLC β2 and 3 were a kind gift from the lab of P. Parker. These sera were prepared in rabbits immunised with the following C-terminus sequences:-

PLC β2	QDPLIAKADAQESRL
PLC β3	GADSESQEENTQL

A mouse monoclonal antibody against PLC β1 was a kind gift from the lab of Sue Goo Rhee.

2.2 Methods

2.2.1 Culture of HL60 cells

HL60 (186) cells were grown using RPMI 1640 medium supplemented with 10% Foetal Calf Serum, 2mM L-glutamine, 50 i.u. of Penicillin and 50μg/ml of Streptomycin, in a 5% CO_2 incubator. Cultures were allowed to reach a density of roughly 1 x 10^6 cells per ml before splitting.

2.2.2 Differentiation of HL60 cells into a neutrophil-like state using dibutyryl c-AMP

Confluent cultures of HL60 cells were treated with 300 μM dibutyryl c-AMP for forty eight hours (187). At the end of this time the cells were seen to have undergone a considerable degree of morphological change, with reduced size, irregular shapes and the formation of considerable numbers of processes.

2.3 Preparation of peripheral blood neutrophils

Whole heparinised blood, or neutrophil enriched buffy coat residues were diluted 1:1 with a 2% solution of dextran in PBS, pH 7.2 and maintained at room temperature for 15-20 minutes, until agglutination of the erythrocytes had taken place. At the end of this time, the cloudy supernatant was taken and layered onto a Ficoll cushion and centrifuged at 750g for 20 minutes.

The supernatant was aspirated off the pellet and the cells gently resuspended in the residual liquid by means of gentle agitation. Contaminating erythrocytes were lysed by the addition of 5ml of distilled water and allowed to stand for 2-3 seconds. Isotonic conditions were then restored by the addition of an equal volume of 2x concentrated PBS. The cells were again pelleted and the lysis process repeated if necessary. The purified neutrophils were then pelleted and resuspended in the appropriate buffer, depending upon the procedure to be undertaken.

2.4 Preparation of rat brain cytosol

Approximately 10g of rat brain tissue was homogenised in 10-15 ml of 20mM PIPES, 3mM KCl supplemented with the following protease inhibitors:-

EGTA	5mM
EDTA	5mM
Benzamidine	10mM
DTT	1mM

Pepstatin A	2μM
Aprotinin	5μg/ml
TLCK	100μM
Leupeptin	0.1mM
PMSF	1mM

This was carried out using a glass homogeniser maintained on ice. The material was then centrifuged at $100x10^3$ g for one hour at 4°C. The supernatant was retained for immediate use, or aliquotted and frozen for later use.

2.5 Reconstitution of PLC activity in SLO permeabilised HL60 cells

HL60 cells were grown up to near confluence in RPMI 1640 medium from Sigma, supplemented with 12.5% FCS, as already described. The cells were then transferred to medium 199, supplemented with 2mM L-glutamine, 5μg/ml of insulin and 5μg/ml of transferrin. To this was added 1μCi of tritiated inositol per 50 ml of culture. The cells were labelled in this manner for 48 hours prior to use in the assay.

At the end of labelling, the cells were spun down by gentle centrifugation at 450g for five minutes, washed twice, and resuspended in 4.5ml of the following buffer:

PIPES	20mM
NaCl	137mM
KCl	3mM
D-glucose	1mg/ml
BSA	1mg/ml
pH 6.8	

After incubation at 37° for 30 minutes the 0.6 i.u. final concentration of SLO were added, in 500μl of the following buffer:

SLO 6i.u./ml	150µl
pCa7 buffer	150µl
1mM MgATP	150µl
20mM PIPES	50µl

Dependent upon the nature of the reconstituting factor under investigation the cells were left to permeabilise at 37°C for 30 minutes, or as indicated. Once permeabilised the cells were pelleted by centrifugation at 450g, and the supernatant removed. A good indicator of successful permeabilisation at this point was the formation of loose, 'fluffy' looking pellet of cells The appropriate volume of the following buffer was added to the cells:

20mM	PIPES pH 6.8
4mM	MgCl2
4mM	Mg ATP
20mM	LiCl
pCa 6 buffer	

The material for the assay was then dispensed into the appropriate number of Eppendorf tubes and maintained in a bath of ice water until all additions had been made and the assay was ready to begin:

Sample 20µl
Cells 20µl
100µM GTPγS or PIPES

The assay was then transferred to a 37°C water bath and incubated for 30 minutes. Quenching of the assay used 1ml of ice cold 0.9% saline. The tubes were capped and centrifuged at $2x10^3$ g for 5 minutes. 900µl of supernatant was removed and run down a column containing 1ml of Dowex ion exchange resin. This was washed with 3x2ml of water to remove free inositol, and 3x2ml of 60mM sodium

tetraborate and 5mM sodium formate solution to remove glycerophosphoinositol. After washing the columns they were transferred from their rack directly into scintillation vials and the bound inositol phosphates eluted from the column using 3x1ml of 1M ammonium formate/0.1M formic acid (188). 7ml of Ultima Flo AR scintillant from Canberra-Packard was added and the activity determined using a Canberra-Packard scintillation counter.

2.6 Assay of PLC activity

2.6.1 Using phosphatidylinositol-4,5-bisphosphate substrate

The whole reaction was carried out in the following buffer:-

20mM	PIPES
100mM	NaCl
0.6%	Na Cholate
2mM	$CaCl_2$
5mM	β-mercaptoethanol

pH 6.8

Phosphatidylinositol-4,5-Bisphophate substrate was made up as follows. 5mg of PIP_2 was dissolved in 1ml of chloroform:methanol 1:1 and placed in a 50 ml glass tube. To this was added 12μCi of ^{3}H-PIP_2 in chloroform:methanol:H_2O 20:10:1. This mixture was then dried down under a stream of dry nitrogen gas. The lipids were resuspended in 28 mls of 2.8 times concentrated sample buffer and sonicated using an MSE probe sonicator. The material was then aliquotted and stored at -20°C until used (189).

An appropriate number of labelled Eppendorf tubes were placed in a rack suspended in ice water. 30μl of the material to be tested for PLC activity was placed in each of the tubes. To this was added 20μl of the substrate cocktail. The assay was then transferred to a 37°C water bath and incubated for 10-20 minutes dependent upon the activity of the samples to be assayed.

The assay was quenched by the addition of 250µl of $CHCl_3$:CH_3OH:HCl 50:50:1 and subject to a vigorous round of vortexing. The assay was acidified by the addition of 75µl of concentrated HCl and again vortexed. The whole was then centrifuged at 2,000g in a chilled Heraeus centrifuge. 100µl of the upper aqueous phase was then removed and the radioactivity present measured by the addition of scintillant and counting in a scintillation counter.

2.6.2 Using phosphatidylinositol as substrate

The assay was conducted in a similar manner to that described above. The PI substrate was prepared as follows. 2.5mg of unlabelled PI and 20µl of ^{3}H PI at 50µCi/ml were dried down under a stream of N_2. This was then sonicated on ice in 1ml of 50mM Tris/maleate buffer at pH. 5.5 containing 2mM $CaCl_2$. This was then placed in a separate tube. 1ml of the above buffer was then added to the original tube and the sonication repeated, in order to remove any residual lipid. The two samples were then pooled and made up to a final volume of 2.5ml. This yielded a final concentration of 1.16mM PI. The substrate was then utilised in the assay as described above, or stored at -20°C until required.

2.7 PI-transfer assay

2.7.1 Preparation of ^{3}H-inositol labelled microsomes:

Fresh rat livers were chopped into small pieces and homogenised in a Potter-Elvehjeum dounce homogeniser, in 0.25M sucrose, 1mM EGTA, 10mM Tris-HCl pH 7.4, (SET buffer) to give a final volume of 200ml. Unbroken cells were removed by centrifugation at $2x10^3$g for 5 mins. Mitochondria were then removed by further centrifugation at $10x10^3$g for 60 mins. Microsomes were then pelleted by centrifugation of the post mitochondrial supernatant at $100x10^3$g for one hour and the resultant pellet resuspended in 50mM Tris and 2mM $MnCl_2$ pH 7.4, in a final volume of 40ml. The material was then again resuspended in an homogeniser and 300µCi of ^{3}H-inositol was added and incubated at 37°C for 90 mins before being pelleted again at

$100x10^3g$ for 90 mins. at 4°C. The pellet was then resuspended in 100ml of 10mM Tris-HCl, 2mM inositol pH 6.8 and pelleted again. This wash step was repeated before the microsomes were resuspended in SET buffer sufficient to adjust the protein concentration to 10-20mg/ml.

2.7.2 Preparation of liposomes

235µl of PC at 100mg/ml was mixed with 100µl of PI at 10mg/ml in chloroform. The mixture was dried down under N_2 in a glass tube. The lipids were then sonicated together in 30ml of SET buffer to give a total lipid concentration of 1mM.

2.7.3 In vitro PI-transfer assay:

The assay was conducted by incubating 50µl of sample diluted in SET buffer with 100µl of microsomes and 100µl of liposomes at 25°C for 30 mins (144). The microsomes were precipitated and removed from the assay mixture by the addition of 50µl of ice cold 0.2M sodium acetate pH 5 in 0.25M sucrose and centrifuging at $11.5x10^3g$ for 15 mins at 4°C. 100µl of the resultant supernatant was then removed, placed in a scintillation vial with 3ml of scintillation fluid and the radioactivity determined using a Canberra-Packard scintillation counter.

2.8 SDS-PAGE electrophoresis and Western Blotting

2.8.1 Gel compositions: Preparations of protein were resolved on the basis of molecular weight using SDS-polyacrylamide gel electrophoresis, derived from the method of Laemelli et al (190).

Dependent upon the molecular weight ranges to be resolved, resolving gels were prepared according to the recipes described in the accompanying table. The recipes

provided enough material for two mini-gels. Large gels were prepared by increasing the volumes appropriately.

In order to ensure that the proteins entered the resolving gel as a compressed single band, a low percentage, non-seiving stacking gel was cast on top of the resolving gel and the protein samples loaded into wells in this gel.

2.8.2 Sample preparation: Samples of protein were prepared and run under denaturing, reducing conditions. As a result proteins resolved purely on the basis of molecular weight. In order to achieve these conditions material was treated with the appropriate volume of a three times concentrated buffer containing sodium dodecyl sulphate (SDS) to denature the protein and β–mercaptoethanol to provide reducing conditions and break disulphide bonds. The material was then heated to 95°C for one minute before being carefully loaded onto the gels which were run in a vertical position. Standard preparations of mixtures of proteins of known molecular weight were run in parallel with unknown samples for the purposes of sizing and locating bands of interest. Typically mini-gels were run at 150V for one and a quarter hours prior to either staining to reveal total protein, or Western blotting for further analysis using immunoreagents.

2.8.3 Composition of resolving gels

% polymer	8%	10%	12%	14%	16%
40% acrylamide/bis mixture	2.3ml	3.1ml	3.9ml	4.4ml	5.0ml
1.5M Tris pH 8.8	3.1ml	3.1ml	3.1ml	3.1ml	3.1ml
Water	6.9ml	6.1ml	5.0ml	4.8ml	4.3ml

10% SDS	125µl
10% Ammonium persulphate	100µl
TEMED	10µl

2.8.4 Composition of stacking gel

30% Acrylamide/Tris	1.3 ml
1M Tris pH 6.8	3.1 ml
Water	5.0 ml
10% SDS	50 µl
10% APS	25 µl
TEMED	10 µl

2.8.5 Electrophoresis Buffers:

10x Running Buffer Composition

Tris	150g
Glycine	720g
Water	50g

Composition of 3x sample buffer

Tris	1.5g
Glycerol	20ml
Water	36ml
SDS	4.0g
Bromophenol blue	2.0mg

pH 6.8

2.8.6 Gel Staining: Dependent upon the relative abundance of the proteins to be investigated gels were stained using any of three techniques depending on loading.

Standard Coomassie blue staining (0.1-0.5μg): Gels were run and immersed in a solution of Coomassie Brilliant Blue as below for a minimum of four hours, or overnight. Destaining to reveal discrete bands of protein within the gel was achieved by successive washes in the dyes solvent base in the presence of pieces of foam rubber which preferentially took up the dye from solution. Gels were then photographed and dried down.

Water	400 ml
Acetic acid	100 ml
Methanol	500 ml
Coomassie Brilliant Blue R250	2.5 g

Colloidal Coomassie staining (0.05-0.1μg): After running, proteins in the gel were immobilised by fixing in a solution of 20% ammonium sulphate. The gel was then transferred to the following solution:

Water	498 ml
Ammonium sulphate	30 g
Orthophosphoric acid	2 ml
Coomassie Brilliant Blue G-250	0.5 g

After incubation over night, the gel was destained by gently agitating in distilled water. The gel was then photographed and dried down.

Silver staining (1-10ng):

This was achieved using the following protocol, at room temperature with solutions and incubations as described below:-

Fixing solution: Incubation 30min to overnight

Ethanol	400ml
Acetic acid	100ml
Water	500ml

Incubation solution: Incubation: 1 hour to overnight

Ethanol	75ml
Sodium acetate	17g
Glutaraldehyde	1.3ml (25% w/v solution)
Sodium thiosulphate	0.5g
Water	to 250 ml final volume

After this incubation the gels were subject to three five minute washes in distilled water.

Silver solution: Incubation 40 minutes

Silver nitrate	0.25g
Formaldehyde	25µl
Water	250 ml

Developing solution: Incubation: until sufficiently developed (Usually 5-10 mins)

Sodium carbonate	6.25g
Formaldehyde	25µl
Water	250ml

Stop solution: Incubation at least 30 mins

Sodium EDTA	3.65g
Water	250ml

At the end of these processes the gels were photographed and dried down.

Copper staining

In addition to the three routinely used protocols described, copper staining was also employed for the purpose of detecting proteins in gels from which antigens for inoculation were to be prepared. This entailed immersing the gel in a solution of 0.3M $CuCl_2$ overnight. The copper ions entered the gel and formed an insoluble complex with the dodecyl sulphate moiety of the SDS present. In areas of high protein concentration this complex was not so efficiently formed and was visible in the gel as a clear area against an hazy background. Destaining of the material excised form the gel was performed by incubating the gel slice in several changes of 100mM EDTA, 100mM EGTA, until destained, with a final incubation in water to remove the chelating agents.

2.8.7 Western Blotting

Depending upon the molecular weight of the proteins to be transferred to the membrane either of two methods were used for electrotransfer onto Immobilon (PVDF) membrane of proteins resolved by SDS PAGE. For relatively high molecular weight species, above 60kDa, a wet transfer method was found to be most effective, while for lower molecular weights a faster semi-dry method was more convenient.

Wet transfer: This was carried out using a BioRad wet transfer system, which was run at 30V overnight, or at 60V for 1.25 hours. The composition of the buffer system used in the tank was similar to that described for running of the PAGE gels, with the exceptions that no SDS was added to the blotting buffer and that it was supplemented with 10% methanol.

Semi-dry transfer: This was carried out using a BioRad semi-dry transfer apparatus. The buffer system used was that of Bjerrum Schaffer-Nielsen as described below. The procedure was optimised by studying the transfer of proteins from a series of standardised gels of rat brain cytosol. Conditions were established at 5V for 15 minutes.

After proteins had been transferred by either of the two methods described above, the position of discrete bands on the blot were located by staining the blots with a 1:1000 dilution of India ink. This process was found not to interfere with further characterisation of the bound proteins using immunological methods.
N.B. For the purposes of this thesis, Western blotting refers specifically to the electrotransfer of proteins from a gel to a membrane solid phase.

2.8.8 Immunological probing and development of blots:

After electrotransfer of proteins to Immobilon P membrane and location of the bands by India Ink staining, blots were transferred to a 150 ml screw capped sample pot and washed with PBS-Tween to remove excess ink and then blocked for one hour with a 5% suspension of milk protein supplemented with 2.5% goat serum in order to prevent non-specific binding of immunoreagents to unoccupied sites on the membrane. This was followed by a five minute wash to remove excess blocking buffer before the addition of the primary antibody, diluted as necessary and incubated for a period appropriate to the individual preparation being used. The primary antibody was removed and the blot washed twice to remove unbound antibody. Appropriately diluted HRPO-conjugated secondary antibodies were then added in blocking buffer and incubated for 30 minutes at room temperature. Again, the solution was poured off and the blot subject to at least five rounds of extensive washing totalling one hour. After this development of the blot was performed using either the proprietary method of Enhanced Chemiluminescence, ECL™ from Amersham International Ltd, or an in house system, constituted as below:-

Luminol solution

2.5mM luminol

0.4mM p-coumaric acid

0.1M Tris pH 8.5

Hydrogen peroxide solution

5.4mM Hydrogen peroxide

0.1M Tris pH 8.5

To use, equal volumes of the above solutions were mixed and incubated with the blot for 1-2 minutes. Excess liquid was poured off from the blots and the pieces of membrane wrapped in plastic film. Membranes were then laid onto Amersham ECL film and exposed for various time periods, before the film was developed using conventional X-ray chemistry.

2.9 Quantification of proteins using the Bradford assay:

100mg of Coomassie brilliant blue G250 (Sigma Ltd.) were dissolved in 50ml of 95% Ethanol. 100ml of concentrated orthophosphoric acid were also added and the whole made up to a final volume of 200ml with distilled water. This material constituted a five times concentrated stock and was stored at 4oC for up to six months. When required the concentrate was reconstituted to working strength with distilled water. This was then filtered using a 0.45mm syringe filter.

5µl samples of BSA (Pierce Ltd.) ranging from 50 to 600µg/ml were used as standards and compared to similar volumes of unknown samples, diluted suitably so as to bring absorbance values within the linear range of the titration. 200 µl of the diluted Bradford reagent was then added to the sample in a flat bottomed microtitre plate (Falcon) and the absorbance of the samples determined at 620nm using a Multiscan plate-reader from Lab Systems Ltd.

2.10.1 Preparation of recombinant PI-TP:

E. coli expressing PI-TPα, and β were used to make the protein. The gene for PI-TPβ had been derived from a rat brain DNA library and cloned into the plasmid pET-21. The gene for PI-TPα was obtained from an human DNA library from HepG2 cells and similarly introduced into plasmid pET-21. Both were expressed as thrombin-cleavable fusion proteins with an hexahistidine tail used for purification of the recombinant protein. These were used to transfect the *E. coli* strain BL21DE3 from Novogen. Glycerol stocks of this modified organism were maintained at -70°C (152, 191).

Luria broth was prepared using powdered base from Gibco BRL. 2.5g of powder was used per 100ml of medium required and the solution autoclaved at 115°C for 15 minutes. Once the solution had cooled to room temperature it was supplemented with 50μM Ampicillin from Gibco BRL.

Initially 200 ml of bacterial culture were grown up overnight at 30 °C in the presence of 0.05 mM ampicillin, having been seeded with 25μl of glycerol stock. This starter culture was then expanded by adding it to a further 800ml of LB broth, again containing ampicillin. After being allowed to grow for 1.5 hours at room temperature, expression of the recombinant protein was induced by the addition of 0.1μM IPTG and the bacterial culture maintained for 4 hours.

2.10.2 Purification of the recombinant protein:

At the end of the induction period, the cells were pelleted by centrifugation and resuspended in a buffer containing 50mM Na_2HPO_4 and 300mM NaCl at pH 8.0. Lysozyme was added at a rate of 1mg/ml and the cells incubated on ice for 30 mins. At the end of this incubation, the cells were sonicated, 6x1minute with a probe sonicator, before being centrifuged at $10x10^3$g for 30 mins at 4°C. The supernatant was then incubated with Ni^{2+}-NTA resin (Quiagen Ltd.) for 30 mins at 4°C and the slurry of broken bacteria and resin poured into pre-prepared columns. The resin was then washed through with 12 bed volumes of 50mM Na_2HPO_4, 300mM NaCl and 10% glycerol, pH 6.0. The resin was then washed with 6 bed volumes of a similarly

constituted buffer containing 525mM NaCl. The final washing step with 6 bed volumes used the same buffer supplemented with 25mM imidazole. The recombinant protein which was bound to the Ni^{2+} resin was then eluted using wash buffer supplemented with 250mM imidazole. The protein was then buffer exchanged into 20mM PIPES, 137mM NaCl and 3mM KCl pH 6.8 using desalting columns. This material was then Bradford assayed, aliquotted and stored at -20°C and used as required.

2.11 Purification of antibodies

2.11.1 Purification of antibodies from tissue culture supernatants.

Hybridomas were cultured in $175cm^2$ tissue culture flasks in RPMI 1640 supplemented with 10% foetal calf serum with 2mM L-Glutamine and penicillin/streptomycin. Once the medium in the flask had become yellow and the cells reached confluence, the medium was harvested by centrifugation to remove the cells and debris and the cells remaining in the flask diluted ten fold. Unless the supernatant was to be used immediately for antibody purification, protease inhibitors were added and the material stored at -20°C.

The pH of undiluted tissue culture supernatant was adjusted to approximately 7.4, before being loaded onto a 10ml Prosep-G column at a rate of 5ml per minute. Batches of up to 400ml of supernatant were processed in this way. The flow through from loading was retained in a fresh vessel. Ten column volumes of phosphate buffered saline containing 0.5M Glycine, pH 7.4 were then passed through the column and once the UV trace had returned to basal levels, the bound immunoglobulins were eluted using 0.1M Glycine pH 3 and the resulting column fractions immediately restored to pH 7.4 by the addition of 1.5M Tris, pH 9.0. Samples taken across the UV peak were then spot assayed for protein using the Bradford assay and those fractions containing protein were pooled and concentrated using 10kDa Centricon concentrators.

After each run, the column was regenerated by treatment with dilute hydrochloric acid at pH 1.5 and re-equilibrated with PBS-Glycine pH 7.4 to which was added 0.02% sodium azide as a preservative.

2.11.2 Purification of polyclonal antibodies

Initial purification of the polyclonal antibodies was achieved using PI-TPα/GST fusion protein resolved from a crude bacterial lysate, and adsorbed onto PVDF membrane by Western blotting. Antibodies bound to this material were eluted by treatment with 0.1M citrate buffer at pH 3.0, and neutralised with Tris.

Subsequent purifications were achieved by binding 5mg of recombinant PI-TPα to a 1ml NHS-activated Sepharose column from Pharmacia, as per the manufacturers instructions. This column was then loaded with sera diluted with PBS pH 7.4 at a rate of 1ml/min using an FPLC. Once the sample was loaded, the column was then washed with PBS pH 7.4 until a steady base was achieved on the UV absorbance monitor. The bound antibodies were then eluted from the column using 0.1M glycine pH 3.0, and 1ml fractions were collected. The material was then neutralised using 1.5M Tris pH 8.8, titred, aliquotted, and stored at -20°C until required.

Chapter 3

Characterisation of the G-protein coupled phospholipase C-β isozymes present in HL60 cells

3.1 Introduction

The initial aim of the project described in this thesis was the characterisation of the enzymes involved in G-protein-coupled phospholipase C signalling in HL60 cells and by extension, neutrophils. With this in view, fast protein liquid chromatography, *in vitro* PLC assays and immunological methods were brought together to identify the PLC-ß isozymes present.

3.2 Preparation of HL60 cytosol and resolution using Heparin Sepharose chromatography:

Large volumes of HL60 cells were grown up for this purpose according to the method already described in Chapter 2. Typically $500x10^6$ cells were cultured, pelleted and washed in 20mM HEPES buffer containing 137mM NaCl, 3mM KCl and 100μM EGTA. The cultured cells were then treated with 2mM DFP on ice for 5 minutes. The cells were then washed and resuspended in a homogenisation buffer comprising 250mM sucrose, 0.1M EGTA, 0.1M EDTA, in 20mM PIPES at pH 6.8. Cells were then disrupted using a MSE probe sonicator. A post nuclear supernatant was prepared by centrifugation at 750xg for five minutes. The supernatant was then further clarified by ultracentrifugation in a Beckman Ti60 rotor at $50x10^3$ rpm for 1 hour at 4°C. At the end of this, the clarified cytosol was dialysed overnight against several changes of 3mM KCl, 20mM PIPES pH 6.8, concentrated to approximately 5ml in an Amicon concentrator, before being filtered through a 0.45μM filter and chromatographed using 4, 1ml Hi-Trap Heparin sepharose columns from Pharmacia.

Initial attempts at the chromatographic resolution of the phospholipases C present yielded what initially appeared to be a single peak of PLC activity on the basis of the PIP_2 hydrolysis assay. Comparing the PLC activity profiles obtained using PI,

or PIP_2 in individual assays it was concluded that there were at least two distinct activities present, yielding a broader peak of PIP_2 hydrolysing activity, superimposed upon a narrower, slightly shifted peak of PI hydrolysing activity. PIP_2 is the main *in vivo* substrate for the activity of PLCs (145). While under *in vitro* conditions, PLCs can be forced to utilise PI by elevating Ca^{2+} levels, under the conditions employed in the PLC assays under consideration, only PLCsγ are able to efficiently hydrolyse PI (144). It was therefore decided to adjust the FPLC protocol, by running a more shallow gradient in order to resolve the peaks of activity further.

A representative PLC activity profile are illustrated in Fig. 3.1A. The material was loaded onto four 1ml Heparin-Sepharose columns, at a rate of 1ml per minute. After loading, the columns were run at 2ml per minute with a salt gradient from 0 to 1M being applied, 5 minutes after loading and reaching maximal salt concentration at 25 minutes. 2ml fractions were collected.

The fractions were assayed for the presence of PLC according to the protocol described in Chapter 2. Both PI and PIP_2 were used as substrate in separate assays, in order to help differentiate between the various PLCs present on the basis of substrate specificity. The results of the PLC assay are shown in Figure 3.1 A. As can be seen, utilising PIP_2 as substrate, the fractions display three distinct peaks of activity which are designated as peaks 1 to 3. Using PI as substrate, a single prominent peak of activity is observed, corresponding exactly to peak 2 in the PIP_2 assay. This suggests the presence of a PLCγ isozyme (145). as observed by S. Cockcroft et al.

3.2.1 Reconstitution of PLC activity in cytosol depleted HL60 cells

Active fractions, as determined by PLC assay were further examined for their ability to reconstitute GTPγS-stimulated PLC activity in SLO permeabilised HL60 cells. It had previously been observed that the cytolytic bacterial toxin SLO was able to produce a decay in the responsiveness of PLCβ isozymes to GTPγS and receptor stimulation in HL60 cells. This decay is a consequence of the loss of cytosolic components from the cell through toxin-generated pores in the plasma membrane. The ability of purified components to reconstitute PLC activity could be assessed by means of adding them back to ^{3}H-inositol labelled, cytosol depleted cells in the

presence of GTPγS. The resultant level of activity of the PLC isozymes present could then be measured in terms of the release of tritiated inositol polyphosphates. The dephosphorylation of the released inositol phosphates is prevented by the presence of lithium in the reaction buffer.

The results of this assay are illustrated in Fig. 3.2. As can be observed, those peaks from the PLC assay designated as peaks 1 and 2 demonstrated an ability to stimulate the hydrolysis of inositol lipids in the presence of GTPγS. This assay was conducted according to the protocol described in Chapter 2. At the time of performing these experiments, it was believed that the reconstituting activity observed was a consequence of the presence of G-protein coupled PLCβ isozymes (145).

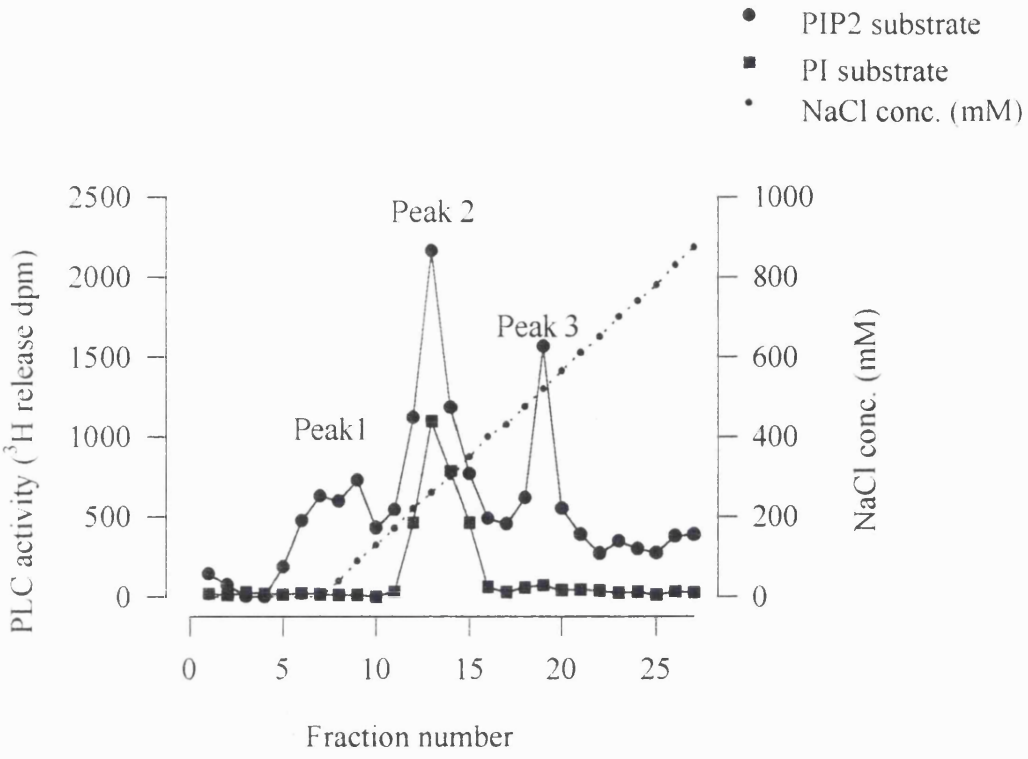
3.2.2 Western Blotting of resolved PLC activities:

The peaks of activity identified on the basis of PLC assay and reconstitution were run out on 8% polyacrylamide gels as described in Chapter 2 and transferred onto Immobilon P, PVDF membrane. The blots were then probed with antibodies against PLCs β1 and 2. The results are shown in Fig. 3.1 B. It was immediately apparent from the results that a strongly immunoreactive protein was being recognised by the anti PLC β2 antibodies at a molecular weight of approximately 100kDa. It was initially believed that this represented a proteolytic fragment of the full length PLCβ2 enzyme.

After the initial chromatography run, further preparations incorporated a cocktail of protease inhibitors in addition to pre-treatment of the cells with DFP, a serine protease inhibitor. None of these treatments was able to abrogate the release of the 100kDa fragment. A similar observation had been reported by P. Gierschick (Personal communication). From these observations it was concluded that PLC β2 was labile and subject to proteolysis in the course of preparing the sample.

A calpain cleavage site was known to be present in the sequence of PLC β1 (54) and it was proposed that PLC β2 may also be cleaved by this activity. To this end, further runs incorporated a set of calpain inhibitors. These however were

A.



B.

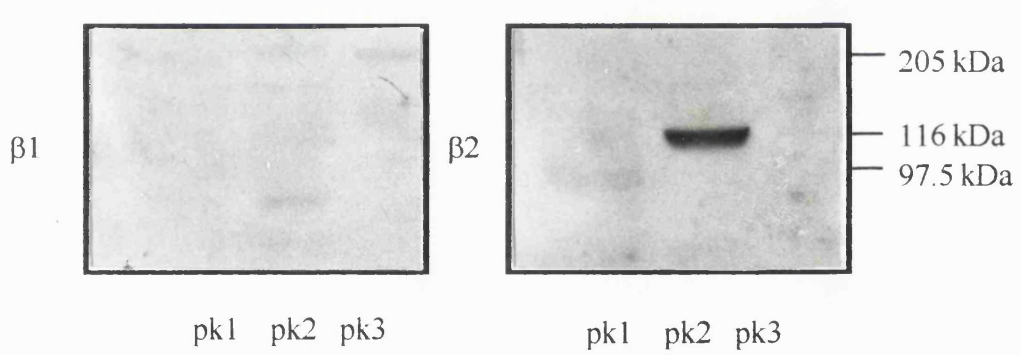


Fig 3.1 Heparin-sepharose chromatography of HL60 cytosol and Western blotting of the resolved PLCs with PLC-β isozyme specific antibodies.

HL60 cytosol was prepared by sonication, and centrifugation This material was then resolved using four 1ml heparin sepharose columns coupled in series, and the resultant column fractions assayed for PLC activity using both PI, and PIP_2 as substrate (A.) Active fractions were pooled and 80μl samples Western blotted. The blots were probed with antibodies against PLCs β1,and 2.(B.).

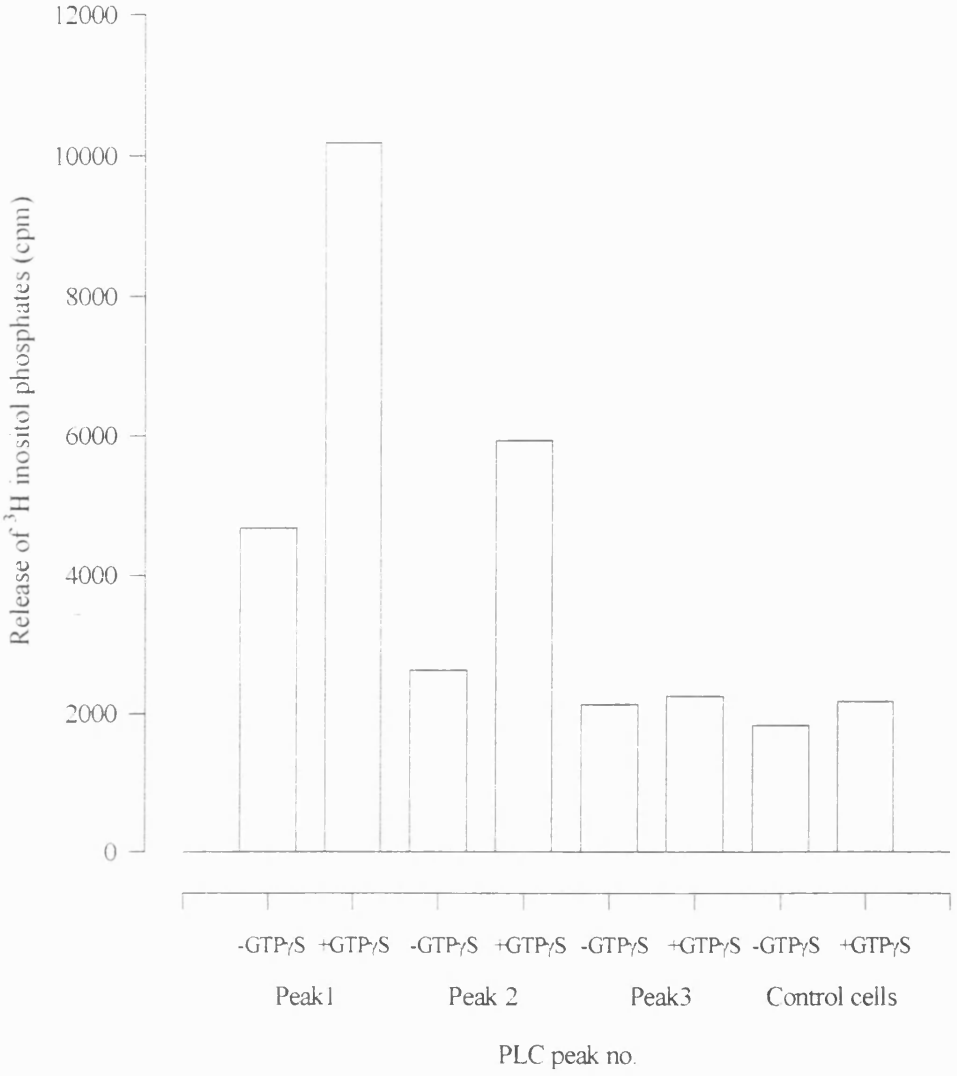


Fig. 3.2 PLC reconstitution using Heparin-sepharose FPLC resolved PLC peaks from HL60 cytosol.

Samples from pooled fractions constituting peaks one to three on the previous figure were assayed for PLC reconstitution according to the standard protocol described in Chapter 2. In brief, HL60 cells were permeabilised with SLO for 30 mins, and washed to removed leaked proteins. GTPγS stimulated PLC activity was thus strongly reduced. Exogenous protein from the column fractions were then introduced to measure their PLC reconstituting ability. Reconstitution can clearly be observed using fractions from peak 1, and peak 2 in response to stimulation with 5μM GTPγS. Peak 3 does not restore activity.
The plotted values represent means of duplicates from a single experiment. The results varied by less than 10% of the calculated mean value

equally unsuccessful in preventing the apparent proteolysis of the PLC. The calpain cleavage site identified in PLCβ1 is present in the carboxy terminal portion of the molecule - thus, if calpain were responsible for the cleavage of PLCβ2, the cleavage site does not represent a sequence conserved between PLC β 1 and 2.

3.2.3 Preparation and Western blotting of HL60 cytosol and membranes by freeze thawing:

In order to definitely identify the PLCs present, in the light of the apparent lability of the PLC β2 enzyme and the consequent inability to obtain a full size band on Western blots even in the presence of multiple protease inhibitors, it was decided to employ an alternative method for the preparation of the cytosol. In order to make the process as rapid as possible and reduce the time available for the activity of proteolytic enzymes, freeze/thawing was arrived at as the method of choice. This had the added advantage that by the very nature of the process, the sample was maintained at low temperatures - use of the probe sonicator resulted in quite discernible warming of the sample and care was taken to ensure that the sample was periodically cooled on ice between treatments.

HL60 cells ($100x10^6$) were cultured as previously described, resuspended in 20mM PIPES, 137 mM NaCl, 3mM KCl pH 6.8 and treated with DFP. The cells were then rapidly frozen using liquid nitrogen. The cells were allowed to thaw at 4°C, before the process was repeated. In total the cells were subject to three rounds of freeze thawing in this manner. The material was then given a low speed centrifugation to remove nuclei and undisrupted cells. The cloudy supernatant was then centrifuged at $100x10^3$g, at 4°C for one hour and the resultant supernatant resolved on 8% polyacrylamide gels and probed with antibodies to PLC β2 and additionally PLC β 3.

In addition to investigating the cytosolic presence of PLCβ 2 and 3, the association of the PLC enzymes with the cellular membranes was investigated by the preparation of crude membrane material. The crude membrane material pelleted in the final ultracentrifugation step described above was retained and then resuspended in homogenisation buffer and washed twice in the same. This was regarded as

representing a crude preparation of cellular membranes. The material was then probed by Western blotting for the presence of PLC β 2 and 3, as it had previously been demonstrated that PLCβ1 was not detectable in the cytosol of HL60 cells.

The results from these investigations are illustrated in Fig. 3.3A As can be seen, cytosol prepared in this manner exhibits an anti-PLCβ2 reactive band at 150kDa as the main signal. The pelleted membranes produced a signal containing both the full length PLCβ2, as well as the 100kDa fragment, with the latter fragment representing the bulk of the observed signal. Nuclear pellet produced an image almost devoid of full length PLCβ2. Probing blots prepared using identical fractions with antiserum specific for PLCβ3 produced a weak signal for this protein in the cytosolic and membrane fraction. This was significantly weaker than that observed using the PLCβ2 antibody.

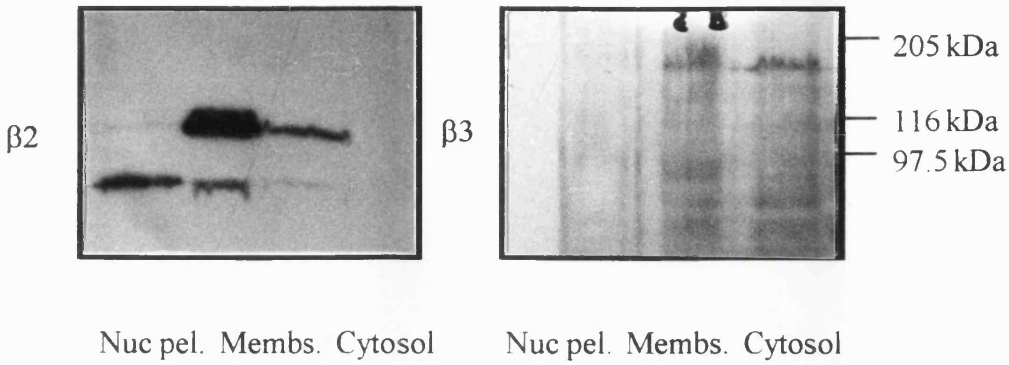
3.2.4 Examination of peripheral blood neutrophils:

In order to determine that the complement of PLCβ isoforms in human neutrophils reflected the situation observed in HL60 cells, neutrophils were prepared from peripheral blood, by a process of agglutinating erythrocytes and Ficoll gradient centrifugation, followed by hypotonic lysis of contaminating erythrocytes. This protocol is fully described in Chapter 2.

The resultant cells were then DFP treated and cytosol prepared for SDS-PAGE by three rounds of freeze thawing and centrifugation. Because of relatively low yields of cells, cytosol alone was examined for the presence of PLCβ 2 and 3.

The results of these blots are shown in Fig. 3.3 B. The blot shows four dilutions of neutrophil cytosol resolved on SDS PAGE gel and probed in parallel with a sample of HL60 cytosol as positive control. As can be observed, a distinct band is seen in the material probed with the PLCβ2 specific antiserum, with a somewhat fainter band at approx. 150kDa being visible in the anti-PLCβ3 blot. It is not possible to draw any valid conclusions upon the relative abundance of the two proteins on the basis of such Western blots, as the blots were each probed with

A.



B.

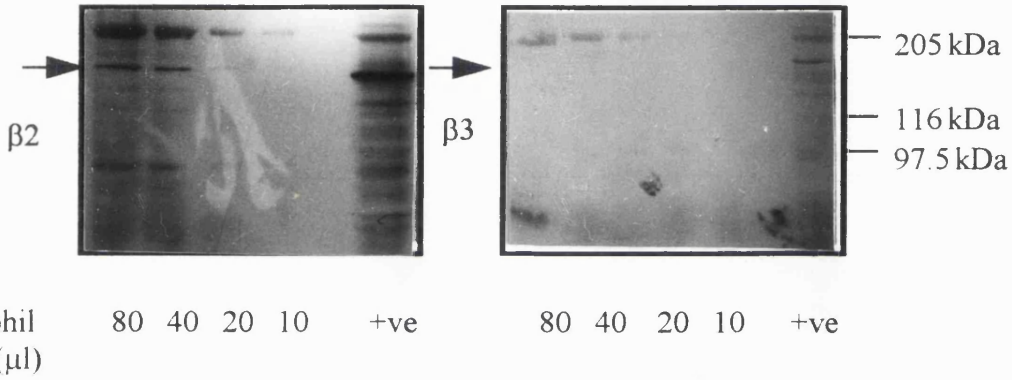


Fig. 3.3 Immunological probing of HL60 cells (A), and peripheral blood neutrophils (B.) for PLCs β 2, and 3.

A. Large volumes of HL60 cells were prepared as previously described, and after treatment with DFP, subject to three rounds of freeze thawing. A post nuclear supernatant was prepared, with the resultant pellet being retained. The PNS was then subject to ultracentrifugation, and the resultant crude membrane prep washed a further two times. Samples of these three fractions were then resolved using SDS-PAGE, and Western blotting. The blots were probed with antibodies specific for PLCs β2, and 3.

B. Peripheral blood neutrophils were prepared from 50ml of blood from a volunteer donor, as described in Chapter 2. These cells were then subject to freeze thawing, and the resultant cytosol probed for the presence of PLC β2 and 3. 80, 40, 20, and 10μl samples of cytosol were loaded in parallel with 80μl of HL60 cytosol as a positive control.

different antibodies which do not necessarily display similar affinity for their target antigens.

3.2.5 Discussion:

By Western blot analysis of material from both HL60 cells and neutrophils, the presence of two G-protein coupled PLC isozymes, PLC β2 and 3 has been demonstrated. It has further been shown that there is an association between these enzymes and the cell membrane. This is in agreement with observations made by C.P. Downes et al utilising turkey erythrocytes and avian homologues to the G-protein coupled receptor systems of mammals - it was observed that PLC was associated with cytoskeletal components in close juxtaposition with the plasma membrane (18). The presence of the other then identified PLCβ isozyme, PLCβ1, however was not demonstrated. This protein has been shown to be strongly expressed in brain tissue, but also to be represented in a variety of other cell types, though at varying levels. PLCβ2 was originally cloned from the cell line HL60, cells of haematopoietic origin and has a limited tissue distribution. Phospholipase Cβ3 was initially identified on the basis of probing the rat thyroid cell line FRTL for PLCβ–like sequences. It has subsequently been demonstrated as being quite ubiquitous in distribution.

Other peaks of PLC activity which were not immunoreactive using any of the antibodies available were observed - their identity remains indeterminate. It has however been previously demonstrated that PLCγ2 is present in cells of haematopoietic origin including neutrophils, though this protein was not visualised using antiserum raised against a carboxy terminal peptide of PLCγ2 (9).

Bearing in mind the carboxy terminal origin of the sequences used for the production of the anti-PLC β2 specific sera the obvious conclusion from the Western blotting data is that the N-terminus 45-50 kDa portion of the PLC β2 is being proteolytically cleaved. The observation that those fractions showing immunoreactivity when probed with β2 specific antibodies also demonstrated PLC reconstitution in response to GTPγS suggests a number of alternative explanations:-

1. The truncated PLC β2 retains structural motifs required for coupling to the appropriate heterotrimeric G-protein. As illustrated in Fig. 1.2 which schematically

depicts the three families of PLCs, PLCs β differ from the other members of the superfamily by possessing an extended C- terminus sequence, through which interaction with Gq α subunits is believed to occur (192). Clearly in the case of the truncated portion of the molecule, this C-terminal sequence is still present, possibly enabling this entity to mediate the observed PLC reconstitution. This effect was recently demonstrated by Kim et al, examining a series of deletion mutants of PLCβ-1 (192). This in itself constitutes an interesting observation. It has been demonstrated that the activity of some members of the PLC β family are influenced by the βγ subunits of heterotrimeric G-proteins (193). These interactions have been demonstrated as taking place at the N-terminus of the phospholipase. It has been proposed that the effect of the βγ subunits on PLC βs serve not as the primary route of stimulation, but as a mechanism whereby the activity of the signalling system can be subtly modulated by interaction with βγ subunits from other G-protein coupled receptor sets. The different PLCβ isozymes display differential sensitivity to βγ subunits. A similar mechanism has been observed in the case of the adenylate cyclases, with differential sensitivity of individual isozymes to specific G-protein subunits. A study of such a truncated, but apparently fully functional PLC and its activity in response to receptor stimulation may throw further light upon the importance of regulatory interactions at the N-terminus of the PLC.

2. At the time of performing the chromatographic resolution of the PLCs, specific antiserum to the PLCβ 3 isoform was not available in this lab. The ability of fractions to produce reconstitution of PLC activity in response to GTPγS may well therefore reflect the presence of fully functional PLCβ3.

3. While the existence of PI-TP had at this stage been known of for a number of decades, the significance of this protein to PLC signalling was not appreciated. The role of this protein in PLC mediated signalling was initially identified on the basis of a cytosolic component which could be chromatographically resolved from PLC activity, being able to elevate the responsiveness of PLC β to GTPγS stimulation in SLO permeabilised HL60 cells (2, 194). Reconstitution of PLC activity may therefore not be mediated by the detected PLC activity in the *in vitro* assay, but may be a consequence of PI-TP elevating the indigenous response, or acting synergistically in combination with any PLC present in the sample. Retrospective examination of

frozen samples for the presence of the mammalian phosphatidylinositol transfer protein α (PI-TPα), failed to demonstrate detectable levels of this protein in the samples available. (See Chapter 4 for details of the preparation of the rabbit antibodies directed against PI-TP). For reasons that will be clarified in Chapter 7 however, this result is unsurprising with the benefit of hindsight.(The predominant isoform of PI-TP in HL60 cells and neutrophils will be demonstrated as being PI-TPβ). Work conducted in this laboratory by Dr. E.M. Cunningham suggested that on Heparin Sepharose chromatography, PI-TP elutes from the column at relatively low salt concentrations (2). Thus, reconstituting activity present in Peak 1 of the chromatographed cytosol is most likely to be a consequence of the presence of PI-TPβ.

Attempts to partially elucidate the distribution of PLCβ2 within the cell by examining washed membranes produced by freeze thawing of cells and the debris remaining from preparation of the PNS reveal the presence of PLC β in the washed membrane fraction, as well as the cytosol. The nuclear material also contains a significant amount of anti-PLCβ2 immunoreactive protein. The signals from the membranes and nuclear material are obtained after washing of these samples with buffer - whether or not the signal reflects contamination with cytosolic proteins remains to be answered definitively. However, the observation of PLCβ in the nuclear material may well reflect the active nuclear inositol cycle which has been characterised in other systems (195-198). It was interesting to note the distinct degrees to which the material was degraded with respect to PLCβ2. This is not a function of the time taken to prepare the samples, as the nuclear pellet which displays the greatest degree of truncation of the PLCβ2 and was the quickest of the three fractions to prepare. It is suggested that the mixture of organelles included in what is described as the nuclear pellet include a variety of proteases some of which are responsible for the cleavage of this protein. Both the membrane and cytosolic preparations were separated from the source of these proteases fairly early in the course of preparation of the sample and it is suggested that this is the reason for the relatively low level of proteolysis displayed in these samples. Alternatively, proteolysis may lead to the release of an hydrophobic entity which is preferentially taken up by membranes in the membranes and nuclear pellet.

Chapter 4

Investigation of the kinetics of PI-TP release from SLO permeabilised HL60 cells using a polyclonal antibody raised against a PI-TPα/GST fusion protein.

4.1 Introduction:

Work in this laboratory revealed the importance of the mammalian phosphatidylinositol transfer protein in the functioning of the PLC signalling pathway (2, 3, 171). With the establishment of this fact, a number of questions were posed about the nature of the protein which included :-

1. Identification of possible regulatory modifications to PI-TP in the stimulated cell, in particular phosphorylation.
2. Identification of any proteins with which PI-TP may be interacting.
3. Translocation events within the stimulated cell - is PI-TP recruited to membranes in HL60 cells?
4. Quantification of PI-TP in HL60 cells in order to establish that the PLC reconstituting activity detected earlier takes place at physiologically relevant concentrations of the protein.
5. Identification of lipids bound to the transfer protein in HL60 cells.
6. Immunolocalisation.

In order to attempt to answer some of these questions it was decided to utilise immunological methods and raise a panel of rabbit anti-sera against the protein. It was hoped that these reagents would have application in Western blotting, immunoprecipitation studies to examine phosphorylation, lipid loading and protein interactions, as well as immunofluorescence.

Because of the limited amount of chromatographically purified protein available it was decided to utilise a PI-TP/GST fusion protein as the source of antigen. To this purpose a cohort of three New Zealand White rabbits was immunised with

preparations of the fusion protein to produce a panel of sera which were immunoreactive towards the native PI-TP.

This set of reagents were then used to study various parameters of the proteins behaviour. Comparison was made between the kinetics with which PI-TP and PLC β isoenzymes leaked from cells upon permeabilisation with SLO. In addition attempts were made to detect whether or not the kinetics of leakage were affected by stimulation of the cells with GTPγS, or phorbol esters.

4.2 Inoculation of rabbits with a PI-TPα/GST fusion protein

The cohort of three female New Zealand White rabbits (Designated as animals D, E and F) was established in the animal house and allowed to settle in for two weeks prior to the primary immunisations. During this time bleeds were taken from them and the sera frozen down to act as pre-immune negative controls.

Bacteria expressing the rat brain PI-TPα in the form of a fusion protein with glutathione-S-transferase (GST) were grown up in Luria broth as described in Chapter 2. Samples of the bacteria were taken after induction with 0.1 mM IPTG and lysed in SDS-PAGE sample buffer under reducing conditions. The PI-TP/GST fusion protein was extremely insoluble and was predominantly found in intracellular granules. In order to obtain an efficient release of the protein from the pelleted bacteria, extensive heating and sonication of the sample was necessary. Once this had been performed, insoluble material remaining was removed by centrifugation and the clarified supernatant removed from the pellet.

The supernatant was then run out on a 10% acrylamide SDS-PAGE gel. In order to visualise the protein copper staining using copper II chloride was used as described in Chapter 2. The recombinant protein was revealed to be the predominant protein and was excised from the gel with a scalpel. The excised gel strip was then destained using several changes of a 100mM solution of EDTA/EGTA. At the end of this process the gel was equilibrated with distilled water.

The material prepared above was then homogenised in Freund's complete adjuvant, using a Polytron homogeniser and was used as the basis of the primary

inoculations. Material was injected into the backs of the rabbits in such a manner as to produce subcutaneous depots of antigen.

The animals were allowed to recover and initiate an immune response for a period of days before test bleeds were taken and further antigen injected. In order to avoid extreme anaphylactic responses, subsequent immunisations utilised Freund's incomplete adjuvant, as the mycobacterial antigen present in the complete preparation induces an extreme state of hypersensitivity in susceptible animals.

During this period, test bleeds of approximately 10 ml were taken three days after each immunisation. The blood was maintained at 4°C overnight in order for efficient clotting to take place and then centrifuged at 2,000 rpm using a bench top centrifuge. The clarified sera were removed from the clot, assayed as described below and aliquotted into 500µl volumes and frozen down for later use. Immunisations and test bleeds were conducted by members of the Animal House staff.

4.3 Assaying the sera for anti PI-TP activity

In the absence of pure recombinant PI-TP and because of the labour intensiveness of chromatographic purification from brain tissue it was decided to screen the sera for anti-PI-TP antibodies using Western blots rather than the more convenient ELISA method. To this purpose, a reference batch of rat brain cytosol was prepared as described in Chapter 2 and frozen down in 500µl volumes. This material was then thawed as required and electrophoresed on 12% acrylamide, 1mm thick, single well gels using a BioRad mini-Protean II apparatus. Proteins were transferred to Immobilon P blotting membrane by wet transfer at 30v overnight. At the end of this process, the transferred proteins were visualised by staining using Ponceau S stain and the position of the molecular weight markers fixed using an ordinary graphite pencil. The blot was then cut into 7mm strips, blocked as described and incubated with dilutions of the sera. Binding of antibodies was determined as described in Chapter 2 using Enhanced Chemiluminescence™ from Amersham.

Using this method, the titre of antibodies was followed over the course of the immunisations and was found to peak at a detection limit of 1:2000 dilution of the sera.

A consistent pattern of spurious bands was however observable at all dilution assayed using Western blots and the first confirmation of the sera's specificity was obtained when they were used to probe protein partially purified from a gel filtration run. Thus it was determined that affinity purification of the antibodies was necessary if they were to become a truly useful immunoreagent.

The results of this blot are shown in Fig. 4.1 in parallel with the results from an in vitro assay of PI-TP activity conducted as part of the routine purification of PI-TP from bovine brain cytosol. 80µl samples of alternate column fractions, treated with 3xSDS PAGE sample buffer were taken from across the peak of PI-transfer activity, These samples were then resolved by SDS PAGE on a 12% polyacrylamide gel and blotted onto PVDF membrane. The blot was then probed with pooled sera from the three animals, diluted 1:1000 and developed using ECL chemistry. A single strong band was detected at a molecular weight of approximately 35 kDa. This peak of immunoreactivity aligned perfectly with the profile of PI-transfer activity as determined by in vitro assay of column fractions from a Superdex-75 gel filtration column.

4.4 Affinity purification of the antibodies

Initial purification of the antibodies was achieved utilising Western blots of the same material used to raise the sera. Blots were prepared using 100µl volumes of the GST/PI-TP fusion protein. This was then stained and the predominant band excised. This was then cut into small strips, transferred to a syringe, blocked with milk protein and incubated with sera diluted 1:10 in blocking buffer. The membrane chips were then washed several times in Phosphate Buffered Saline pH 7.4, before bound antibodies were eluted using 100mM citric acid pH 3.0. The eluted material was neutralised using 1.5 M Tris pH 8.8. Fig. 4.2 illustrates the results obtained by probing various cytosols and the bacterial material used for the immunisations with the resultant antibody preparation. As can be seen, all of the mammalian cytosols

Fig. 4.1 Demonstration of PI-TP specific antibodies in the sera of rabbits immunised with a PI-TP/GST fusion protein.

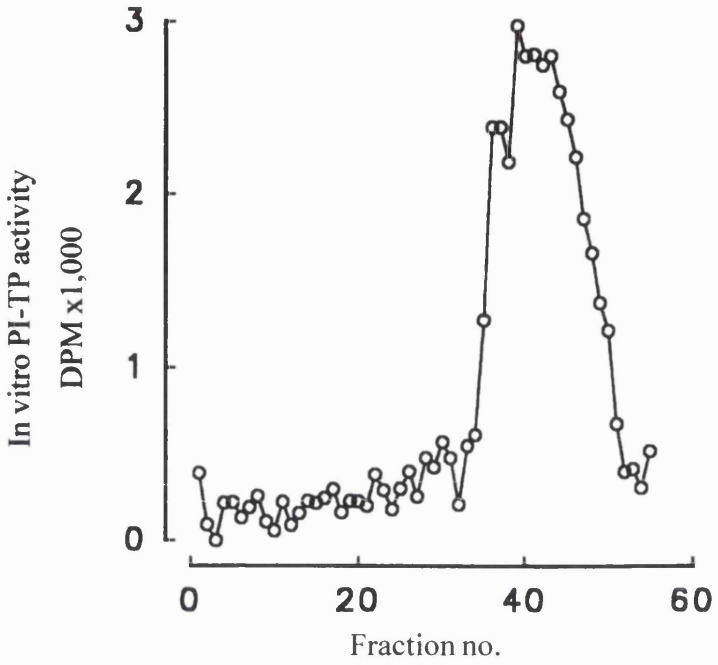
Fig. 4.1 represents the first unequivocal proof that the sera of the immunised cohort of rabbits contained antibodies specific for PI-TP. The sera of the animals had been monitored for antibody production by Western blotting a standardised preparation of rat brain cytosol specifically set asides for this purpose. While apparently specific bands were observed to be produced over the course of the immunisations the data were always clouded by the presence of a number of spurious bands. On the basis of the putative positive data it was decided to screen the pooled sera of the animals using partially purified PI-TP from bovine brain.

Fig. 4.1A represent the results of a PI-transfer assay conducted on column fractions from gel filtration chromatography of partially purified bovine brain PI-TP. This assay was conducted by Dr. E.M. Cunningham in this laboratory in the course of a routine chromatographic purification of the protein.

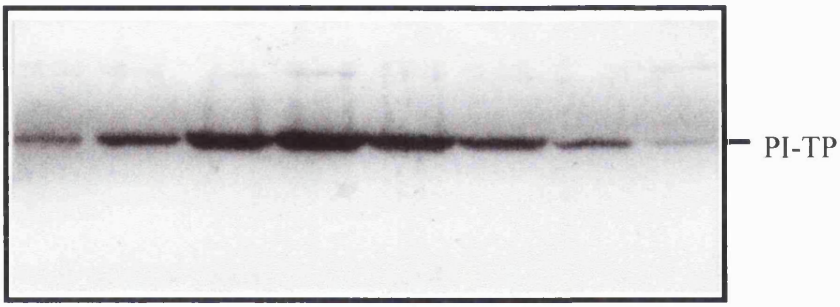
Fig. 4.1B shows the results obtained by probing a Western blot of 80µl of alternate column fractions diluted with 3xSDS PAGE sample buffer. Perfect alignment between the two sets of data in Figs. 1A, and 1B can be seen.

Fig. 4.1C. shows the results of probing an IEF gel of rat brain cytosol. The two loadings represent 50, and 25µl volumes of rat brain cytosol at approximately 15mg/ml. While at the higher loading both bands appear of approximately the same intensity, at the lower loading, the PI-loaded protein is visibly predominant. This discrepancy is probably a consequence of image saturation by the larger sample, leading to a non linear response.

A.



B.



Frac. no. 37 39 41 43 45 47 49 51

C.

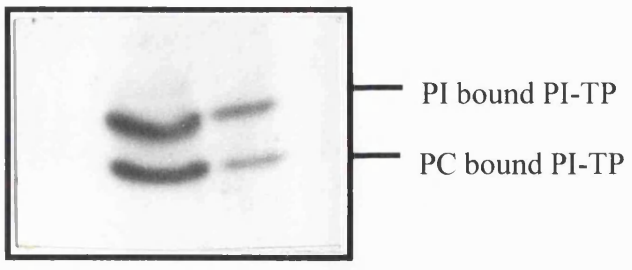


Fig 4.1

produce a single clear band at the appropriate molecular weight. The bacterial material produces a complex pattern of bands, presumably as a consequence of degradation products and the 'carry over' of specific antibodies against other bacterial proteins which may be present.

With the advent of recombinant PI-TP, this material was used to generate an affinity column by binding 5 μg of recombinant protein onto a 1ml NHS-activated sepharose column from Pharmacia, as described in Chapter 2.

The combination of the molecular weight of the resultant bands produced on Western blots of rat-brain cytosol and the coincidence of immunoreactivity with PI-transfer activity in chromatographic fractions confirmed that the antibodies were in fact directed against PI-TP. Further proof of this was obtained by examining rat brain cytosol resolved on isoelectric gels. It had previously been demonstrated in the literature that PI-TP from mammalian tissues, when resolved using isoelectric focusing produced two distinct bands which corresponded to PI and PC bound states, with the PI bound protein migrating to a more acidic region of the gel than the more basic PC bound form (1, 154, 156). Attempts were made to determine whether any factors could produce a shift in the pattern of lipid occupation observed in PI-TP released from HL60 cells. To this end preparations of HL60 cytosol were resolved on isoelectric gels in parallel with rat brain cytosol. However, the protein was not sufficiently strongly represented in the HL60 cytosol for detection with the antibody. Blotting of rat brain cytosol however produced a pattern of bands which appeared to correspond to the isoelectric points already reported for PI-TP (5.5 and 5.3). The results of blotting samples of rat brain cytosol probed with the anti-PI-TP antibodies is illustrated in Fig. 4.1C. 50 and 25μl samples of rat brain cytosol were loaded in parallel with preparations of HL60 cytosol. The HL60 cytosol failed to produce any signal, presumably in consequence of limited amounts. It was not possible to increase the loading of the gel, as the capacity of the IEF gels beyond which serious distortion of the bands occurred was low. The bands produced by resolving rat brain cytosol were regarded as a further proof of the specificity of the antibody produced. The results of probing a variety of cytosols with the affinity purified antibody are illustrated in Fig. 4.2.

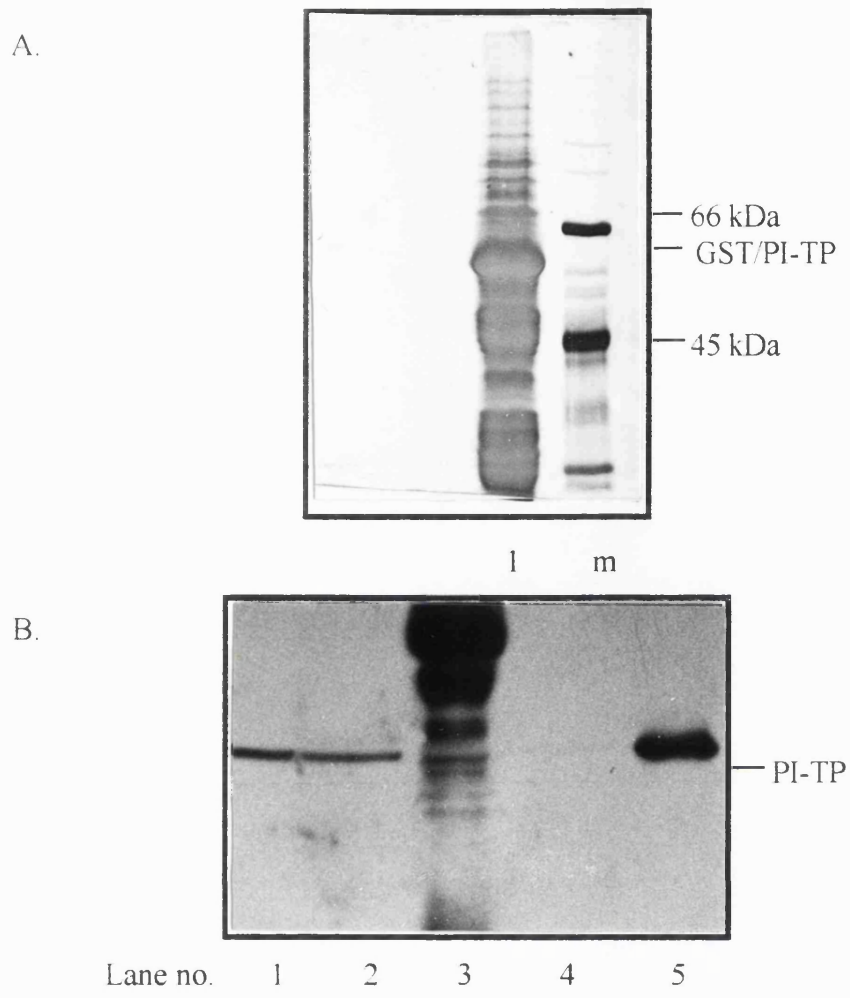


Fig 4.2 SDS PAGE resolution of the crude innoculum, and demonstration of affinity purified polyclonal antibody specificity.

Fig. 4.2 A shows a sample of the GST/PI-TP fusion protein resolved on a 10% PAGE gel. A small sample of the induced, pelleted bacteria was solubilised in SDS PAGE sample buffer by sonication, and heat. Insoluble material was removed by centrifugation. The GST/PI-TP fusion protein can be visualised as the predominant band at approximately 55 kDa in lane 1. This material was utilised as the immunogen, as well as ligand for the purification of antibodies. The lane designated m contains molecular weight standards.

Fig 4.2 B shows a Western blot performed on various cytosols. Lane 1, and 2 contain purified bovine, and rat brain PI-TP, lane 3 contains a sample of the original material used for immunisation (GST/PI-TP fusion protein), lane 4 contains HL60 cytosol, and lane 5 contains 5µl of rat brain cytosol.

4.5 Studies of protein release from permeabilised cells

The use of SLO permeabilised HL60 cells to study various reconstituting factors in both the PLC and PLD signalling pathways has been established in this lab and the process extensively characterised. Treatment of cells with the bacterial toxin SLO produces lesions in the cell membrane such that the soluble components of the cytosol are able to leak out, leaving behind membrane bound signalling components (199).

The above effect is illustrated by Fig. 4.3. Undifferentiated HL60 cells were cultured as described and permeabilised according to the protocol in Chapter 2. Both supernatant from the permeabilisation and the remaining cell pellet were then examined by SDS-PAGE. Fig. 4.3A. shows the accumulation of cytosolic proteins, which are free to diffuse from the cell, in the permeabilisation supernatant. The strong band at 66 kDa represents both SLO and BSA from the permeabilisation buffer which co-migrate, while the other strong band observed at 44 kDa probably represents actin. The changes in Fig. 4.3B are more subtle - while the cytosolic proteins are able to leave the cell, many membrane associated proteins are retained. Thus only relatively few bands can be seen to be diminishing with time of permeabilisation.

Various purified components can then be added back to the system to assess their ability to restore the process being examined. It was on this basis that PI-TP was initially established as playing a major role in the operation of the PLC system (2).

4.5.1 Release of the PLCs β 2 and 3 from permeabilised HL60 cells:

$50x10^6$ HL60 cells were prepared for permeabilisation according to the protocol described in the methods section. 500µl samples were removed at time points t=0, 1, 5, 10, 15, 20 and 30 minutes and the cells immediately pelleted by a pulse spin in a benchtop microfuge and the supernatant removed. This was then treated with 3x sample buffer and heated to 96°C for three minutes. The proteins in

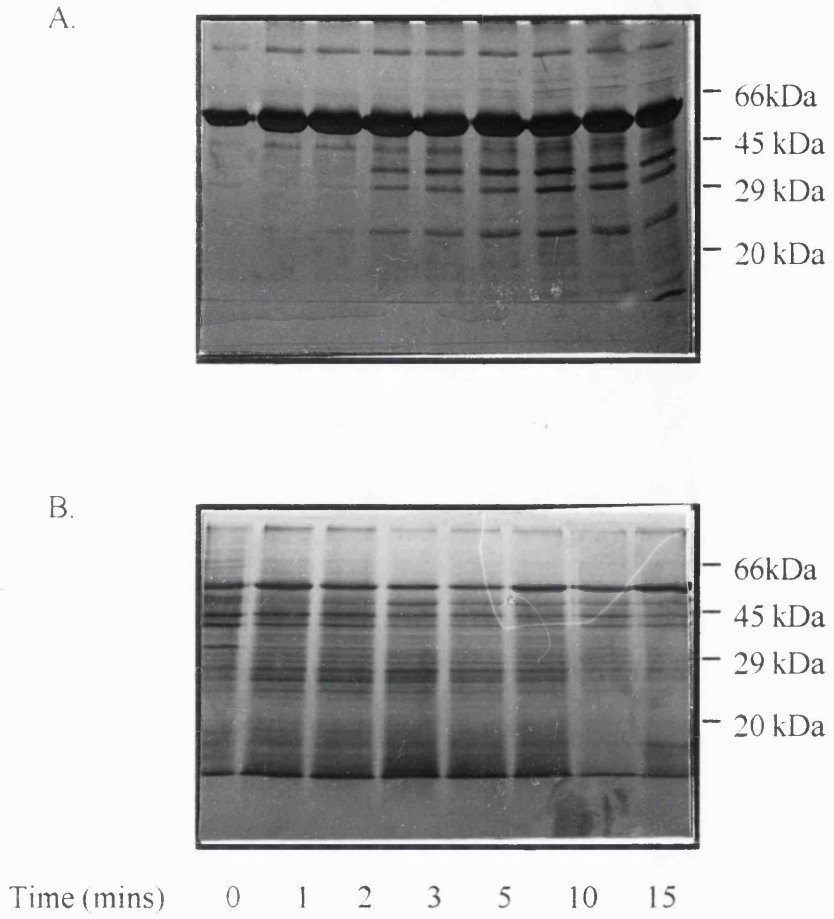


Fig 4.3 SDS PAGE examination of permeabilised HL60 cells.

$50x10^6$ cells were prepared as described, and permeabilised according to the standard protocol for PLC reconstitution assays. Samples of material were removed from the permeabilisation at time points as shown in the figure. Supernatant (Fig. 4.3A) was immediately treated with sample buffer and resolved. The cell pellets (Fig. 4.3 B) remaining after centrifugation was RIPA extracted on ice for 15 mins prior to resolution of the proteins on 12% SDS-PAGE gels. The gels were then silver stained using the protocol described in Chapter 2.

the sample were resolved by electrophoresis on an 8% polyacrylamide gel and Western blotted as described previously. The proteins were then transferred to Immobilon P by wet transfer and the blot probed with antibodies against PLC βs 2 and 3.

The results of these blots are shown in Fig. 4.4A. As can be seen, there is a gradual accumulation of both PLCβ-2 and 3 in the permeabilisation supernatant which continues to increase for at least 45 minutes. In the case of PLC β-2, the accumulation of the full length protein is accompanied by an increase in the amount of immunoreactive material at approximately 100 kDa which is believed to represent a proteolytic fragment of PLCβ-2. PLCβ-3 follows a similar pattern, but is only visible as a single band at approximately 150 kDa. The final lane in this series of blots, labelled m, represents the results of probing membranes prepared from HL60 cells prepared by sonication, low speed centrifugation to pellet nuclei and undisrupted cells and ultracentrifugation to pellet the membranes. The membranes were then washed twice in PIPES buffered saline solution. The resultant preparation of crude membranes was then dissolved in SDS-PAGE sample buffer and resolved in parallel with the supernatant from the permeabilisation. Full length PLC β2 can be seen as a faint band. Fig. 4.4B shows a 'composite' picture of the release of various activities from permeabilised cells, as determined using *in vitro* biochemical assays.

4.5.2 Release of PI-TP from permeabilised HL60 cells:

$50x10^6$ HL60 cells were prepared as above for permeabilisation. Samples were removed from the permeabilisation reaction at time points t=0, 15, 30, 45, 60, 120, 300 seconds. The cells were again pelleted and the supernatant retained for study, the constituent proteins being resolved on a 12% polyacrylamide gel before electrotransfer. Initial attempts to probe for PI-TP were unsuccessful as a result of the relatively low abundance of this protein in the preparation (Later studies showed that the predominant isoform of PI-TP present in HL60 cells is PI-TPβ. These antisera were raised against the only know isoform at the time, PI-TPα). Consequently the procedure was repeated and the proteins in the supernatant precipitated by adding trichloroacetic acid to a final concentration of 5%. After refrigeration at -20°C for 60 minutes the precipitated proteins were washed in 5% TCA and then resolubilised in

1M sodium hydroxide and neutralised by the addition of an equal volume of 1M Tris pH 9. After treatment with sample buffer the proteins were again resolved using similar conditions to those above.

The results of these investigations are illustrated in Fig. 4.4A. As can be seen, there is a rapid release of PI-TP from the permeabilised cell by comparison with the rate of loss of PLCβ-2 and 3, with maximal release of PI-TP apparently observed at approximately 5 minutes after permeabilisation commenced and no visible presence of the protein at t=0.

Attempts were also made to determine whether, as in the case of the phospholipases Cβ, PI-TP was retained within the cell. Initially this was performed by treating the residual cell pellet left from permeabilisation with SDS-PAGE sample buffer. This proved to be unsuccessful as a result of the large amount of nuclear material left behind and the consequently thick nature of the sample obtained precluding efficient resolution of the constituent proteins on an SDS PAGE gel despite attempts to shear contaminating DNA by repeated passage through a fine gauge needle and sonication.

Further attempts made use of RIPA* buffer treatment of the cell pellet to solubilise the proteins present in the residual material, followed by a similar TCA precipitation step to that already described. $50x10^6$ HL 60 cells were prepared as in preceding experiments and permeabilised according to the standard protocol. The supernatant and cell pellet separated by pulse centrifugation. The residual cell pellet was treated with 500µl of RIPA buffer for 15 minutes at 4°C prior to pelleting insoluble material at $10x10^3$g for 20 minutes. The RIPA extract was then TCA precipitated according to the same protocol as the permeabilisation supernatant and dissolved in 50µl of SDS PAGE sample buffer. Samples of the RIPA treated cell pellet and supernatant from the actual permeabilisation were then resolved and blotted in parallel and probed with the polyclonal rabbit anti-PI-TP antibodies. Using this method, the loss of protein from the cell appeared to be total and in the cell pellet at the end of a five minute permeabilisation period, no traces of PI-TP were detectable by Western blot analysis using the antibodies then available. The results of these experiments are illustrated in Fig. 4.5.

*RIPA buffer was constituted as follows: 150mM NaCl, 1% NP40, 0.5% Deoxycholate, 0.1% SDS, and 50mM PIPES pH 6.8.

Fig. 4.4 Investigation of the kinetics of PLC β, and PI-TP leakage from SLO permeabilised cells.

Fig. 4.4 A, show typical results from examining the kinetics of PLCβ, and PI-TP release using the immunoreagents already referred to. HL60 cells were prepared as described in Chapter 2. The cells were then permeabilised with the bacterial toxin SLO according to the standard protocol in Materials and Methods. During the time course of the permeabilisation, samples of permeabilisation mixture were removed, and the cells removed by pulse spinning them in a bench top microfuge. The resultant supernatant was retained, and resolved on SDS-PAGE gels. Each of the lanes represents the equivalent of $2.4x10^6$ HL60 cells. Both sets of data agree as to the time course with which PI-TP, and the PLCβ enzymes are released from the cell. The results support the premise that release of PI-TP from the permeabilised cell is the crucial factor in the rapid decay of GTPγS stimulated activity that is observed, since at 5-10 minutes, the release of PI-TP into the medium appears to be reaching equilibrium, with little further release being apparent. PLC β release is however still ongoing, with a visible rise in extracellular amounts of this protein being visible even after 30 minutes of permeabilisation.

Fig. 4.4B. shows the results of a series of biochemical investigations to determine the rate with which PLC activity, PI-TP, and lactate dehydrogenase activity leave the permeabilised HL60 cell. The open triangles represent the decay of GTPγS stimulated PLC signalling in the permeabilised cell. These data were furnished by Dr. E.M. Cunningham from this lab. Of particular interest is the close symmetry between the release of PI-transfer activity from the cell, and the decay in GTPγS stimulated PLC activity.

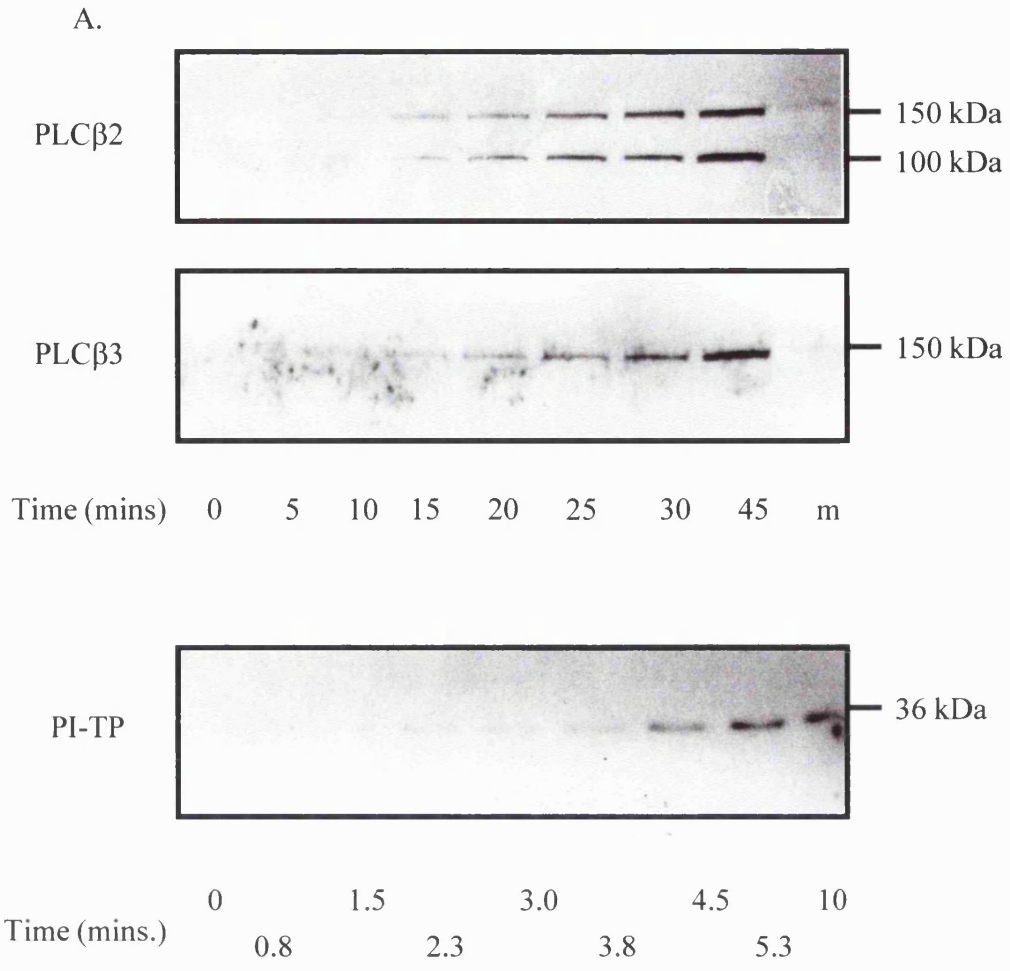

A.
PLCβ2
150 kDa
100 kDa
PLCβ3
150 kDa
Time (mins) 0 5 10 15 20 25 30 45 m
PI-TP
36 kDa
Time (mins.) 0 0.8 1.5 2.3 3.0 3.8 4.5 5.3 10
B.
LDH
PI–TP
PLC
% release
100
80
60
40
20
0
0 10 20 30 40 50 60
Time (mins.)

Fig. 4.4

Attempts were also made to assess the impact of stimulation of cells with GTPγS and the phorbol ester PMA. No significant differences were observed in the kinetics of leakage of PI-TP at this time. As a consequence of extensive handling and the necessity of TCA precipitation only poor data is available from these studies using the polyclonal antibodies and is not shown. This question is more comprehensively addressed in Chapter 7 using suitable immunoreagents to detect the predominant PI-TPβ isoform.

4.6 Immunoprecipitation of PI-TP using sera D, E, & F:

In an attempt to investigate other proteins with which PI-TP may be interacting and other factors influencing the activities of this protein including possible alterations in the phosphorylation state of the protein, various strategies were assessed for the immunoprecipitation of PI-TP using the three sera in the panel, either individually, or in combination. The strategies used include:

I. Immunoprecipitation using Protein A sepharose as capture phase
II. Immunoprecipitation using Protein G sepharose as capture phase.
III. Using Polyethylene glycol to selectively precipitate immune complexes

None of the above strategies yielded a reliable, unequivocal immunoprecipitation.

4.7 Quantification of PI-TP by ELISA:

Attempts to quantify PI-TP by establishing an ELISA system were unsuccessful as a consequence of the antisera, even after affinity purification, being unable to function as a capture phase when coated onto the plastic of ELISA plates. Biotinylated PI-TP was used to probe the antibody coated plates and binding of the biotinyl protein was measured using Extravidin-HRPO with OPD substrate. No

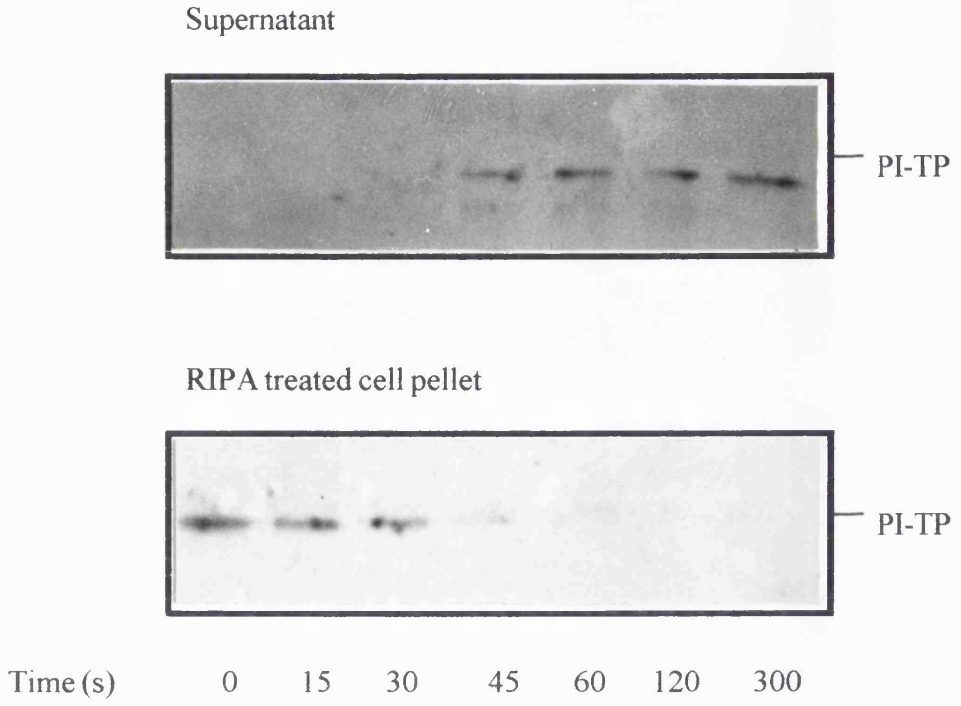


Fig 4.5 Examination of the retention of PI-TP in SLO permeablised HL60 cells

In order to investigate the loss of PI-TP from permeabilised HL60 cells further, HL60 cells were permeabilised according to the standard protocol, and both the supernatant, and the extracellular medium probed for the presence of PI-TP. Earlier attempts to carry out these investigations were hindered by the viscous nature of the cell sample when solubilised in SDS PAGE sample buffer. It was therefore decided to treat the permeabilised cell pellet with RIPA buffer, in order to strip away proteins bound to cellular membranes, while leaving the nuclear material as insoluble matter. Each lane corresponds to approximately $5x10^6$ HL60 cells.

significant capture was detected. The ELISA strategies considered are schematically represented in Chapter 7, Fig. 7.1.

4.8 Discussion:

Studies of the kinetics with which the phospholipase C β isozymes leak from the permeabilised cell reveal that by comparison with the similarly sized lactate dehydrogenase, a truly cytosolic protein, PLC leakage is considerably retarded, with the process only being completed after at least 45 minutes from the start of permeabilisation. This observation is a confirmation of results made using biochemical methods in a variety of different cell types (200, 201). Even at the end of this period it appears likely that some at least of the PLCβ present in the cell remains associated either with the cell membrane itself, or as suggested by the work of C.P. Downes (16), the underlying cytoskeleton. The ability to reconstitute PLC activity without the addition of exogenous protein, all be it at reduced levels, in extensively permeabilised cells also supports the premise that some of the activity remains in the cell and is dependent upon the maintenance of some degree of structural integrity - purified membranes from disrupted cells produce an extremely poor PLC response to GTPγS stimulation in comparison to SLO permeabilised cells.

Examination of PI-TP and the kinetics with which it leaves the cell reveals a somewhat more rapid process. This effect is illustrated in Fig. 4.4. Fig. 4.4 B illustrates results obtained from earlier work conducted by Dr. E.M. Cunningham in this laboratory using biochemical assays to determine the release of PI-TP, PLC activity and LDH release from permeabilised HL60 cells. These experiments were conducted in parallel with stimulation of cells with GTPγS to determine the rate at which PLC activity decays in the stimulated cell. As can be seen, PI-transfer activity accumulated in the supernatant quite rapidly, with 80% release having occurred within 10 minutes of permeabilisation. This process is closely paralleled by the loss of LDH from the cell. It is particularly worthy of note that the responsiveness of PLC in the permeabilised cell to GTPγS decays over a very similar time course. These biochemical observations are confirmed by investigating permeabilisation using

immunological methods. In Fig. 4.4 A the kinetics of PLC leakage from permeabilised cells follows very closely the kinetics demonstrated by in vitro assays for the individual activities. This supports the model in which PI-TP plays an important role in the function of this signalling pathway in intact cells (2).

Attempts to study the extent to which the protein leaked from the cell by Western blotting supernatant and residual cell pellets from permeabilisation experiments revealed that to all intents and purposes, PI-TP completely leak from the cell within a period of approximately five minutes.

Recent work has identified the existence of another isoform of PI-TP within mammalian cells with very high degree of conservation, designated as PI-TPβ (146). The sequences of α and β PI-TP can be found in Chapter I, Fig 1.5. Immunoreagents have been raised against the α isoform and a panspecific antibody has also been raised in the lab of K. Wirtz and have been used to study the subcellular localisation of PI-TP in Swiss 3T3 fibroblast cells (168, 202-204). In this case, the differential retention of detectable levels of the two proteins in various membrane compartments including the nucleus and Golgi has been demonstrated even after extensive permeabilisation. The apparent discrepancy between these observations and Western blot analysis may be explained as a result of the different methodologies used, or as a function of the cells used in the two different studies. It should also be remembered that the reagents used in these experiments were raised against the PI-TPα and that while considerable homology exists between the two proteins, the antibodies raised against PI-TPα will not be completely cross reactive with the other isoform. Thus it is conceivable that this reagent will yield results skewed in favour of the α-isoform and may misrepresent, or even fail to detect the presence of the β isoform.

Chapter 5

PI-TPα is phosphorylated upon stimulation with PMA or fMLP :

5.1 Introduction:

Because of the failure of the rabbit polyclonal antibodies raised against PI-TPα to immunoprecipitate and to ensure a continuing supply of immunoreagents it was decided to raise further antibodies. As a result of the relative ease with which the protein could be partially purified using an alternative, shorter protocol and the small amounts of material necessary for the immunisations it was decided to attempt to raise a panel of mouse monoclonal antibodies, rather than peptide antibodies raised in rabbits. With this in mind, a simplified purification protocol for PI-TP was arrived at which offered the additional advantage of producing a mixture of both PI-TP and the small G-protein Arf, with the possibility of producing reagents specific for both of these interesting proteins.

5.2 Raising, characterisation and application of mouse monoclonal antibodies against the mammalian phosphatidylinositol transfer protein α

5.2.1 Preparation of antigen for immunisations:

Material for use in a series of primary and boost inoculations was prepared by partial chromatographic purification of PI-TP from bovine brain according to the following protocol.

Bovine brain tissue was homogenised using a Phillips food blender in 20mM Tris, 3mM KCl pH 7.4, containing EDTA, EGTA, DTT, and the protease inhibitors approtinin, pepstatin, benzamidine, TLCK, and DFP. The material was centrifuged at 10,000 rpm in a Sorvall GS-3 rotor, at 4° C overnight. The clarified supernatant was removed and the residual pellet frozen for future use. The supernatant was then subject to Ammonium sulphate precipitation. Proteins precipitating between 40 and 60% saturation were retained and pelleted by centrifugation at 10,000g for one hour. The resultant pellet was then solubilised in 50 ml of homogenisation buffer, and dialysed overnight against the same in order to

remove residual Ammonium sulphate. Once the conductivity of the solution had dropped below the equivalent of 25mM NaCl the proteins were further resolved by DE-52 weak anion exchange chromatography.

The material was loaded onto a 200ml DE52 packed column equilibrated with 20mM Tris, 3mM KCl, 0.02% NaN3. It was then washed with 3 column volumes at a rate of 2 ml/min. A NaCl gradient was then applied from 0.0 to 1.0 M and 71, 8ml fractions collected. Alternate fractions were assayed for phosphatidylinositol transfer activity, both neat and diluted ten fold. Active fractions were pooled and concentrated down to a total of 5ml prior to loading onto a Superdex 75 gel filtration column.

The column was equilibrated with 20mM PIPES, 3mM KCl, 0.02%NaN3, pH 6.8 and the 5ml sample, which was first filtered through a 0.22μm filter, was loaded onto the column, which ran at a rate of 1.5ml/min. After an initial wait of 105 minutes the fraction collector was turned on and collected 1.33ml samples. After a total run time of three hours the chromatography was stopped and alternate fractions assayed for PI-transfer activity. Those found to be positive were pooled and concentrated. This material was then assayed for protein using the Bradford assay and run out on a gel to asses the purity of the preparation. The end result was 400μl of material with a concentration of 240μg/ml total protein. By Coomassie staining of the gel approximately 2/3 of the protein was PI-TP, and the remainder being predominantlyArf (G.M.H.Thomas, oral communication). This material formed the basis of a series of primary, and boost immunisations in a cohort of three balb/c mice.

The process of purification is followed in Fig. 5.1. Graph 5.1 A shows the PI-transfer profile of fractions eluting from the DE-52 column, while Graph 5.1.B shows a similar profile for the Superdex 75 column. Samples of the fractions from the column were assayed for PI-transfer activity as described in Chapter 2. The samples were assayed both neat and diluted 1:5. Photograph 5.1 C shows a Coomassie stained gel of the material at various stages of purification. PI-TP and Arf are arrowed.

Fig. 5.1 Purification of PI-TP from bovine brain tissue for use in immunisations.

Fig 5.1A and B illustrate the results of PI-transfer assays conducted on column fractions of chromatographed, ammonium sulphate cut bovine brain cytosol.

Fig. 5.1A. shows the result of DE52 chromatography, as described in the main text. The column fractions were assayed both neat, and diluted 1:5.

Fig. 5.1B shows the result of a similarly conducted PI-transfer assay on column from gel filtration chromatography.

Fig. 5.1C shows a Coomassie blue stained gel of the material during the process of purification:-

Lane 1	Molecular weight markers
Lane 2	Ammonium sulphate precipitated material
Lane 3	Post DE-52
Lane 4	Post gel filtration

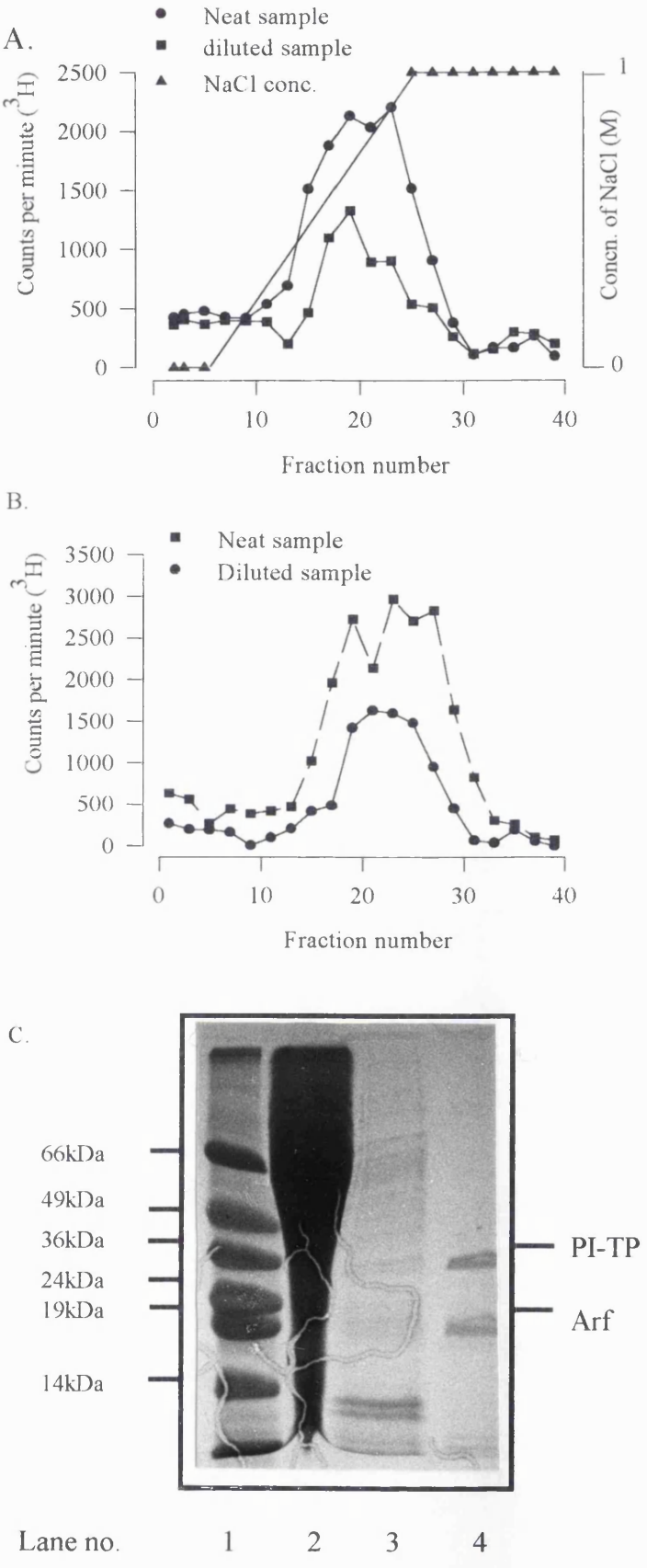

A.
Neat sample
diluted sample
NaCl conc.
Counts per minute (^{3}H)
Concn. of NaCl (M)
Fraction number
B.
Neat sample
Diluted sample
Counts per minute (^{3}H)
Fraction number
C.
66kDa
49kDa
36kDa
24kDa
19kDa
14kDa
PI-TP
Arf
Lane no. 1 2 3 4

Fig 5.1

5.2.2 Development of ELISA for detection of antibodies against PI-TP

In order to detect the presence of antibodies in the sera and later tissue culture supernatants, a convenient and rapid colourimetric method was developed. Previous attempts to develop an Enzyme Linked Immunosorbent Assay (ELISA) to quantify PI-TP based on the polyclonal serum failed when this proved to be impractical as a capture phase in the assay. As a result it was decided to base the assay on PI-TP directly bound to the surface of a 96 well microassay plate despite the increased risk that the protein may undergo denaturation upon binding to the plastic. Initially the polyclonal sera were used for the establishment of the assay for anti-PI-TP activity. The accompanying diagram, Fig 5.2 schematically represents the ELISA strategy. This assay differs from the earlier attempts to utilise the polyclonal antibodies as a capture phase for a quantitative assay, as in this case, PI-TP itself is used as a capture phase for anti-PI-TP antibodies in the supernatant.

5.2.3 Inoculation and screening for antibodies:

Material was purified from bovine brain according to the protocol described above for the preparation of PI-TP and Arf using weak anion exchange (DE52) chromatography and gel filtration. This material when examined by SDS-PAGE and stained using Coomassie R250 was estimated to comprise approximately 60% PI-TP and 30% Arf. The purified proteins were used for a series of primary and boost immunisations for a cohort of three Balc/c mice.

In order to determine that the animals had mounted an immune response against the antigen an ELISA was developed to detect anti-PI-TP antibodies. Purified PI-TP was diluted to a concentration of 2μg/ml in 10mM Na_2HPO_4, pH 7. 100μl of this material was coated onto ELISA plates (Gibco BRL Immunoplates) overnight at 4°C. The antigen solution was then removed from the wells and the plates washed using PBS -Tween 20 (0.02%) pH 7.4. The protein binding sites of the plate were then blocked by incubation with a 2.5% solution of non-fat milk protein in

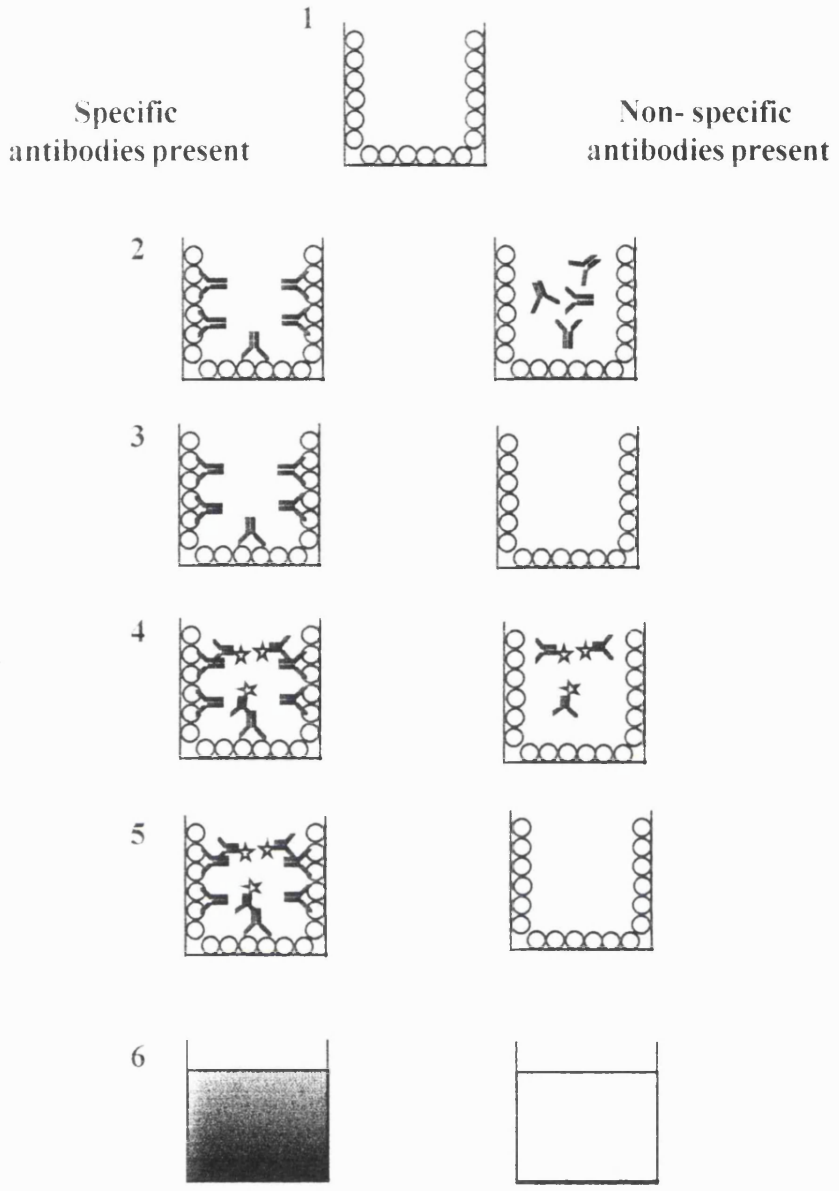


Fig 5.2 Schematic diagram of ELISA strategy to detect PI-TP specific antibodies in sera, and hybridoma culture supernatants.

1. Wells of 96 well plate coated with antigen (PI-TP)
2. Incubated with serum/culture supernatant under study. Specific antibodies bind to antigen.
3. Plate washed with PBS-Tween 20. Specific antibodies remain bound to PI-TP capture phase.
4. Plate incubated with HRPO labelled goat anti-mouse IgG.
5. Plate washed - HRPO conjugate remains associated with bound mouse antibodies.
6. Presence of bound conjugate detected by colour change using OPD substrate.

PBS-Tween for one hour at room temperature. The plate was then washed free of the blocking solution.

The initial screen of the mouse serum for antibodies utilised small quantities of material produced from tail bleeds. Because of this the material was assayed in duplicate doubling dilutions from 1:10 to 1:1280. The plates were incubated with the serum dilutions at 37°C for one hour. After this incubation the samples were carefully removed from the wells by aspiration and the wells washed three times with PBS-Tween 20 before being probed for the presence of bound mouse immunoglobulins using a $1:30x10^3$ dilution of a goat, anti-mouse IgG coupled to Horse Radish Peroxidase (Sigma Immunochemicals). This incubation was for 30 minutes at 37°C. The plates were washed particularly rigorously at the end of this incubation, as contamination of the wells with conjugate would have led to a false positive signal. The final step in the assay was colourimetric development using o-phenylenediamine hydrochloride substrate (Sigma Immunochemicals) at 1mg/ml in 0.1M citrate buffer, pH 5 with the addition of 0.003% H_2O_2. On the basis of this assay, two mice were selected and sacrificed - splenocytes from one of the animals were used for the fusion, while material from the other animal was retained for possible future use. The results of this assay are illustrated in Fig 5.3. An essentially unmodified version of this assay was then subsequently used to screen the resultant hybridomas for the production of relevant antibodies.

5.2.4 Fusion, growth and screening of the hybridomas:

When an appropriate level of response was detected the animals were sacrificed and the spleen aseptically dissected from the carcass, placed in a sterile Petri dish with 2ml of RPMI and teased apart using sterile forceps. This material was then layered onto the top of a further 20ml of RPMI and clumps of cells allowed to settle out of suspension. The remaining cell suspension was then transferred to a sterile Universal tube and the cells gently pelleted at 1000rpm in a bench top centrifuge. The supernatant was aspirated from the pellet, which was then gently tapped loose and treated with 1ml of red blood cell lysis buffer and gently mixed in the pipette. The cells were then added to 20ml of RPMI and washed. The resultant

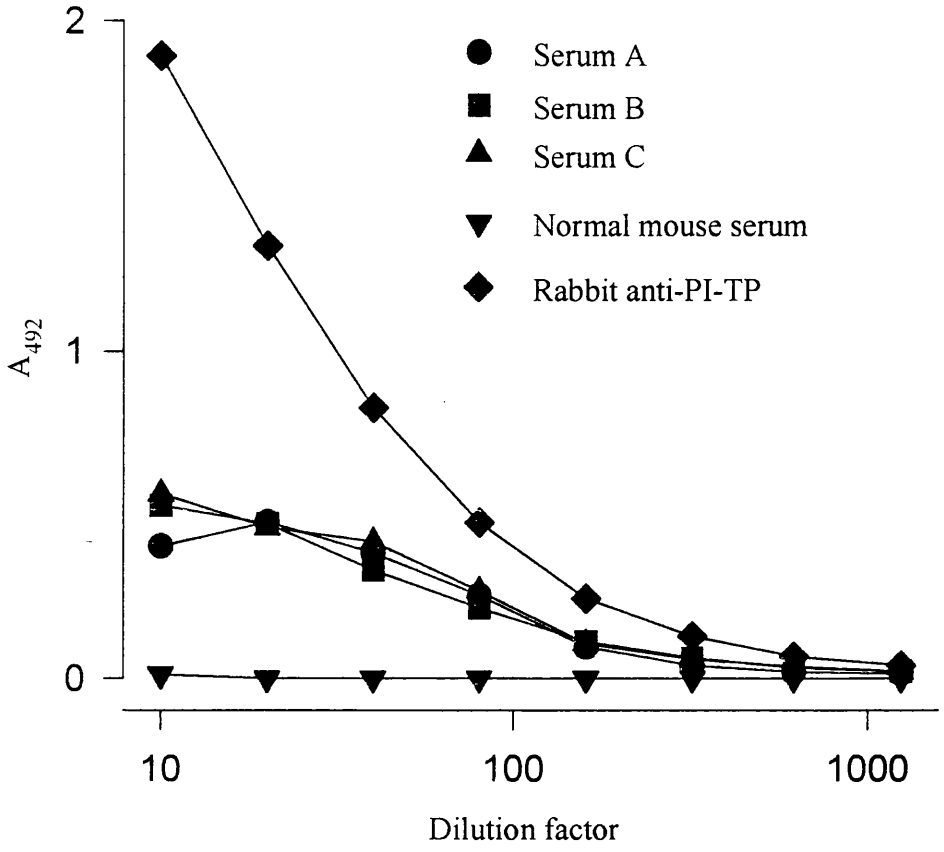


Fig 5.3 Screening for the presence of PI-TP specific antibodies in the sera of immunised animals prior to use in fusion to produce antibody secreting hybridomas.

This graph shows the results from an ELISA to determine the relative responses of the mice to the injected antigen. Material for assay was prepared from blood obtained by tail bleeds of the immunised mice. The resultant sera were then diluted as illustrated in the figure above, and assayed in an ELISA utilising immobilised PI-TP as the capture phase for the antibodies. The binding of antibodies was detected using a goat anti-mouse-HRPO conjugate, and OPD substrate. On the basis of these data two animals were selected at random, and sacrificed, one for fusion, and the other for possible later use. Polyclonal rabbit anti-PI-TP antibody was included as a positive control.

cell pellet was again gently tapped loose and resuspended in 10ml of RPMI - any clumps of cells were removed. The cells were counted and left to stand until required.

The JK fusion partner cells were counted and the necessary volume to give a 1:4 ratio of JK:splenocytes calculated. The cells were then taken and pelleted by gentle centrifugation and washed in 20ml of RPMI, after which they were resuspended in 10 ml of RPMI and transferred to the splenocyte tube and all the cells present pelleted at 1000 rpm for 10 minutes. The pellet was loosened and 1ml of a 50% w/v solution of PEG (mw 3000-3700) was added dropwise with gentle shaking over one minute, prior to the addition of 1ml of RPMI in similar fashion. A further 20 ml of RPMI was added, again with gentle agitation over a period of four minutes. The tube containing the cells was then inverted three time and the cells pelleted at 800 rpm for eight minutes. The supernatant was aspirated, the cell pellet resuspended in RPMI supplemented with 10% foetal calf serum, L-Glutamine and Penicillin/Streptomycin, and the whole incubated at 37 °C for three to four hours.

At the end of the fusion procedure the cells were then plated out onto 96 well sterile tissue culture plates, which had previously been seeded with a feeder layer of MRC-5 cells. After being allowed to grow for 4 days in HAT supplemented medium 100µl of medium was removed from the wells and replaced. After 7 days the supernatants from the plated were screened for appropriate antibodies as described for the mouse sera. These procedures were conducted by the staff of the Monoclonal Antibody Unit at University College London, Messrs. Terry Jowett and Alan O'Shea

Positive cultures were then subject to two round of cloning by limiting dilution to provide pure hybridomas and expanded sufficiently to form the basis of frozen stocks. Three clones of each hybridoma were selected on the basis of the titre achieved and frozen down in FCS supplemented with 8% tissue culture grade DMSO (from Sigma).

5.3.1 Characterisation of the monoclonal antibodies:

Cloned hybridomas were cultured according to the method described in Chapter 2. The isotype of the individual monoclonal antibodies was determined using a kit from Sigma. The results showed that mAbs 5F12 and 3A7 are IgG1, mAb 5B2 is an IgG2 and 1A4 is an IgM. Further to this, samples of tissue culture supernatant

from the growing hybridomas was used to probe Western blots of a standardised rat brain cytosol preparation. 66µl of rat brain cytosol was treated with 33µl of three times sample buffer and heat treated at 96°C. This material was then loaded onto a single well, 1mm thick, 12% polyacrylamide-SDS gel and electrophoresed under reducing conditions. The resolved proteins were then transferred onto Immobilon P (PVDF) from Millipore, by overnight blotting at 30V. The blot was then blocked as usual, divided into equal strips and incubated with 10 fold dilutions of the supernatants, with the rabbit polyclonal antibody against PI-TP already described and an irrelevant antibody raised against PLC γ1. The strips were then washed and probed for bound antibody using Enhanced Chemiluminescence reagents from Amersham International and developed using Hyperfilm ECL from the above company. Using this method it was determined that mAbs 5F12 and 1A4 were useful in Western blotting (Fig 5.4).

5.3.2 Further characterisation of the mAb 5F12

This antibody was of particular interest because of possible applications in Western blotting and the ease with which it could be purified from tissue culture supernatants by virtue of being IgG1a. It was noted however that the antibody failed to detect the presence of a C-terminal His tagged recombinant PI-TPα that was recognised by the polyclonal antibodies prepared against a PI-TP-GST fusion protein and confirmed to be present by in vitro PI-transfer assay. The alternatively N-tagged r-PI-TP was however recognised by this monoclonal antibody and hence the recognised epitope was ascribed to the carboxy terminal. The results of these investigations are illustrated in Fig. 5.5. 50 and 25µl volumes of C, or N-terminal tagged rPI-TPα at 1µg/ml were loaded in parallel with samples of N-terminal tagged PI-TPβ at similar dilution, resolved by SDS PAGE, Western blotted and probed with mAb 5F12. As can be seen, only the N-terminally tagged protein and rat brain cytosol which was included as a positive control produced a signal.

Because the antibody 5F12 was effective both in the ELISA and in Western blots it was decided that the epitope recognised by the antibody was probably a

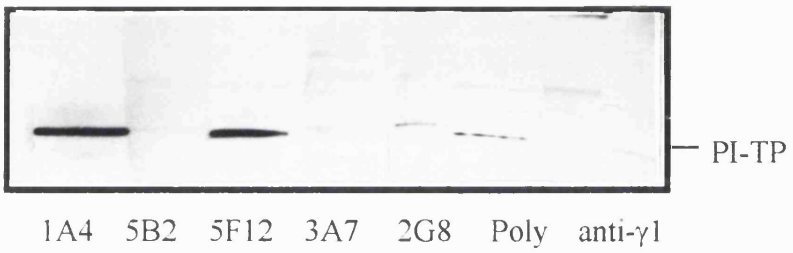


Fig 5.4 Testing of monoclonal antibodies against rat brain cytosol.

66μl of rat brain cytosol was loaded onto a single well, 1mm thick, SDS PAGE gel. This was then blotted onto PVDF membrane, and probed with supernatants from growing hybridoma cultures, diluted 1:10. Bound immunoglobulin was detected using an HRPO conjugated goat-anti-mouse antibody, and the signal developed using ECL reagents. Polyclonal rabbit anti-PI-TP antibodies were included as a positive control, and a monoclonal antibody against PLC γ1 was included as a negative control.

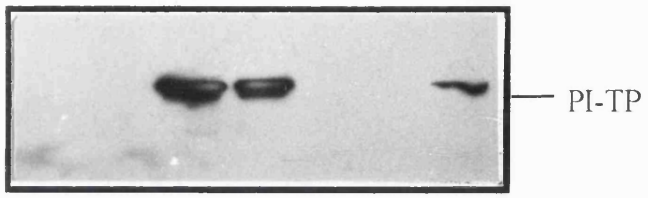


Fig. 5.5 Recognition of C, and N-terminally hexahistidine tagged rPI-TP α and β by mAb 5F12.

50, and 25μl samples of His-tagged recombinant proteins at 1μg/ml were loaded onto a 12% gel, as described in the fig. The gel was then Western blotted onto PVDF membrane, and probed with the monoclonal antibody 5F12. As can be seen, only the N-terminally tagged PI-TPα is recognised by monoclonal antibody. This implies that the antibody is specific for the carboxy terminal of PI-TP, and that the epitope is disrupted by the presence of the hexahistidine tag. 10μl of rat brain cytosol was included as a positive control, denoted by rbc.

primary structural feature, not degraded by the hostile conditions invoked in SDS PAGE.

In collaboration with Shuntaro Hara in this lab, the immunoreactivity of the antibody was assessed against a panel of C-terminal truncation mutants, possessing 5,10 and 20 amino acid deletions from the C-terminus of the α form of PI-TP. Removal of the first five carboxy terminal amino acids completely destroyed antibody binding. The results of this study are shown in Fig. 5.6.

It was decided to further test the conclusion that the antibody was specific for PI-TPα by performing an ELISA using the two recombinant proteins PI-TPα and β. The results are illustrated in Fig 5.7. On the basis of this data it was concluded that the entire panel of antibodies, including 5F12 was specific for the α-form of the protein.

5.3.3 Identification of mAb 5F12 as an immunoprecipitating antibody

The ability of the antibodies to immunoprecipitate PI-TP was first assessed using rat brain cytosol, known to be a particularly rich source of the protein. Initial attempts at immunoprecipitation were performed using a preparation of rat brain cytosol diluted approximately 7 fold to a final total protein concentration 3mg/ml in 20mM PIPES, pH 6.8. Initial attempts to produce significant amounts of the mAbs met with varying degrees of success. As a result of this, the monoclonal antibody designated as 5F12 was the first of the antibodies to be assessed.

Initially purified antibody was added to the diluted rat brain cytosol and incubated at 4^0C for a period of either one hour, or overnight prior to the addition of the solid matrix which was a 50% v/v slurry of Protein-G, or Protein-A beads. This was then incubated for one hour at 4^0C before being subject to five rounds of washing in PBS, pH 7.4. The beads were then pelleted and prepared for electrophoresis by heating to 96^0C in SDS-PAGE sample buffer for three minutes. Bound proteins were resolved on a 12% PAGE gel and blotted onto Immobilon P as described in the methods section. The blots were then probed with either polyclonal rabbit antibodies

Fig. 5.6 Determination of the cross reactivity of monoclonal antibody 5F12 with N, and C-hexahistidine tagged rPI-TPα, substitution mutants of PI-TPα T267A, T267V, deletion mutants of PI-TPα Δ5 – 20, and rPI-TPβ.

Fig. 5.6 A shows the result of SDS-PAGE resolution of wild type, substitution mutants, and truncation mutants of PI-TPα, as well as wt PI-TPβ. Samples were diluted to a total concentration of 1μg/ml in SDS PAGE sample buffer, and 50μl of this material was resolved on a 12% gel, and Coomassie stained. Lanes contain the following samples:-

Lane 1	rPI-TPα wt (N-tagged)
Lane 2	Substitution mutant T267A
Lane 3	Substitution mutant T267V
Lane 4	Deletion mutant Δ5
Lane 5	Deletion mutant Δ10
Lane 6	Deletion mutant Δ20
Lane 7	rPI-TPβ wt (N-tagged)

Fig. 5.6 B shows a similar set of samples to Fig. 5.6 A probed with the affinity purified polyclonal antibody described in Chapter 4. This reagent is panspecific, recognising all of the PI-TPα mutants, as well as the β isoform. The intense signal visible in lane 4 is a consequence of overloading of the gel, and does not reflect a greater affinity of the antibody for this PI-TP mutant (PI-TPα Δ5).

Fig. 5.6 C indicates that the epitope recognised by the antibody 5F12 lies within the C-terminus pentapeptide. Removal of the first five N-terminal amino acids from the protein prevents recognition by the antibody. The specificity of the antibody for the α isoform of the protein is also illustrated by the failure of the antibody to recognise N-terminally tagged PI-TPβ.

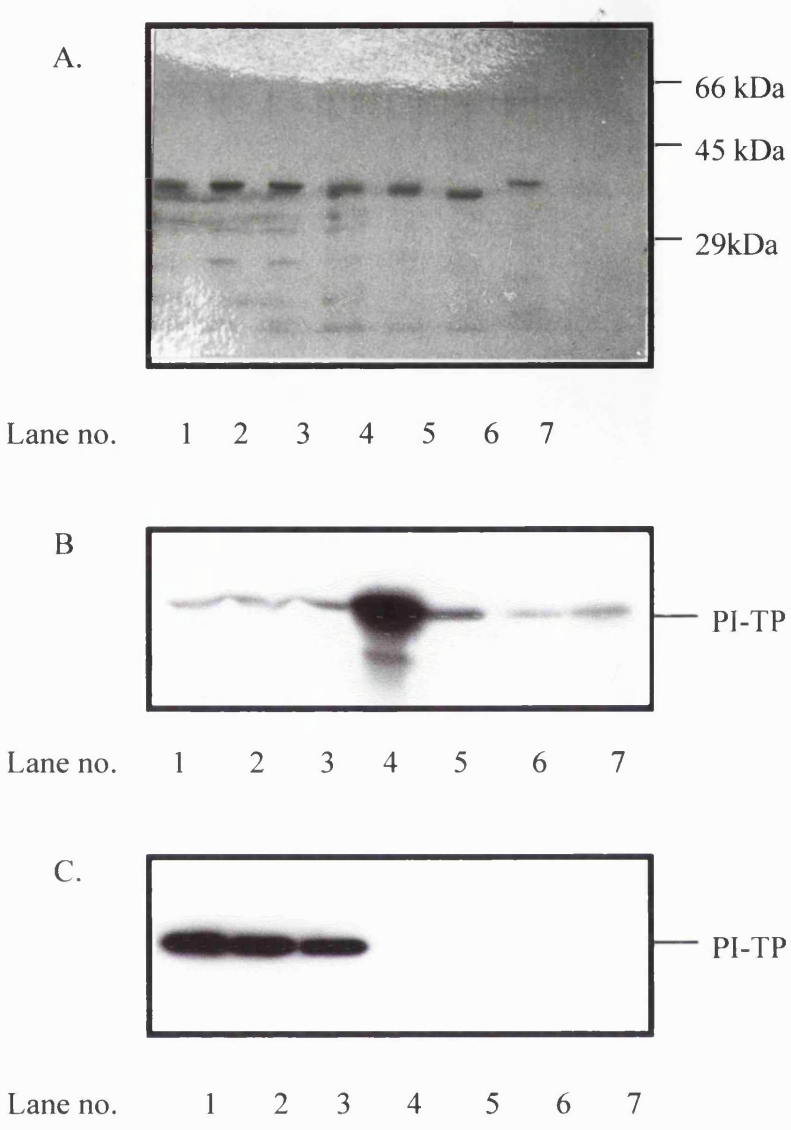

A.
66 kDa
45 kDa
29kDa
Lane no. 1 2 3 4 5 6 7
B
PI-TP
Lane no. 1 2 3 4 5 6 7
C.
PI-TP
Lane no. 1 2 3 4 5 6 7

Fig. 5.6

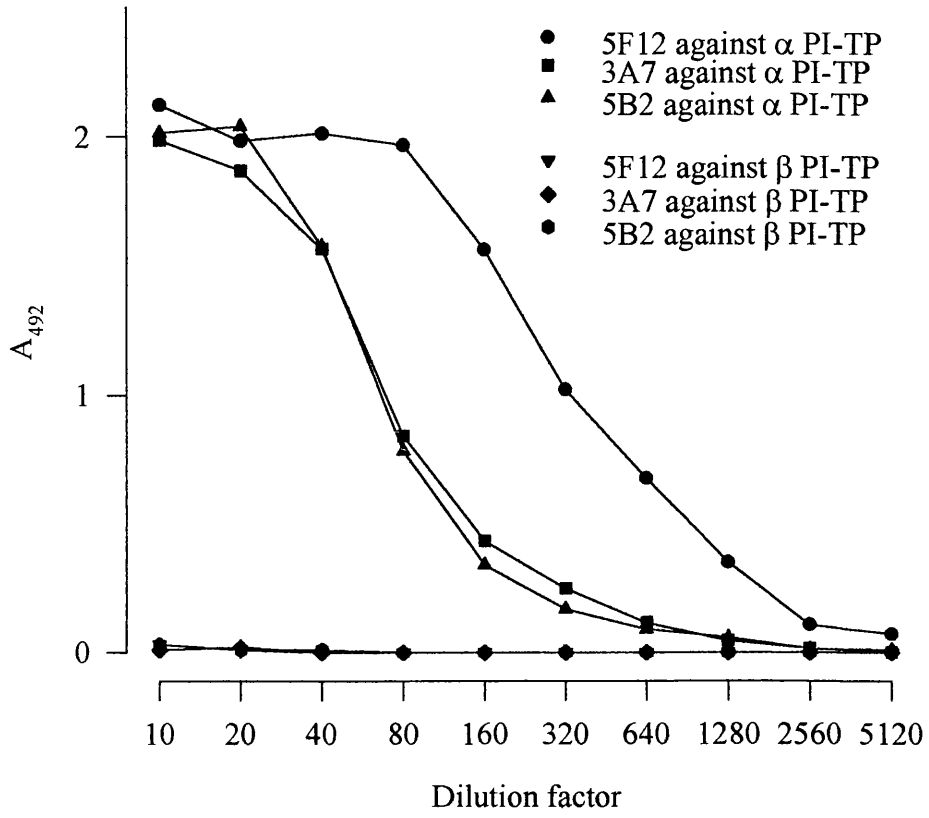


Fig 5.7 Determination of specificity of panel of monoclonal antibodies using recombinant, N-terminally hexahistidine tagged PI-TP α, and β in an ELISA system.

Individual ELISA plates were coated with each r-PI-TP at 2mg/ml in 10mM NaH_2PO_4 pH 7.0. The plates were then blocked with 2.5% milk protein, and probed with dilutions of hybridoma culture supernatant, as detailed in the figure. The signal was developed using an HRPO conjugated goat-anti-mouse antibody with OPD substrate. The plate was then read at 492nm.

against PI-TP, or with the monoclonal antibody 5F12. Initial experiments using this protocol were unsuccessful.

The above experiments were duplicated, but with the addition of 0.5% SDS to the system. Under these conditions it was found that the antibody was very strongly immunoprecipitating. The accompanying figure illustrates the effect of SDS on the efficiency of immunoprecipitation (Fig 5.8). The figure illustrates that concentrations of SDS as low as 0.01% were sufficient to produce a strong immunoprecipitation using diluted rat brain cytosol as the source of PI-TP.

The original goal of these experiments was to determine conditions under which the antibody might be used to co-immunoprecipitate other proteins with which PI-TP may interact. In view of the strongly denaturing nature of the added detergent and the consequent damage to active complexes, it was deemed desirable to add as little of this as possible. As a compromise between the need for low as possible concentrations of SDS to avoid damage to protein/lipid complexes and the minimum requirement for SDS to produce a strong immunoprecipitation, a concentration of 0.02% SDS was chosen.

5.3.4 Direct coupling of antibody to CNBr activated Sepharose versus Protein G as adsorbent

Attempts to immunoprecipitate PI-TP from disrupted HL60 cells yielded signals on Western blots which were not a strong as originally anticipated from the efficiency with which the antibody was able to immunoprecipitate the protein from rat, or bovine brain material. It was suggested that the immunoprecipitation protocol could be modified to make it more efficient and to this end, as an alternative to protein-G sepharose as the adsorbent, cyanogen bromide activated sepharose was used to covalently link the antibody to the beads. A variety of lysis buffers were also assessed for their efficiency in liberating the antigen and permitting efficient immunoprecipitation. The results of these investigations are shown in Fig. 5.9. The composition of the lysis buffers investigated were as follows:-

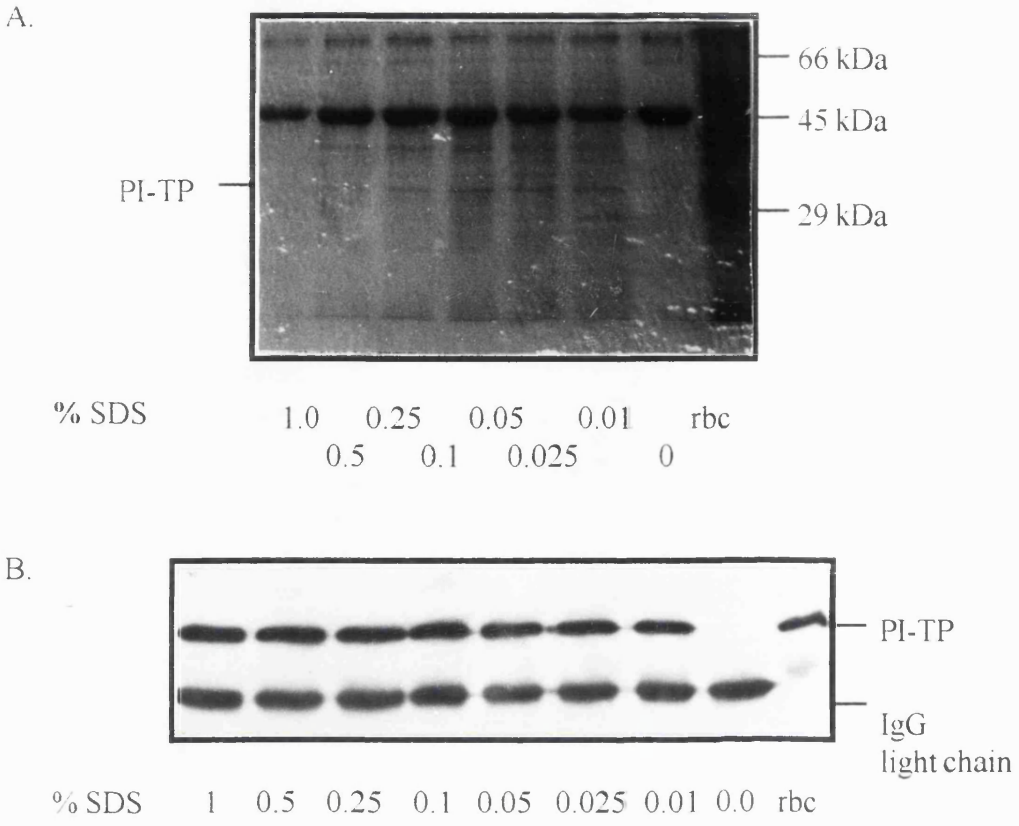


Fig. 5.8 Immunoprecipitation of rat brain PI-TPα using monoclonal antibody 5F12. Titration of SDS into the system.

Fig 5.8 A shows a 12% gel of material immunoprecipitated from rat brain material using mAb 5F12. The initial sample was diluted to 3mg/ml total protein. The material was then divided into 500µl aliquots, and incubated overnight with 50µl of purified antibody at 1mg/ml. 50µl of a 50% suspension of Protein-G spharose was then added, and incubated with gentle agitation for 1 hour at 4°C. The beads were then washed three times in PBS, pH 7.4, before resolution by SDS-PAGE.

Fig 5.8 B shows a Western blot of the same samples, probed with 5F12, and developed using the in house ECL system. As can be seen, the antibody immunoprecipitates effectively at SDS concentration as low as 0.01%. There is however an absolute requirement for SDS in the system, as can be seen from the negative result obtained in the absence of SDS.

Fig. 5.9 Optimisation of detergent disruption system, and immunoprecipitation protocol using PI-TPα specific monoclonal antibody 5F12.

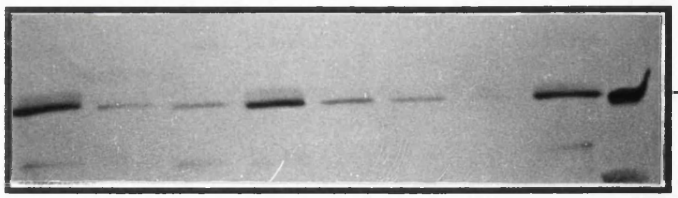
Fig 5.9A. shows the results of immunoprecipitation of HL60 cells disrupted using a variety of detergent systems. The samples were immunoprecipitated using either free antibody, subsequently adsorbed using Protein G sepharose, or monoclonal antibody covalently coupled to CNBr activated sepharose. 0.02% SDS was added to the cell lysate after centrifugation at $10x10^3g$ for 15 minutes to remove insoluble material.

Lane no.	Low salt	NP40	High salt	RIPA buffer	ProtG/CNBr
1	+				CNBr
2	+				ProtG
3			+		CNBr
4		+			CNBr
5		+			ProtG
6				+	ProtG
7			+		ProtG
8				+	CNBr

Lane 9 represents 10µl of rat brain cytosol included as a positive control.

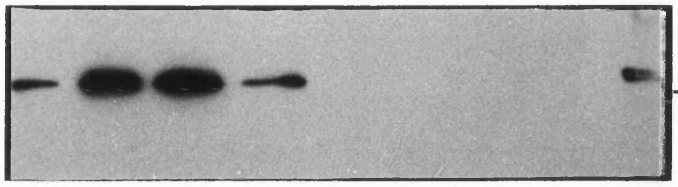
Fig. 5.9 B. shows the result of immunoprecipitating 500µl rat brain cytosol diluted to 3mg/ml with PBS pH 7.4 using different volumes of a 50% suspension of the 5F12 coupled sepharose beads, in the presence, and absence of 0.02% SDS. Again, the dependence of the immunoprecipitating activity on the presence of this detergent is demonstrated. On the basis of these results it was decide that the optimal system for immunoprecipitating PI-TP from disrupted cells was to use a low salt, NP40 based lysis buffer, restricting the volume of 50% v/v 5F12 beads to 25µl to produce maximal precipitation, and minimise non-specific binding. CNBr activated beads were also coupled to control, non-immune mouse IgG purified from whole mouse serum, and consistently failed to bring down the protein.

A.



Lane no.	1	2	3	4	5	6	7	8	9

B.



Volume of resin (μl)	100	50	25	10	100	50	25	10	rbc
SDS 0.02%	+	+	+	+	-	-	-	-	

Fig 5.9

Low salt lysis buffer	1% NP40, 50mM PIPES pH 6.8
NP40 lysis buffer	150mM NaCl, 1% NP40 50mM PIPES pH 6.8
High salt lysis buffer	500mM NaCl, 1% NP40, 50m PIPES pH 6.8
RIPA buffer	150mM NaCl, 1% NP40, 0.5% Deoxycholate, 0.1% SDS, 50mM Tris PIPES pH 6.8

For this process, monoclonal antibody was purified using the protocol described in the methods section and buffer changed from Tris/glyine pH 7.0 into 0.1M borate buffer, pH 8.0 using a PD-10 desalting column. This material was then incubated with cyanogen bromide activated beads at a rate of 5mg of antibody per ml of the hydrated resin, overnight at 4°C. After this incubation, unreacted sites on the beads were inactivated by a further incubation with 100mM ethanolamine for 3h at room temperature. The beads were then washed and used for immunoprecipitations. On the basis of the efficiency with which small volumes of the beads were able to immunoprecipitate PI-TP from rat brain cytosol, this protocol was adopted for future work in preference to the more 'traditional' protein-G coupled beads. For the purposes of disrupting the cells, a low salt lysis buffer was selected, comprising 1% NP40, in 20mM PIPES, pH 6.8. It was further found that addition of SDS to the lysis buffer prior to disruption of the cells led to inefficient immunoprecipitation. The protocol was therefore further modified, with 0.02% SDS being added to the samples for immunoprecipitation only after disruption and pelleting of the insoluble material. It was concluded that this situation arose as a result of the detergent being bound to insoluble components of the cell and hence removed from the system upon preparation of the supernatant.

5.4 Immunoprecipitation of ^{35}S-methionine labelled cells:

One of the areas of most acute interest to be investigated using the antibodies developed in the course of the preceding work was the possible association of PI-TP with other proteins. With this in mind, HL60 cells were cultured in medium

containing a mixture of ^{35}S-methionine and cysteine. This labelled material was obtained from Amersham International under the trade-name Promix.

Initial attempts to immunoprecipitate PI-TPα making use of the antibody 5F12 covalently coupled to cyanogen bromide activated sepharose met with limited success. It was realised at this point that addition of 0.02% SDS to the lysis buffer used for the disruption of the cells led to removal of the detergent from the system when the cytosol was clarified by centrifugation and the protocol was modified, with SDS being added to the clarified supernatant. This led to the efficient capture of PI-TP. Cells labelled using Promix when immunoprecipitated demonstrably brought down detectable levels of PI-TP, as shown by Western blot. Examination of the associated proteins precipitated in this fashion yielded variable results, with no bands consistently appearing in association with PI-TP. It was necessary to expose the phoshorimaging plate for considerable periods of time and it was concluded that PI-TPα represented a small component of the protein present. Cells were cultured in the presence of 1μCi of Promix per ml for 48 hours prior to immunoprecipitation. Differentiated cells were immunoprecipitated after stimulation with 1μM fMLP for 10 minutes, or after 10 minutes pre-incubation with 10nM PMA. No consistent bands were obtained other than those corresponding to PI-TP. The results of these investigations are shown in Fig 5.10.

At the time of conducting these experiments, efficient antibodies against the β isoform of PI-TP had not yet been produced. Hence the only unequivocal observation that could be made at this point was that the monoclonal antibody 5F12 successfully immunoprecipitated ^{35}S labelled PI-TP from labelled cytosols. A further complicating factor was the presence of 0.02% SDS in the material under investigation - it was proposed that this amount of detergent was sufficiently high to disrupt any unstable, transient complexes such as would be invoked by the model of PI-TP chaperoning PI through the sequence of PI-kinases and PLCs involved in this signalling system.

Fig 5.10 Immunoprecipitation of ^{35}S labelled HL60 cells.

Fig 5.10 A shows the phosphorimage of an immunoprecipitation of ^{35}S methionine/cysteine labelled HL60 cells. $50x10^6$ cells were cultured in the presence of 50μCi of Promix labelling reagent from Amersham for 48 hours. The cells were then washed free of the label, and disrupted using the low salt lysis buffer described in the main text. The insoluble material was removed by centrifugation at $10x10^3g$ for 15 mins., and the supernatant made up to 0.02% SDS. The material was then immunoprecipitated using either 5F12 coupled beads (Lane 1), or non-immune mouse IgG coupled to CNBr sepharose (Lane 2). The precipitate was then washed three times with lysis buffer, and treated with SDS PAGE sample buffer. The bound proteins were then resolved on a 12% gel, and Western blotted.

Fig. 5.10 B shows the result of probing the blot with polyclonal antibody against PI-TPα. Lanes 1, and 2 are as above. Lane 3 shows 5μl of rat brain cytosol loaded as a positive control. As can be seen, no PI-TP is detected in the material precipitated with the non-immune antibodies. Lane 1, and 2 in this figure, and 5.10A represent the equivalent of $26x10^6$ cells.

Fig. 5.10 C shows the results of an immunoprecipitation of $150x10^6$ differentiated HL60 cells ^{35}S labelled with 150μCi of Promix either unstimulated (Lane 1), stimulated with 1μM fMLP (Lane 3), or 10nM PMA (Lane 5). Lanes 2, 4, and 6 represent immunoprecipitations of the same material using the negative control resin. Each lane contains the equivalent of $25x10^6$ cells. In all cases a band corresponding to PI-TPα is the only strongly represented signal.

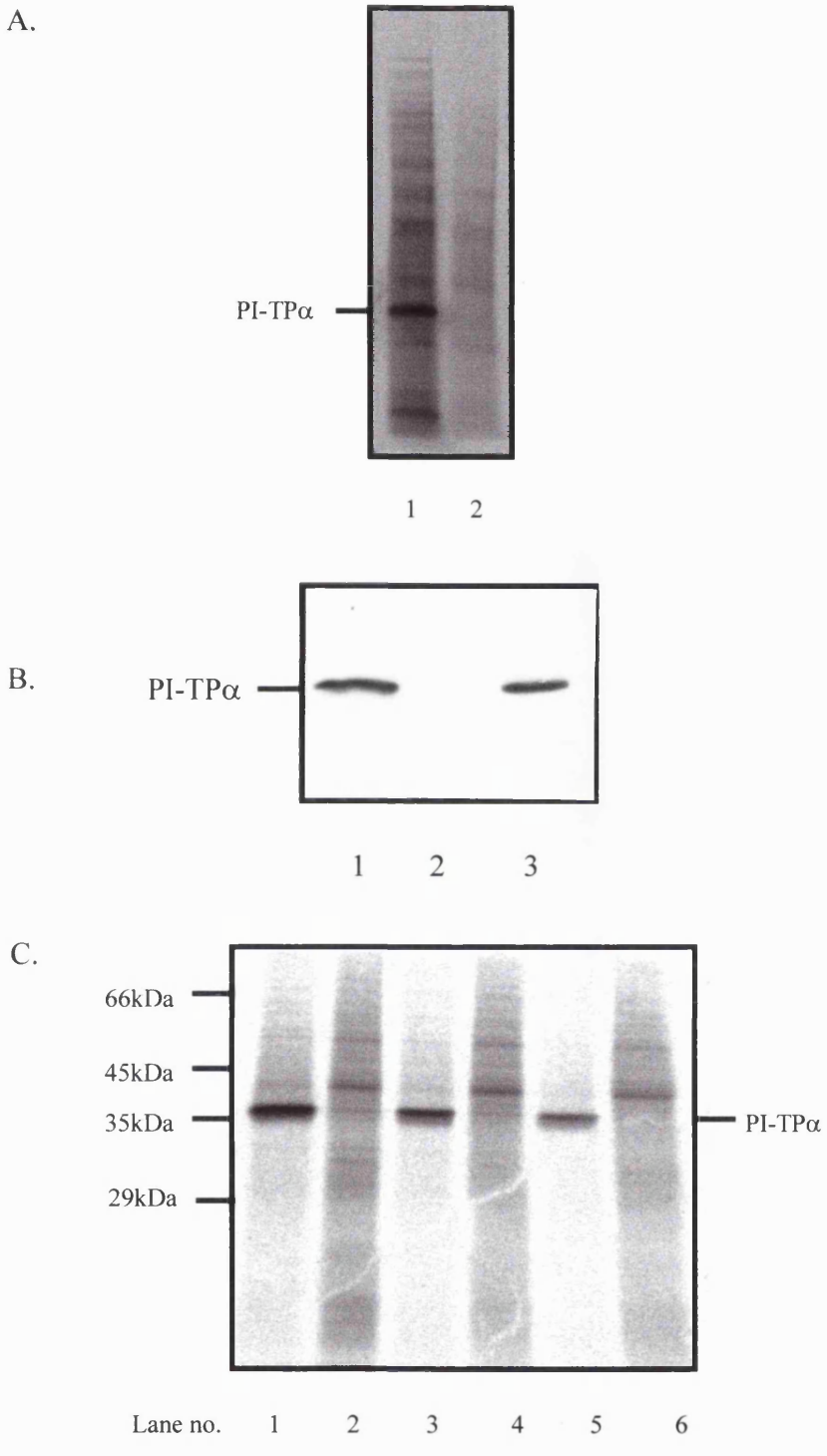

A.
PI-TPα
1 2
B.
PI-TPα
1 2 3
C.
66kDa
45kDa
35kDa
29kDa
PI-TPα
Lane no. 1 2 3 4 5 6

Fig. 5.10

5.5 Phosphorylation studies on PI-TPα

Since co-immunoprecipitation of proteins interacting with PI-TP was demonstrated to be of uncertain merit, it was decided to address the question of phosphorylation of the protein in response to various stimuli. It had previously been demonstrated in the lab of K.W.A. Wirtz that in Swiss 3T3 fibroblasts, PI-TP was phosphorylated in response to both PMA, a stimulator of PKC and also Bombesin. The latter acts through a seven membrane spanning, G-protein coupled receptor mediating activation of PLC in addition to other effectors in a manner analogous to that of the fMLP receptor in HL60 cells and neutrophils. It was therefore decided to investigate the effect of PMA and the chemotactic peptide fMLP stimulation on HL60 PI-TP phosphorylation.

5.5.1 Effect of stimulation with 10nM PMA

$200x10^6$ HL60 cells were cultured as described, washed and resuspended in 2ml of 20mM Tris, 137mM NaCl, 3mM KCl and 1mM $CaCl_2$ and $MgCl_2$. The cells were then maintained at 37°C and incubated in the presence of 1mCi of ^{32}P labelled sodium orthophosphate for one hour, with occasional shaking. At the end of the labelling period, the cells were pelleted by centrifugation at 1500rpm in a bench top centrifuge and the label removed. The cells were then washed a further two times in order to minimise the amount of excess label in the sample and facilitate safe handling of the resultant material.

The labelled cells were then resuspended in 4.5 ml of Tris buffered saline as above and 900µl of the material was dispensed into each of a series of reaction tubes, containing 100µl of 100nM PMA, such that the final concentration of PMA in the 1ml sample was 10nM. The tubes were then incubated at 37°C and samples removed at appropriate time points. The reaction was terminated by the addition of 500µl of the low salt lysis buffer, which contained protease inhibitors and phosphatase inhibitors. Samples were maintained on ice until the end of the time course and allowed to stand for a further 15 minutes to ensure efficient lysis of the cells by the disruption buffer. At the end of this time, the material was pelleted by centrifugation at $10x10^3g$ for 15

minutes and the supernatant retained for immunoprecipitation. This latter was conducted by the addition of 50µl of a 50% v/v suspension of 5F12 coupled sepharose beads, as already described and SDS to bring the final concentration of detergent to 0.02% w/v. The immunoprecipitations were incubated overnight at 4°C and washed three times in cold, single strength lysis buffer before being resolved by SDS PAGE and Western blotted onto PVDF membrane. The blotted material was then autoradiographed using a Fuji phosphorimager. The results of these experiments are illustrated in Fig. 5.11. On the basis of these data it was concluded that PI-TPα in HL60 cells was phosphorylated in response to PMA, a result which was not altogether surprising in the light of similar observations made in Swiss 3T3 cells and the presence of five putative PKC phosphorylation sites on this protein.

5.5.2 Effect of 1µM fMLP

The observation that PMA stimulation led to phosphorylation of PI-TP broached the question of whether a more physiologically relevant stimulus would lead to similar modification of the protein. HL60 cells differentiated using the agent dibutyryl cAMP develop a morphology similar to neutrophils, a cell to which HL60s are believed to represent a primitive precursor. In addition to morphological changes, the differentiated cells also express a receptor for the chemotactic factor fMLP.

$500x10^6$ HL60 cells were differentiated by exposure to dibutyryl cAMP at a concentration of 0.3mM for 48 hours. At the end of this differentiation process the cells were prepared in a similar manner to that described for the study of the effect of PMA. The cells were labelled with 1mCi of ^{32}P orthophosphate and resuspended in Tris buffer as before. 900µl of the cells were then dispensed into reaction tubes containing 100µl of fMLP at a concentration of 10µM, such that the final concentration of this agent in the 1ml sample was 1µM. The reaction was stopped as before at time points as shown in the figure and the sample processed in essentially

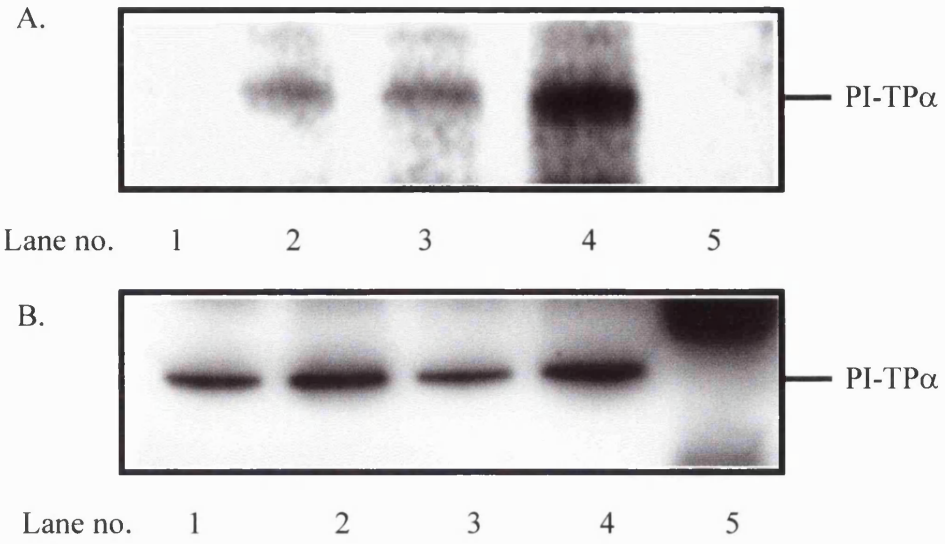


Fig 5.11 Phosphorylation of PI-TPα under the influence of 10nM PMA in undifferentiated HL60 cells

Undifferentiated HL60 cells ($200x10^6$) were prepared according to the usual culture protocol. The cells were then resuspended in 2ml of 20mM Tris, 137mM NaCl, 3mM KCl, 1mM $CaCl_2$, and 1mM $MgCl_2$ pH 7.4. The cells were then labelled by incubating them in the presence of 1mCi of ^{32}P labelled sodium orthophosphate for one hour at 37°C. The cells were then washed free of excess label, and allowed to stand for 15 minutes prior to use. 900µl of the cells were then added to 100µl of 100nM PMA (10nM final conc.) and the assay incubated at 37°C. Tubes were removed from the water bath at time points t=0, 30, 60 and 300 s (Lanes 1-4). The reaction was stopped by treatment with 500µl of low salt lysis buffer supplemented with phosphatase inhibitors, and insoluble material removed by centrifugation. The resultant supernatants were then immunoprecipitated using 5F12 monoclonal antibody coupled to sepharose, washed five time in single strength lysis buffer, resolved by SDS PAGE, Western blotted, autoradiographed, and probed using the monoclonal antibody 5F12. Lane 5 contains material immunoprecipitated using normal mouse IgG coupled resin.

Fig 5.11A shows the resultant phosphorimage, and Fig. 5.11B shows the result of probing the blot with mAb 5F12.

Each lane contains the equivalent materialfrom $40x10^6$ HL60 cells.

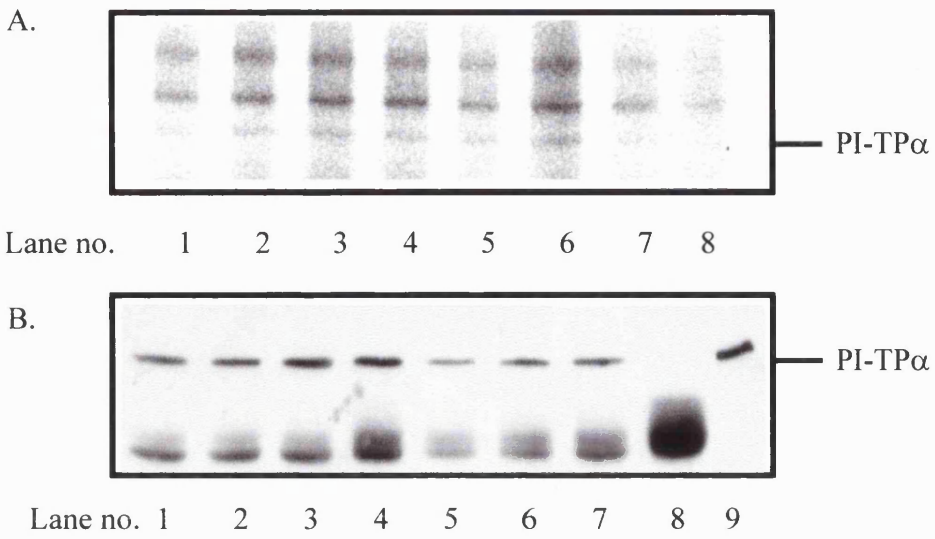


Fig 5.12 Phosphorylation of PI-TPα under the influence of 1μM fMLP

Differentiated HL60 cells ($200x10^6$) were prepared according to the usual culture protocol. The cells were then resuspended in 2ml of 20mM Tris 137mM NaCl, 3mM KCl, 1mM $CaCl_2$, and 1mM $MgCl_2$ pH 7.4 supplemented with 1mg/ml BSA, and 1mg/ml D-glucose. The cells were labelled by incubating them in the presence of 1mCi of ^{32}P labelled sodium orthophosphate for one hour at 37°C. The cells were then washed free of excess label, and allowed to stand for 15 minutes prior to use. 900μl of the cells were then added to 100μl of 10μM fMLP (1μM final conc) and the assay incubated at 37°C. Tubes were removed from the water bath at time points as described in the figure. The reaction was stopped by treatment with 500μl of low salt lysis buffer, and insoluble material removed by centrifugation. The resultant supernatants were then immunoprecipitated using 5F12 monoclonal antibody coupled to Sepharose in the presence of 0.02% SDS, washed five time in single strength lysis buffer, resolved by SDS PAGE, Western blotted, autoradiographed, and probed using the monoclonal antibody 5F12. The autoradiaograph is illustrated in Fig. 5.12A, while the Western blot is shown in Fig. 5.12 B. Lanes 1 to 6 correspond to time points t=0, 1,5, 10, 15 and 30 mins post stimulation. Lane 7 represents unstimulated cells incubated in parallel with the fMLP stimulated cells for 15 mins. Lane 8 contains stimulated cells, lysed at t=15 mins, and immunoprecipitated with negative control beads, coupled to non immune mouse IgGs. Lane 9 in the blot shows 5μl of rat brain cytosol as a positive control. Each lane represents $25x10^6$ cells.

the same manner. The results of these experiments are illustrated by the result in Fig. 5.12.

Examination of the phosphorimage produced led to the conclusion that PI-TPα undergoes a slight detectable increase in phosphorylation in response to fMLP in a similar manner to that observed in Swiss 3T3 cells stimulated with bombesin.

Having established the phosphorylation of PI-TPα was subject to change as a result of stimulation with PMA and fMLP it was hoped to be able to further examine the site or sites of phosphorylation by means of phosphoamino acid analysis and phosphopeptide mapping. A number of attempts were made at this analysis, but in consequence of the low levels of proteins which could be immunoprecipitated, this proved impractical.

Even the best samples obtained using the above protocol were barely detectable using a hand held monitor and the immunoprecipitated phosphorylated PI-TP represented a relatively small proportion of this signal in consequence of other non-specific proteins also coming down. It was necessary to expose phosphorimaging plates for periods of several days to visualise the bands obtained and once excision of the relevant band from the blot had taken place, much of the residual activity present was lost in the form of free phosphate as a result of the acidic conditions involved in the hydrolysis of the PVDF bound protein.

5.6 Discussion:

This section of work describes the production of a panel of monoclonal antibodies against PI-TP and their characterisation in terms of isotype and functional properties. One of the antibodies, designated as 5F12 was of particular interest by virtue of being able to powerfully immunoprecipitate the antigen in the presence of low concentrations of SDS. Because of an inability to recognise PI-TP on Western blots and the difficulties inherent in the purification of IgM class immunoglobulins two of the cell lines in the panel (2G8 and 3H3) were frozen down without further characterisation. The remaining Ig M in the panel, 1A4 proved immunoreactive with

A.

B.

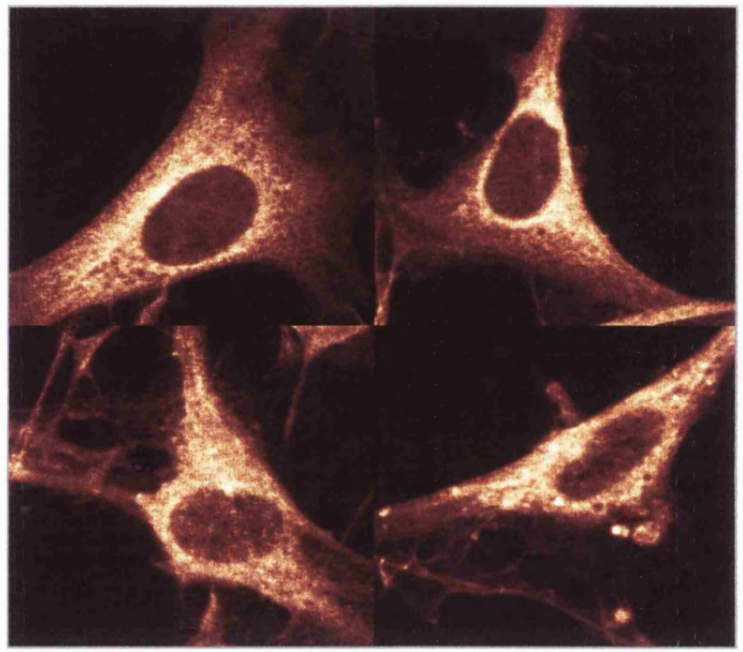


Fig. 5.13 Immunofluorescent staining of PI-TPα in Swiss 3T3 cells using PI-TPα specific mAb 5F12

The above figure shows images prepared in collaboration with A.M. Pendleton using mAb 5F12 to stain Swiss 3T3 fibroblasts. A diffuse, cytosolic staining is observed using this antibody, as has already been documented. Panel A shows intact cells, while panel B shows SLO-permeabilised cells. It has been noted that adherent cells show different leakage kinetics for PI-TPα compared to HL60 cell (Oral communication from A.M. Pendleton).

PI-TP on Western blots and on the basis of this property, stocks of supernatant were prepared for this application. This antibody appears to recognise a similar epitope to that identified for the mAb 5F12 on the basis of Western blots performed using recombinant proteins as described in the main text and could probably represent a class switch variant, produced in the primary stage of the immune response. (Data not shown.)

On the basis of the specificity with which mAb 5F12 reacted with either N-terminal, or C-terminal His tagged PI-TPα it was concluded that the recognition site of the antibody lies at the carboxy terminal of the PI-TP molecule. Further studies using a series of five amino acid truncation mutants supported this hypothesis - the ability of the antibody to recognise SDS-denatured protein also suggest that the antibody is specific for a stretch of primary structure rather than a more complicated secondary, structure and hence that the epitope lies with the carboxy terminal pentapeptide.

In collaboration with Anne Marie Pendleton in the laboratory of Anna Koffer, the potential usefulness of the antibodies for immunofluorescent cellular localisation in Swiss 3T3 cells was assessed. Both 5B2 and 5F12 appeared to produce a specific punctate staining within the cell, as previously described (167). This is illustrated in the case of 5F12 in figure 5.13.

Although mAb 5F12 efficiently immunoprecipitated PI-TPα from rat brain cytosol, application of this antibody to the study of PI-TPα in HL60 cells proved to be difficult, due to the low amounts of this protein detected in immunoprecipitates. The facts that there were high levels of PI-transfer activity detectable in HL60 cells and that the mAb 5F12 was specific for the α isoform of the protein led to speculation that PI-TPβ was the predominant isoform in HL60 cells rather than PI-TPα. This prompted the production of a panel of PI-TPβ specific antibodies.

Chapter 6

Raising of monoclonal antibodies against the mammalian β-phosphatidylinositol transfer protein

6.1 Introduction:

Initial attempts at producing immunoreagents led to the development of a rabbit polyclonal antibody which was subsequently demonstrated to be specific for both α and β isoforms of PI-TP. The limitations of the antibodies produced in this manner (See Chapter 4) led to an attempt to raise monoclonal antibodies against PI-TP purified from bovine brain utilising an improved, rapid purification protocol which produced a mixture of PI-TP and the small GTPase Arf-1, another protein of interest to the research conducted in this laboratory. Characterisation of this panel of antibodies revealed that all of the five antibodies reactive with PI-TP were absolutely specific for PI-TPα either in Western blotting, or on the basis of an ELISA similar to that used in the screening of the original hybridoma supernatants.

Attempts to quantify the amount of PI-TP present in HL60 cell and neutrophil cytosols suggested that in the case of neutrophils there appeared to be significant quantities of PI-TPβ present in the cell. With this in view and information revealing a potential distinct distribution of the PI-TP α and β within the cell (202) it was decided to attempt to raise a panel of monoclonal antibodies specific for the β-isoform. Since some success had been attained previously at raising antibodies against Arf-1 it was decided also to attempt a similar strategy, utilising a mixture of the two proteins in immunisations for the mice.

6.2.1 Preparation of material for immunisation:

r-PI-TP β was cloned from a rat brain c-DNA library as described by Cunningham et al (152). The resultant cells were then used to produce r-PI-TPβ according to the protocol already described in the methods section and purified using a nickel affinity column. For the purpose of raising a panel of antibodies it was decided

to remove the N-terminal His tag from the resultant protein by incubating it overnight at room temperature with 1 i.u of Thrombin per mg of rPI-TPβ. 250 μg of this protein was prepared in 500μl of 20mM PIPES, with 137mM NaCl and 3mM KCl. This material was then used as the basis of a series of primary and boost inoculations into two Balb/c strain mice, according the a similar protocol to that already described in Chapter 5.

6.2.2 Screening of sera for anti-PI-TPβ:

Because of the absence of a reliable capture phase antibody on which to base an assay, the proteins of interest were directly coated onto a solid support phase. This had previously been demonstrated to work efficiently in the case of PI-TPα and the main concern remained one of whether binding of the protein to the plate would lead to denaturation of the protein, potentially leading to exposure of epitopes not normally exposed on the native protein under physiological conditions.

ELISA plates were coated with r-PI-TPβ, or Arf-1 at 2μg/ml in a 0.1M solution of Na H_2PO_4 at pH 7.0 overnight at 4°C, or at room temperature for one hour and blocked with 2.5% milk protein in PBS-Tween 20 for one hour at room temperature.

Sera for screening were obtained from the experimental animals by tail bleeds, which produced limited quantities of material. The assay was conducted in triplicate and in order to provide the required volume of sample necessary for the assay, an initial dilution of 50 fold in PBS-Tween 20 was necessary. 200μl of the sample was added to each of the wells at the head of the column and the sample then subject to sequential two fold dilutions in PBS-Tween 20. The final dilution of the sample was 3,200 times. The robustness of the immune response against PI-TPβ was reflected by the fact that the titre of antibody in the sera of both animals did not start to tail off significantly until a dilution factor of 200 was attained (See Fig. 6.1). The sera was also screened for activity against PI-TPα. Unsurprisingly in consideration of the highly conserved nature of the proteins, a significant degree of cross reactivity was detected. The titre at which the signal for the PI-TPα started to decay was however significantly lower by comparison to that for PI-TPβ.

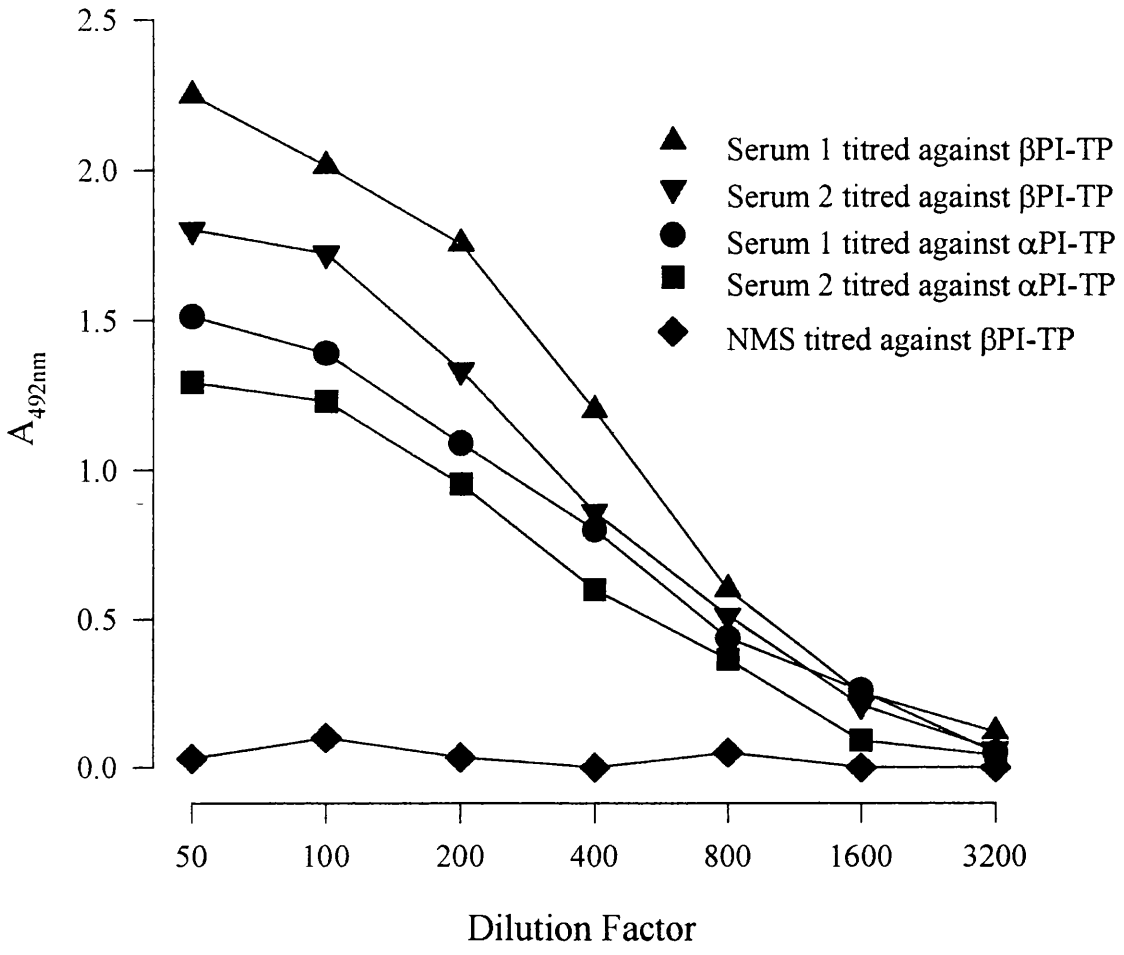


Fig. 6.1 Demonstration of PI-TP specific antibodies in sera of immunised animals.

The figure above illustrates the results from an ELISA designed to detect both PI-TPα, and β specific antibodies. ELISA plates were coated with the recombinant proteins, probed with dilutions of sera derived from tail bleeds of the experimental animals, and the signal developed using the chromogenic substrate OPD, as described in the text. Antibodies reactive towards both proteins were detected. Normal mouse serum (NMS) was included as a negative control.

The absorbance values represent means of triplicates which individually varied from the mean by less than 10%.

On the basis of these data, the highest responding animal was selected for sacrifice to provide splenocytes for the fusion. This was performed four weeks after the commencement of immunisation and followed the method already described in Chapter 5.

6.2.3 Screening of tissue culture supernatants for the presence of anti-PI-TPβ antibodies:

An essentially unmodified protocol was followed for the purpose of screening the tissue culture supernatants. The samples were however diluted only four fold to ensure the detection of any specific antibody present. Also because of the large number of samples (756 in total) to be screened in the initial assay, single points only were taken.

The initial screen produced large number of potential positive cultures (118 in total). The large numbers involved meant that only the most strongly positive cultures were selected for continued consideration. This reduced the total number of positive cultures for consideration to 55.

Because of the existence of an anti-PI-TPα panel already, it was decided to concentrate on those cultures demonstrating a marked specificity for the β isoform. To this end, those 55 cultures originally selected were re-assayed against both of the PI-TP isoforms in parallel. This reduced the number of positive cultures for consideration to 24 and these cultures were expanded from the microtitre plate to 24 well tissue culture trays. These cell lines were again tested and on the basis of the results, the total number of cultures was reduced to 12.

Both the α and β isoforms of PI-TP utilised in the assays and the material used for the immunisations were prepared using His tagged material which was then subsequently cleaved using thrombin. One persistent cause of concern was the possibility of generating antibodies specific for the His tag, rather than the protein of interest. This was in part allayed by the observation that the screened supernatants

appeared to have a specific affinity for the PI-TPβ compared to the α form despite the presence of the His tag in both preparations.

In order to help demonstrate that the antibodies were reacting specifically with the relevant proteins, supernatants from the cultures were tested in parallel with PI-TPα and β as well as rArf1 as a negative control. The signals produced by each protein were plotted as a histogram. Those antibodies which demonstrated a cross reactivity between all three proteins were discounted from further consideration. The accompanying graph (Fig. 6.2) shows the comparative signal produced against both PI-TPα and β by those antibodies demonstrated as being specific for these proteins.

6.3 Screening supernatants for function in Western blotting:

After expansion of the cultures into the larger tissue culture trays, the cells were allowed to grow for four days at the end of which time 500μl of tissue culture supernatant was removed and used for the purpose of screening in Western blot detection. To this purpose, 100μl of both of the PI-TP isoforms at a concentration of 1μg/ml were individually resolved on 12% SDS PAGE gels, blotted onto PVDF membrane at 30v overnight, cut into strips as previously described and probed with a 1:10 dilution of the supernatant in PBS and developed by means of the luminescent method described in Chapter 2. Figure 6.3 shows the results obtained. One point of particular interest was the observation that a member of the panel appeared to specifically recognise PI-TPα, but not PI-TPβ, against which the panel of antibodies was originally raised., having previously demonstrated an equal affinity for both proteins in the screening ELISA.

In order to investigate the ability of the antibodies to recognise PI-TPβ from eukaryotic cells, samples of rat brain and human neutrophil cytosol were individually resolved and blotted as above. The resultant image is shown in Fig. 6.4. It was noted that only a relatively small number of the antibodies produced a strong signal from Western blots of this material. These antibodies were selected to constitute a panel used for later investigations of HL60 and other mammalian cell lines.

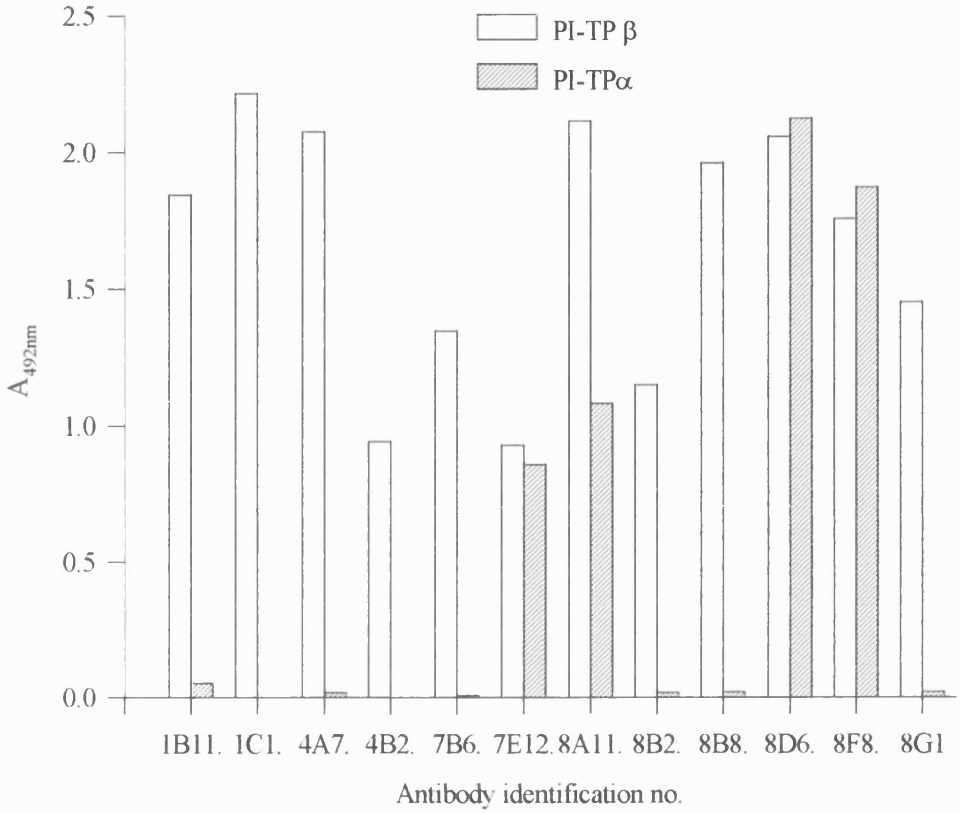
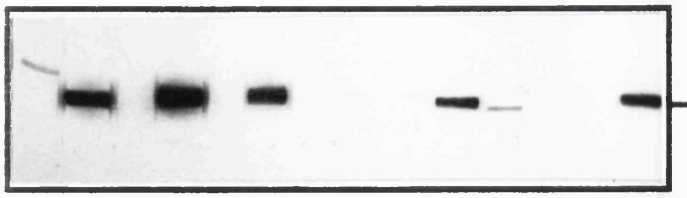
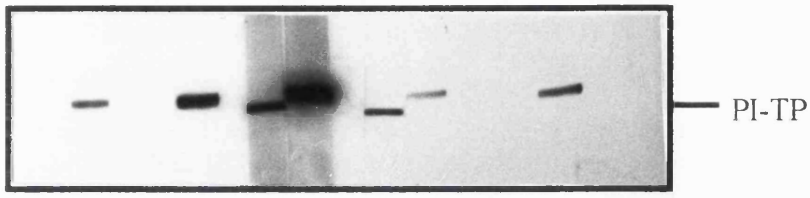


Fig 6.2 ELISA to demonstrate the specificity with which the strongest reacting hybridomas from the original rounds of screening recognise rPI-TPα, and β.

ELISA plates were coated with the individual recombinant proteins, and blocked as per the usual screening assay. The previously identified hybridomas were again screened. Samples of hybridomas supernatant were diluted 1:3 before being loaded onto the coated plates. In view of the pre-existence of PI-TPα specific monoclonal antibodies, those hybridomas producing PI-TP β specific antibodies were selected for particular attention.



Antibody I.D.	1B11	1C1	4A7	4B2	7B6	7E12	8A11
PI-TP isoform	α β	α β	α β	α β	α β	α β	α β



Antibody I.D.	8B2	8B8	8D6	8F8	8G1
PI-TP isoform	α β	α β	α β	α β	α β

Fig. 6.3 Immunoblotting, and detection of recombinant PI-TP isoforms using members of the monoclonal antibody panel raised against rPI-TPβ.

100μl of the recombinant PI-TPs adjusted to a concentration of 1μg/ml were resolved by SDS PAGE, and transferred onto PVDF membrane. The blot was then blocked with milk protein, cut into strips, and probed using 1:10 dilutions of hybridoma supernatants, previously demonstrated as being responsive to PI-TP in ELISA.

mAbs 1C1,4A7,7B6,8A11,8B2,8B8, and 8G1 were PI-TPβ specific.
mAb 1B11 showed a low level of cross reactivity.
mAb 8D6, and 8F8 were panspecific for both isoforms.
mAb 7E12 only recognised PI-TPα.
mAb 4B2 failed to produce a significant signal.

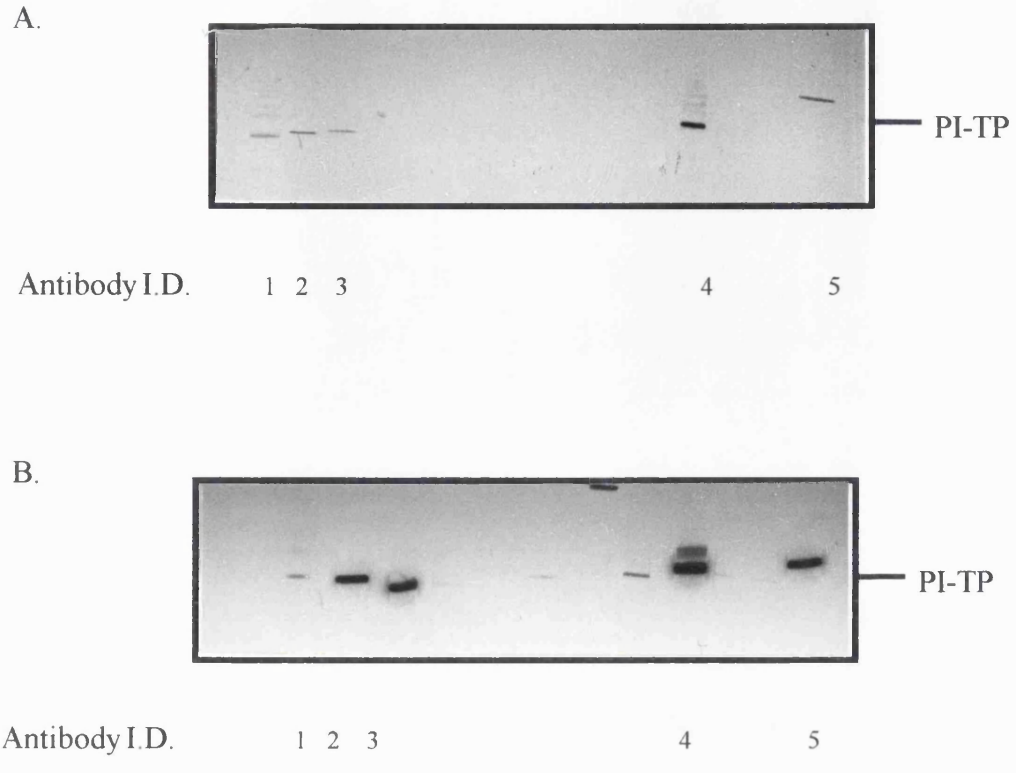


Fig 6.4 Probing human neutrophil, and rat brain cytosol with monoclonal antibodies raised against PI-TPβ.

In order to assess the ability of the monoclonal antibodies to recognise the proteins produced in eukaryotic cells, samples of neutrophil cytosol (A), and rat brain cytosol (B) were resolved on 12% SDS PAGE gels, and blotted onto PVDF membrane. The blot was then cut into strips, probed with the individual antibodies, and the image visualised using the in house chemiluminescence method.

The labelled lanes correspond to the antibodies as follows:-

Lane 1 = 1B11
Lane 2 = 1C1
Lane 3 = 4A7
Lane 4 = 8B8
Lane 5 = mixed mAbs

6.4 Assessment of immunoprecipitating ability:

The ability of the antibodies to immunoprecipitate the recombinant proteins was initially assessed using crude tissue culture supernatant that had previously been screened for immunoreactivity against the α and β isoforms of PI-TP. 0.5ml of standard preparation of the recombinant PI-TPβ, at a concentration of 0.5μg/ml was incubated with 50μl of the supernatant overnight at 4°C before being precipitated using Protein-G sepharose from Pharmacia for one hour at 4°C. The resin was then washed three times in PBS pH 7.4, before being treated with SDS-PAGE sample buffer and any bound proteins resolved on a 12% gel prior to Western blotting and probing with a mixture of monoclonal antibodies which had previously been demonstrated to specifically recognise the β isoform of PI-TP. The assay was performed both in PBS, pH. 7.4 and in RIPA buffer, which contains 0.1% SDS, a condition that had previously been demonstrated to be favourable for the immunoprecipitating activity of the antibody 5F12. The results of this screen are shown in the accompanying figure (Fig 6.5).

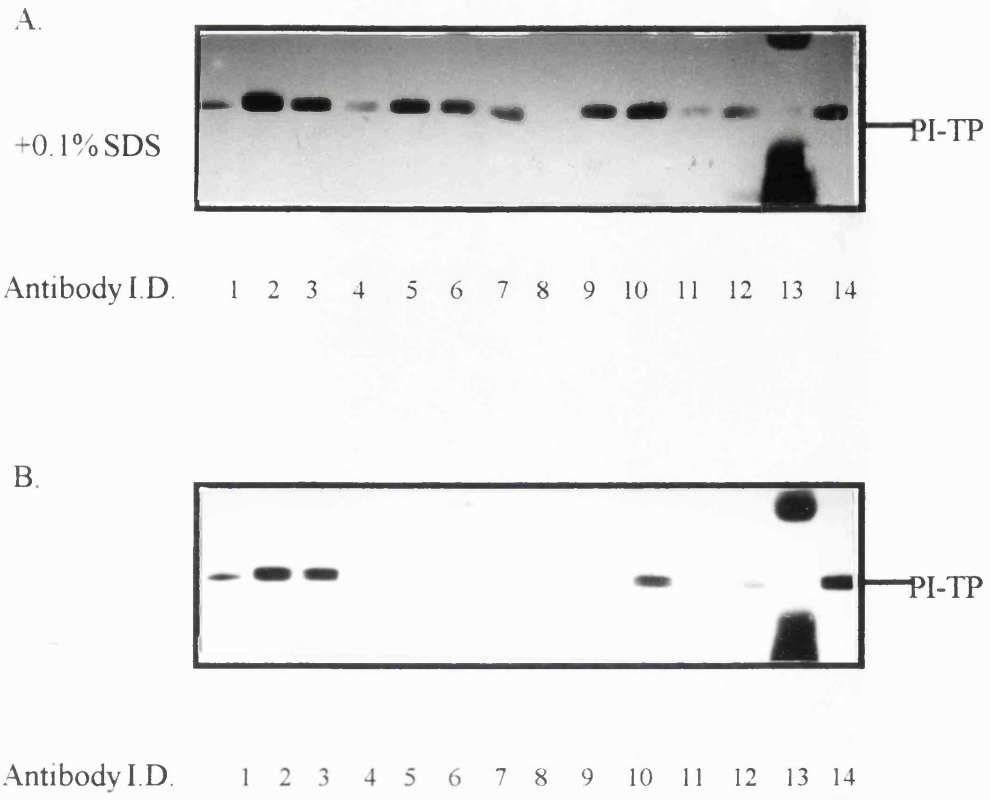


Fig. 6.5 Immunoprecipitation of recombinant PI-TPβ using monoclonal antibodies.

rPI-TP was diluted to 500ng/ml in PBS, pH 7.4. 500µl of this material was then incubated with 50µl of hybridoma supernatant at 4°C overnight. This was performed in the absence, and the presence of 0.1% SDS. 50µl of a 50% suspension of protein G-sepharose beads in PBS was then added , and incubated at 4°C for one hour prior to pelleting of the beads by centrifugation, and washing three times in PBS. The beads were then treated with SDS PAGE sample buffer at 96°C, and resolved, and blotted onto PVDF, before being probed with a mixture of monoclonal antibody supernatants, 1B11, 1C1, 4A7, and 8B8. The labelled lanes correspond as follows:-

Lane 1 = 1B11	Lane 5 = 7B6	Lane 9 = 8B8
Lane 2 = 1C1	Lane 6 = 7E12	Lane 10=8D6
Lane 3 = 4A7	Lane 7 = 8A11	Lane 11=8F8
Lane 4 =4B2	Lane 8 = 8B2	Lane 12=8G1

Lane 13=non-specific mouse IgG Lane 14=0.5µg rPI-TPβ

6.5 Discussion:

The use of recombinant protein as a source of antigen for the raising of this panel of monoclonal antibodies enabled the development of a powerful tool for the further investigation of the functional properties of the PI-TPβ isoform. In addition, a number of the antibodies raised in the course of this exercise have been characterised as being pan specific.

Of particular note is the ability of the antibodies in the panel to immunoprecipitate, almost without exception, in the presence of SDS and in a number of cases, in the absence of SDS. These reagents now throw open the intriguing possibility of investigating the interaction of PI-TP with other proteins by means of co-immunoprecipitation studies, as well as allowing the study of phosphorylation studies on the β isoform, which had previously been impossible as a result of the demonstrated specificity of the 5F12 antibody for the C-terminus decapeptide of the α isoform. It was also noted that in immunoprecipitation there was evidence of some degradation of the rPI-TPβ, as illustrated by the fact that it resolved as a doublet on the Western blot. Whether or not this represents the loss of the hexahistidine tag from the protein, or some other form of proteolytic cleavage, antibody 1B11 consistently does not recognise the higher molecular weight moiety - hence it may be concluded that this antibody is specific for a terminal sequence. That this antibody recognises the native protein in Western blots suggests that an analogous situation exists between this antibody and the monoclonal antibody 5F12 i.e. that this antibody recognises an epitope that is disrupted by the presence of the hexahistidine tag, suggesting that 1B11 may be specific for the N-terminal of PI-TPβ.

Chapter 7

The major isoform of PI -TP in neutrophils/HL60 cells is identified as PI-TPβ

7.1 Introduction:

One of the questions which it was felt necessary to answer was the amount of PI-TP present within the cell. The earlier studies which had identified PI-TP as the PLC reconstituting factor in HL60 cells had entailed the addition of PI-TP back to cytosol depleted cells. The relevancy of the amounts of PI-TP added to this system had at this time yet to be established.

The PI-TPα specific panel of antibodies was raised first and using these reagents, attempts were made to quantify this protein in HL60 cells and neutrophils. In addition, taking advantage of the demonstrated ability of this antibody to immunoprecipitate PI-TP from rat and bovine brain cytosols, attempts were made to investigate the phosphorylation state of PI-TPα under various conditions of stimulation.

The advent of the panel of PI-TPβ specific reagents allowed the further characterisation of the system in HL60 cells and neutrophils and also explained some of the problems encountered in the studies carried out using the PI-TPα specific reagents.

In addition to an examination of the state of affairs extant in HL60 cells and neutrophils, the antibodies also enabled an examination of the distribution and relative abundance of the two PI-TP isoforms in other cell lines, specifically RBL 3H3, a rat cell line resembling mast cell and PC12 cells, which closely resemble adrenal chromaffin cell.

7.2 Quantification of PI-TP α and β in HL60 cell and neutrophils

7.2.1 ELISA strategies:

With the development of immunoreagents against PI-TPα, initial attempts at quantification of the protein revolved around the development of an ELISA (Enzyme Linked Immunosorbent Assay) system. The basic strategies considered for this assay are schematically represented in Fig. 7.1.

Identification of potential capture phase antibodies:

Initial attempts to quantify PI-TP in HL60 cells and other material were based on the development of a sandwich ELISA, in which it was anticipated that an antibody, or antibodies from the panel would be bound to the plastic of an ELISA plate and be used to trap PI-TP from the sample under investigation. It was then hoped to be able to detect this bound PI-TP by making use of another PI-TP specific antibody, tagged with biotin. Detection of the complex bound to the ELISA plate would then be achieved using HRPO conjugated Extravidin.

In order to identify potential capture phase antibodies, preparations of Protein G purified IgG class antibodies from the PI-TPα specific panel were diluted to a concentration of 2μg/ml and coated onto ELISA plates overnight at 4°C. The antibody solution was then removed from the plate and the wells blocked by incubation with a 2% BSA solution in PBS-Tween, pH 7.4 for one hour at room temperature.

The plates were then incubated with a preparation of biotinylated rPI-TPα, diluted over a range from 1mg/ml to 8μg/ml. This material was prepared by incubating recombinant protein with N-hydroxysuccinimidyl biotin overnight at 4°C in a borate buffer at pH 8.0. The biotinyl protein was then dialysed extensively to remove unbound biotin from the reaction mixture. This was necessary as a result of the anomalous behaviour of biotin in solution, which though being a relatively low

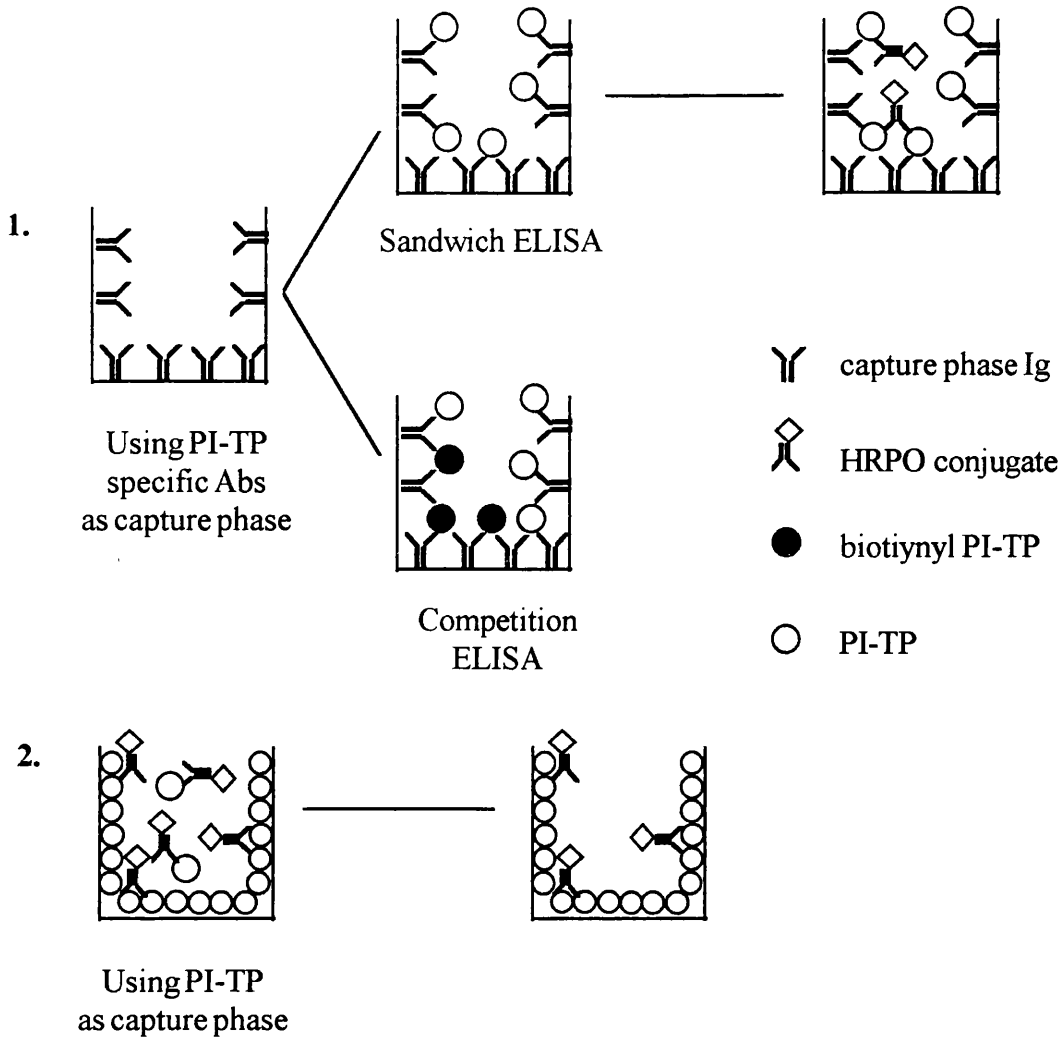


Fig 7.1 Schematic representation of potential ELISA systems to detect, and quantify PI-TP.

Section 1 illustrates the two possible methods making use of anti-PI-TP Abs as a capture phase to bind PI-TP from the sample under investigation. In the sandwich assay, PI-TP bound to the capture phase antibody is detected using another HRPO, or biotinylated antibody directed against an alternative epitope. Binding of the labelled antibody is proportional to the amount of PI-TP captured- hence more PI-TP produces a higher signal.

In the competition ELISA, a known amount of biotinyl PI-TP competes for binding sites with PI-TP in the sample under investigation. Hence higher concentrations of PI-TP in the unknown sample reduce the binding of labelled protein, lowering the observed signal.

Section 2 shows a competition ELISA using PI-TP as a capture phase for labelled PI-TP specific Abs. PI-TP in the sample competes with the capture phase for biotinylated, or HRPO conjugated antibody. Again, higher concentrations of PI-TP in the sample diminish the intensity of the signal.

molecular weight molecule, diffuses and dialyses as a much larger entity. The plates were incubated for one hour at 37°C before being washed three times with PBS-Tween. The plate was then probed with HRPO coupled extravidin from Sigma diluted $1:10x10^3$ in PBS-Tween and incubated for 30 mins at 37°C. The plate was then washed free of extravidin and subject to five washes. Colour development was performed using OPD substrate which was incubated for 30 mins at room temperature. The reaction was arrested by the addition of 50µl of 1M HCl. The absorbance of the wells determined using a Multiscan plate reader set to 492nm.

The results of this assay are illustrated in Fig. 7.2. As can be seen, some degree of capture is observed with all of the antibody preparations assayed. Monoclonal antibody 3A7 appeared to give the greatest degree of capture, with the mixed antibodies, 5B2 and 5F12 performing less well.

Biotinylation of proteins is a commonly used strategy to amplify the signal intensity in a variety of methods, including ELISA. Given the signal obtained using concentrations of biotinyl PI-TP as high as 1mg/ml and the expected lower values in HL60 cytosol it was concluded that the use of the antibodies in a sandwich ELISA was not a viable proposition. At this time the PI-TPβ specific antibodies were not available - it is possible that some of these reagents, many of which are pan-specific, may act as a basis for ELISA systems to identify both isoforms of PI-TP. It is suggested that if pan-specific antibodies could be used as a 'universal' capture phase for both isoforms, the ratio of the individual proteins could be determined by probing the captured protein with isoform specific antibodies.

Alternative competition strategies to the sandwich ELISA were considered. It was decided however that the establishment of the more rigorous conditions required for such an assay to be set up were too demanding. It was therefore concluded that another method would provide more satisfactory results.

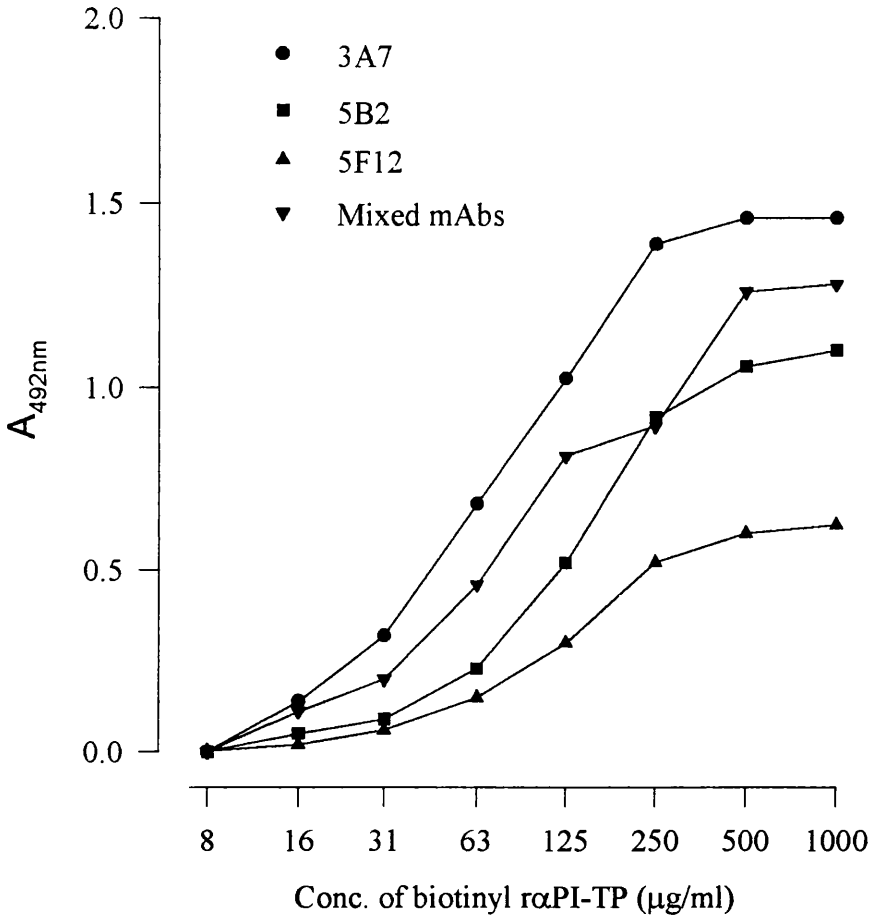


Fig 7.2 Identification of optimum capture phase for use in ELISA to detect PI-TPα.

The above figure illustrates attempts to identify a capture phase antibody for use in an ELISA system to detect, and quantify αPI-TP. Protein G purified monoclonal antibodies were diluted to a final dilution of 2μg/ml on a 0.1M NaH_2PO_4 pH 7 buffer, and used to coat ELISA plates overnight. The coated wells were then blocked by incubation with 2% BSA in PBS-Tween, pH 7.4. The plate was then exposed to serial two fold dilutions of biotinylated rPI-TPα from 1mg/ml to 8μg/ml. The plates were then washed with PBS-Tween, and probed with HRPO coupled Extravidin™ from Sigma. The plates were washed, and the colour developed using OPD substate. The absorbance values represent means of triplicates which individually varied from the mean by less than 10%.

7.2.2. Quantitative Western blotting PI-TP

Utilising monoclonal antibodies against α and β PI-TP

The failure of the initial panel of antibodies to function as the basis of an ELISA system made the adoption of an alternative strategy necessary. Consequently it was proposed that the two proteins should be quantified using Western blotting and densitometry. To this end, bulk standard preparations of the two isoforms of PI-TP were prepared in SDS PAGE sample buffer and frozen down to maintain consistency throughout. Samples of cells were prepared according to two alternative strategies:-

1. Cells were grown/prepared according to the appropriate protocol, suspended in 1ml of an phosphate buffered saline and treated with DFP at 10μM for 5 minutes on ice, prior to undergoing three rounds of freeze thawing as described in Chapter 3. The efficiency of the disruption was assessed using a microscope. The cytosolic component of the preparation was then obtained by centrifuging the material at $10x10^3$g for 30 minutes and removing the supernatant. The remaining solid material which comprised mainly membranes, undisrupted cells and nuclei was retained separately, washed twice with PBS and then extracted using RIPA buffer.

2. Cells were prepared as above and DFP treated. At the end of this treatment they were sonicated using a probe sonicator from Materials and Sonics Ltd.. The efficiency of the treatment was assessed by visually inspecting a small quantity of the material under a microscope. The sample was then given a low speed spin in a bench top microfuge to remove nuclei and undisrupted cells. The resultant post nuclear supernatant (PNS) was then spun at $43x10^3$ rpm in an Beckman Ti 45 rotor for one hour at 4°C. The supernatant was then removed from the pelleted membranes and stored under refrigeration until used. The pelleted membranes were then washed twice by resuspension in 1ml of PBS and repeated centrifugation. The membranes were then solubilised in SDS PAGE sample buffer.

In order to avoid overloading the SDS PAGE gels used for the resolution of the proteins contained in the samples prepared above, the concentration of the total protein loaded was assessed using the Bradford assay, as described in Chapter 2. The total protein concentration within the sample was then adjusted to 1mg/ml in SDS PAGE sample buffer. This was then loaded in parallel with standards of the recombinant proteins and resolved using 12% acrylamide gels and blotted onto Immobilon P membrane as described in Methods and Materials, before being probed with the appropriate antibodies and developed using chemiluminescent methods. In order to improve the linearity of the response from the film (ECL-Hyperfilm from Amersham Int. Ltd.) it was preflashed using a standard photographic flash-lamp, suitably diffused by trial and error to give optimum results.

Once the blots were developed, the images were scanned onto computer discs in TIFF format using a flat bed scanner. Densitometry was then performed on the images using TINA software and the data obtained from the standards plotted out in terms of densitometric units per square millimetre. The plotted data from the densitomety performed and the Western blots examined are shown in Fig. 7.3 to 7.6.

Using these methods it was possible to ascribe an approximate value to the concentration of PI-TPβ within the various cells examined, both in terms of percentage total protein and in terms of μg of PI-TP per cell. In addition it was possible to arrive at an estimate of the concentration of PI-TP in the cytosol of the cell. In the case of HL60 cells, PI-TPβ was estimated as representing 0.03% of total protein, with $5.5x10^{-8}$ μg being present in the cell. This yielded a final concentration of 275μg/ml. In the case of the neutrophil, PI-TPβ was estimated as representing 0.04% of total cellular protein, with $7.7x10^{-8}$μg being present in the cell. This yielded a concentration of 385μg/ml in cytosol. These latter values are estimated on the basis of the volume of HL60 and neutrophils cells being approximately 200fl. This value means that the concentration of PI-TPβ in neutrophil and HL60 cytosol is in the region of 7.5-10μM.

One of the most striking observations to be made using these methods was the fact that PI-TPα was present in both HL60 cytosol and neutrophil cytosol in almost

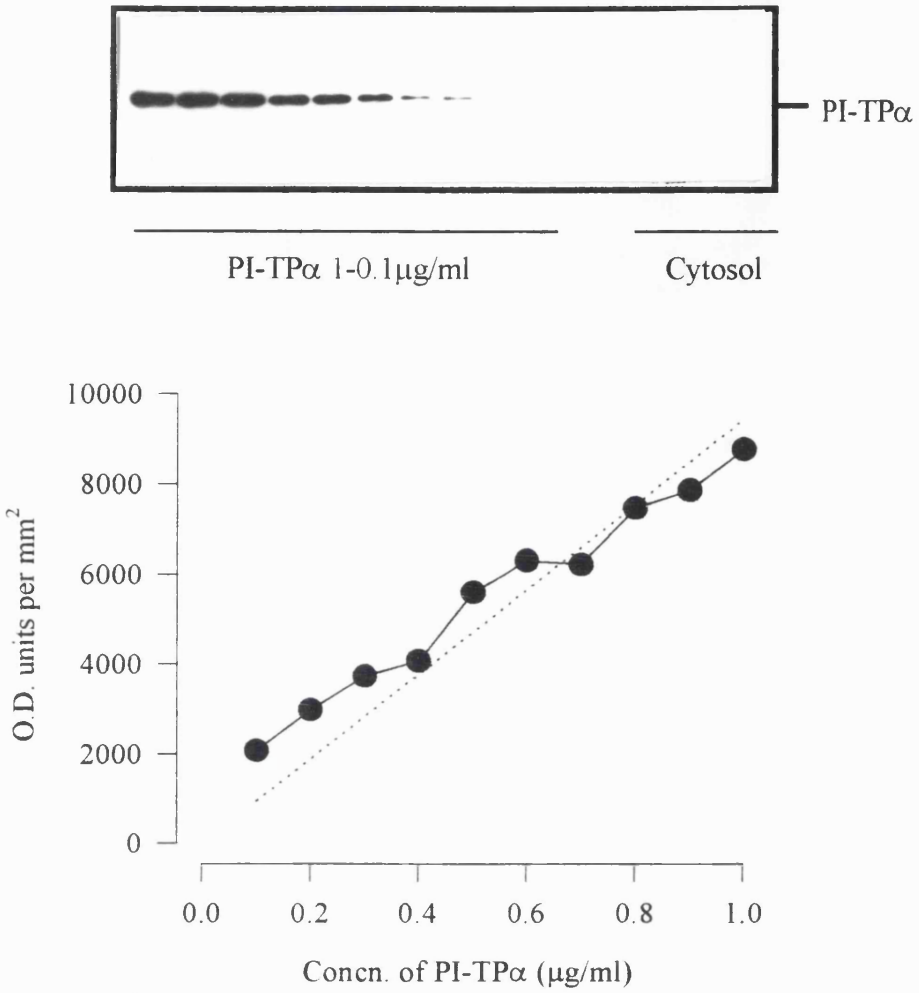


Fig 7.3 Quantification of PI-TPα in undifferentiated HL60 cells.

HL60 cells were cultured as described in Chapter 2, and disrupted by means of a probe sonicator (3x10 s pulses, with cooling on ice between treatments). A sample of material was examined against untreated cells, and the degree of cell disruption assessed visually as being in excess of 95%. Membranes,and undisrupted cells were removed by centrifugation at $10x10^3$xg for 15 mins. The total protein present in the cytosol was measured using the Bradford assay, and the samples diluted to a total concentration of 1mg/ml in SDS PAGE sample buffer. This was then loaded neat, diluted 1:1, and diluted 1:3 This was then resolved, blotted, and probed with the monoclonal antibody 5F12. The resultant blot was then scanned using a flat bed scanner, and the image analysed using TINA software. A standard curve was constructed using the recombinant standards, against which the amount of protein in the prepared cytosol was judged. No cytosolic PI-TPα was detected. The dotted line represents a regression calculated using SigmaPlot on the basis of the data.

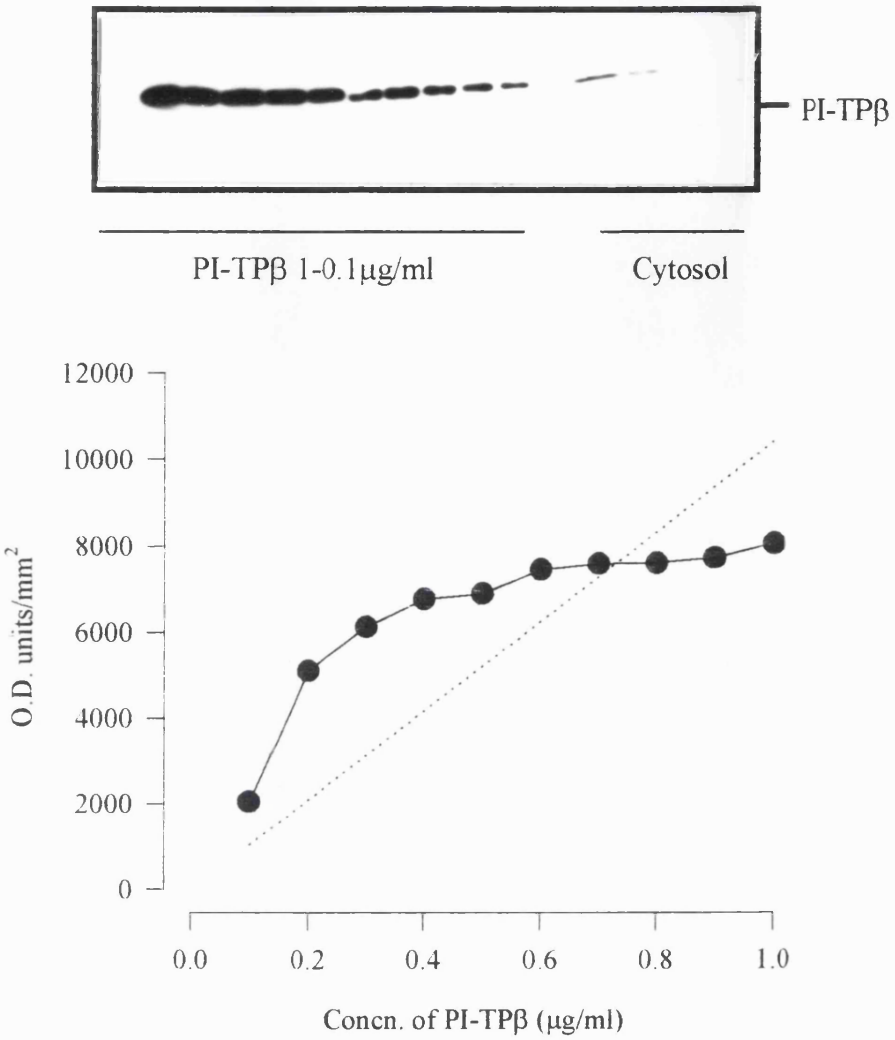


Fig 7.4 Quantification of PI-TPβ in undifferentiated HL60 cells.

HL60 cells were cultured as described in Chapter 2, and disrupted by means of a probe sonicator (3x10 s pulses, with cooling on ice between treatments). A sample of sonicated material was examined against untreated cells, and the degree of cell disruption assessed visually as being in excess of 95%. Membranes, and undisrupted cells were removed by centrifugation at $10x10^3$xg for 15 mins. The total protein present in the cytosol was measured using the Bradford assay, and the samples diluted to a total concentration of 1mg/ml in SDS PAGE sample buffer. This was then resolved, blotted, and probed with monoclonal antibodies specific for PI-TPβ. The resultant blot was then scanned using a flat bed scanner, and the image analysed using TINA software. A standard curve was constructed using the recombinant standards, against which the amount of protein in the prepared cytosol was judged. The dotted line represents a regression calculated by the computer using SigmaPlot software on the basis of the densitometric data. Cytosol at 1mg/ml yielded a value of $6.9x10^3$ O.D units per mm^2.

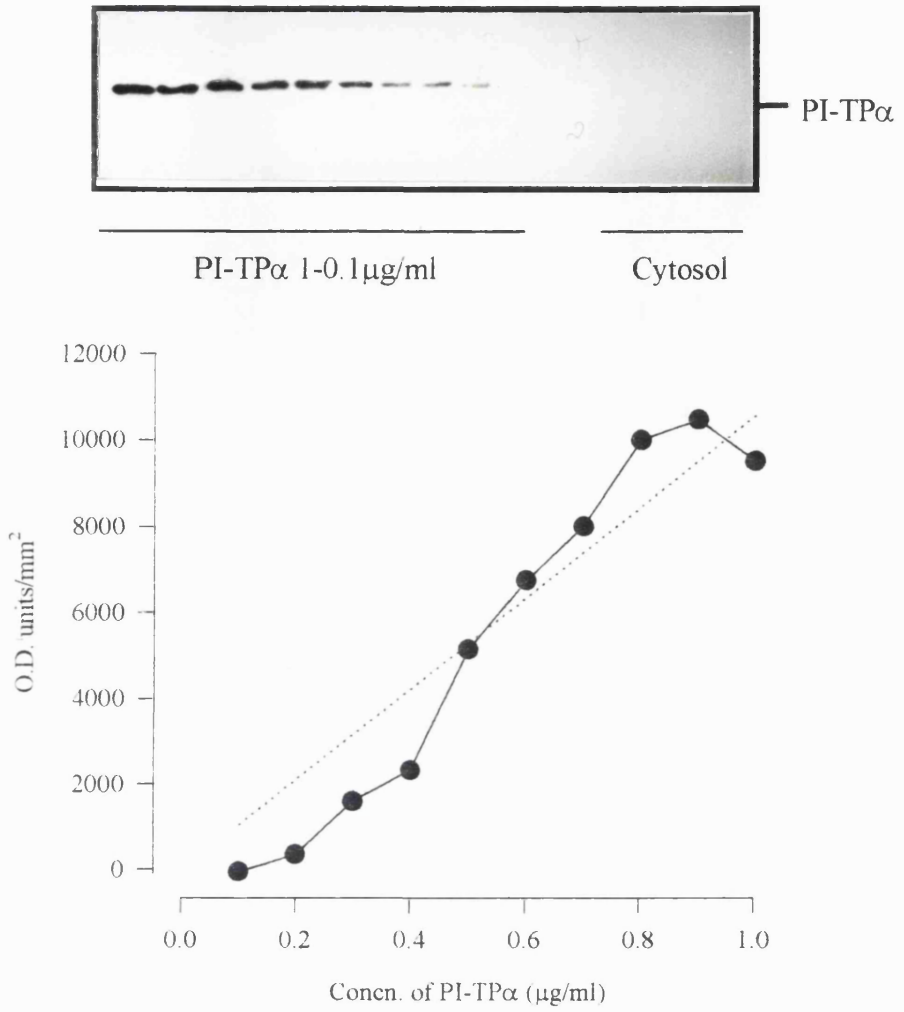


Fig 7.5 Quantification of PI-TPα in peripheral blood neutrophils.

Neutrophils were prepared as described in Chapter 2, and disrupted by means of a probe sonicator (3x10 s pulses, with cooling on ice between treatments). A sample of material was examined against untreated cells, and the degree of cell disruption assessed visually as being in excess of 95%. Membranes, and undisrupted cells were removed by centrifugation at $10x10^3$xg for 15 mins. The total protein present in the cytosol was measured using the Bradford assay, and the samples diluted to a total concentration of 1mg/ml in SDS PAGE sample buffer. This was then resolved, blotted, and probed with the monoclonal antibody 5F12. The resultant blot was then scanned using a flat bed scanner, and the image analysed using TINA software. A standard curve was constructed using the recombinant standards, against which the amount of protein in the prepared cytosol was judged. No cytosolic PI-TP was visualised in these samples.

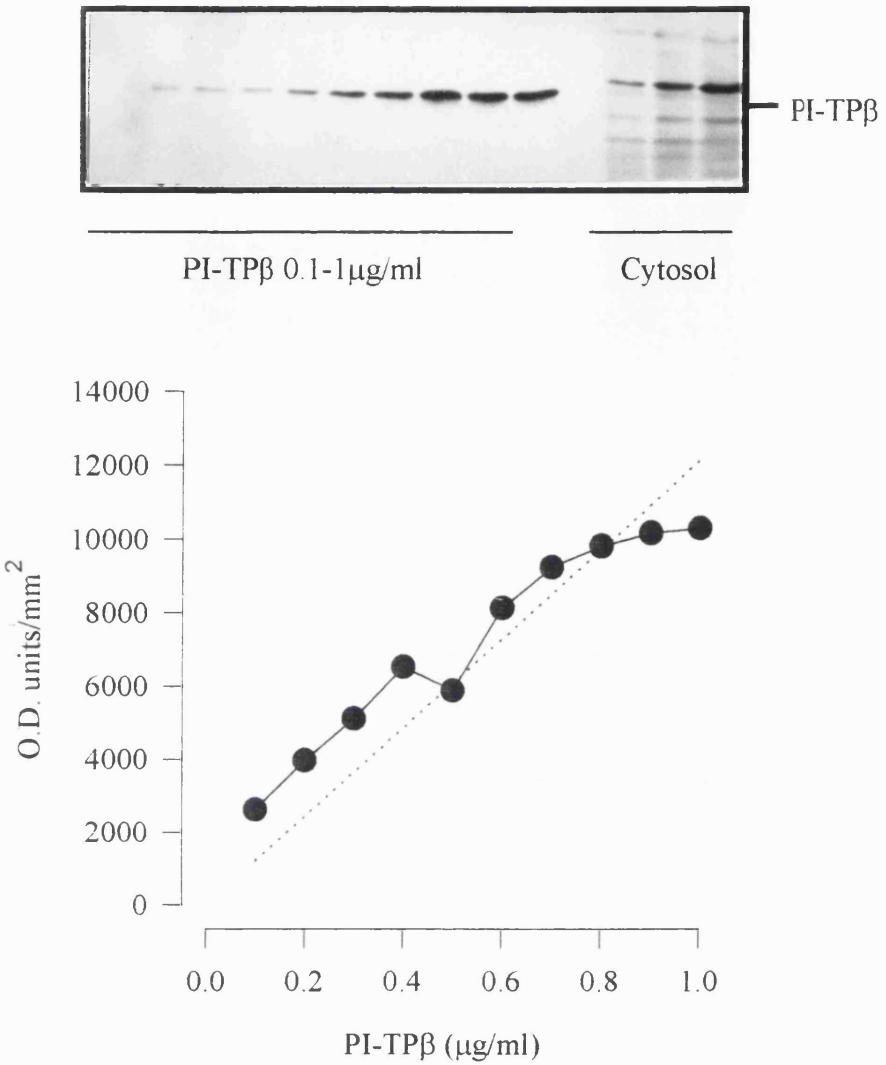


Fig 7.6 Quantification of PI-TPβ in peripheral blood neutrophils.

Neutrophils were prepared as described in Chapter 2, and disrupted by means of a probe sonicator (3x10 s pulses, with cooling on ice between treatments). A sample of material was examined against untreated cells, and the degree of cell disruption assessed visually as being in excess of 95%. Membranes,and undisrupted cells were removed by centrifugation at $10x10^3$xg for 15 mins. The total protein present in the cytosol was measured using the Bradford assay, and the samples diluted to a total concentration of 1mg/ml in SDS PAGE sample buffer. The material was loaded neat, diluted 1:1, and 1:3. This was then resolved, blotted, and probed with a mixture of the monoclonal antibodies 1C1, 1B11, 4A7, and 8B8. The resultant blot was then scanned using a flat bed scanner, and the image analysed using TINA software. A standard curve was constructed using the recombinant standards, against which the amount of protein in the prepared cytosol was judged. Cytosol at 1mg/ml yielded a value of $4x10^3$ O.D. units per mm^2.

vanishingly small amounts. With the data available from the procedures described the extent of the disparity between the two isoforms of PI-TP cannot be accurately determined - a reliable ELISA system may provide better data.

Examination of PC12 and RBL 3H3 cytosol revealed a somewhat different state of affairs, with the PI-TPα being represented in the cytosol of these cells at levels comparable to that found for the β isoform. Levels of PI-TPβ were similar to those observed in HL60 cells and neutrophils, as assessed by eye.

In addition to examining the cytosol of PC12 and RBL 3H3 cells, it was also decided to examine the crude membrane material left in the post nuclear supernatant. To this end, the membrane fraction remaining after ultracentrifugation was further washed and dissolved in SDS PAGE sample buffer for resolution in parallel with the cytosolic material. The results of Western blotting this material from both PC12 and RBL 3H3 cells is shown in Fig. 7.7. Since earlier attempts to detect PI-TP in the membranes of HL60 cells using the polyclonal antibody had previously failed (Data not shown) the membrane fraction from preparations of HL60 cytosol was not initially examined. It was decided to investigate the effect of differentiation of HL60 cells using dibutyryl cAMP, which drives the cell towards a terminally differentiated neutrophil like state. At the same time, membrane fractions from the two cell types were examined. The results of these experiments are illustrated in Fig. 7.8. In order to ensure that equivalent loading of the material derived from differentiated and undifferentiated cells was similar, a 12% SDS PAGE gel was run and stained with standard Coomassie.

As can be observed, the differentiated material appears to contain slightly more PI-TPβ than the undifferentiated cells, an effect that was consistently observed. This may well reflect the fact that upon differentiation, the HL60 cell starts to express membrane receptors for a number of ligands including the chemotactic peptide fMLP. Hence it could be expected that the demands placed upon the differentiated cell, or neutrophil in terms of inositol lipid signalling would be increased, leading to en elevation of the level at which PI-TP is expressed in order to ensure the efficient supply of substrate. This also agrees with the observation that neutrophils have slightly higher concentrations of PI-TPβ than undifferentiated HL60 cells.

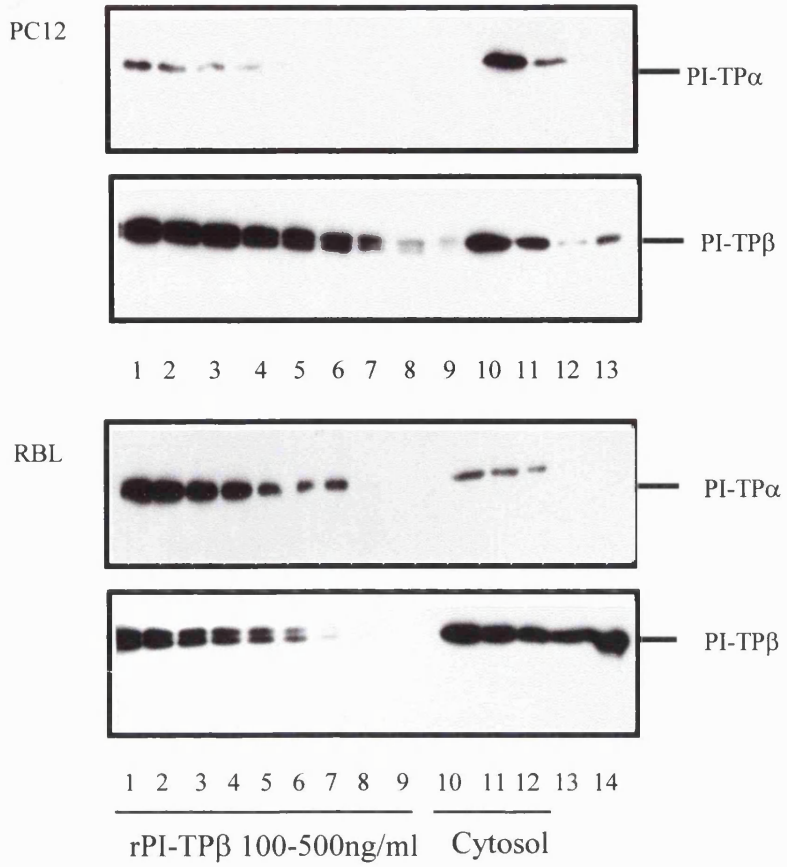


Fig 7.7 Western blot analysis of PC12, and RBL cells - probing for PI-TP α and, β in membranes, and cytosol.

PC12, and RBL cells were scraped, counted, and resuspended in 20mM PIPES, 137mM NaCl, and 3mM KCl. The cells were then subject to sonication in the same manner as the neutrophil, and HL60 cells examined earlier. The total protein concentration was adjusted to 1mg/ml, and the washed membranes from the preparation of the cytosol were dissolved in SDS PAGE sample buffer. This material was then resolved in parallel with rPI-TP standards ranging from 500 to 100ng/ml in 50ng steps. As can be observed, the signal obtained for both isoforms in the two cell types examined is of similar strength. This does not correspond to the situation observed in neutrophils, and HL60 cells, in which PI-TPα is unmeasurable by this method.

PC12: Lanes 1-9 contain the titration of rPI-TP.
Lanes 10-11 contain cytosol, neat, and diluted 1:1 PC12
Lane 12 contains solubilised nuclear pellet
Lane 13 contains solubilised membrane material.

RBL Lane 1-9 contains the titration of rPI-TP
Lane 10-12 contain cytosol, neat, and diluted 1:1, and 1:3
Lane 13 contains solubilised nuclear pellet
Lane 14 contains solubilised membrane material

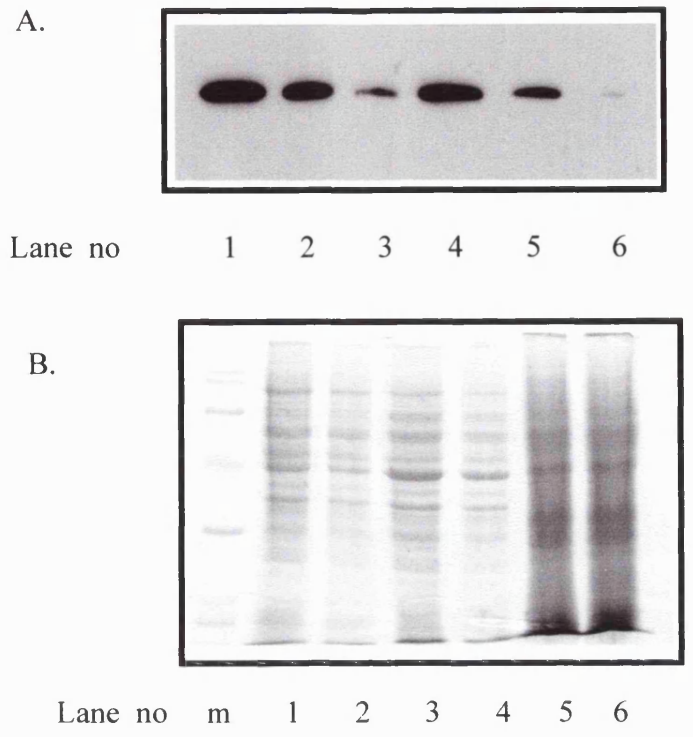


Fig 7.8 Upregulation of PI-TPβ in differentiated HL60 cells.

In order to examine the effect of differentiation upon HL60 cells, and the expression of PI-TPβ, HL60 cells were prepared as described in Chapter 2. $50x10^6$ cells were differentiated using 300μM dibutyryl cAMP for 48 hours. $50x10^6$ undifferentiated HL60 cells were cultured. Both sets of cells were washed, and resuspended in 20mM PIPES, 137mM NaCL, 3mM KCL, and treated with 10μM DFP for five minutes. The cells were then disrupted by sonication, and a post nuclear supernatant prepared by centrifugation at 750xg. This PNS was then clarified by centrifugation at $100x10^3$g at 4°C for 30 mins., and the cytosol removed. The pellets were then resuspended in 1ml of PIPES saline as above, and washed. The resultant crude membrane prep was then resolved by SDS PAGE in parallel with cytosol which had been adjusted to a final concentration of 1mg/ml. Western blots on Immobilon P PVDF membrane were prepared, and probed using a mixture of the monoclonal antibodies 1B11, 1C1, 3A7, and 8B8. In order to assess the equity of loading, a 12% gel of the material was prepared, and stained using standard Coomassie staining. The resultant Western blot is shown in Fig. 7.8A (lane 1, and 2 are 50μl, and 25μl of differentiated cytosol. Lane 3 contains 50μl dissolved membranes. Lanes 4, 5, and 6 are equivalent undifferentiated samples).

A Coomassie stained gel is shown in Fig. 7.8B (Lane 1 and 2 are 50μl, and 25μl of differentiated cells, and lane 3, and 4 are equivalent undifferentiated samples. Lanes 5, and 6 contain solubilised membranes from $50x10^6$ differentiated, and undifferentiated cells respectively.

7.3 Examination of sucrose density gradient resolved membrane fractions:

In an attempt to further characterise the apparent association between membranes and the PI-TPβ isoform, fractions prepared from HL60 cells disrupted using a ball-bearing cell-cracker and resolved into distinct membrane fractions were examined by SDS-PAGE and Western blotting. This however failed to reveal the presence of PI-TP in any fraction other than the cytosol, with no immunoreactivity being detected in any of the membrane fractions investigated. The material for screening in Western blots was provided by Dr. J. Whatmore from this lab. The results of this investigation are illustrated in Fig. 7.9. The cytosolic portion of the gradient is represented by fractions 15-17.

It is suggested that the interaction of this protein with the membranes under investigation is such that it is disrupted by preparing membranes in this fashion and the protein is lost from the membrane to the cytosol. Certainly there is reason to believe that the observed association of PI-TPβ with the membrane is not an artefact, as comparable effects have been observed in studies of the intracellular distribution of the two PI-TP isoforms by immunofluorescence (167), i.e. that the β isoform of PI-TP is observed to be retained in the permeabilised cell to a significantly greater degree than the alternative isoform. That this observation is not purely an artefact of the low levels of PI-TPα expressed in HL60 cells resulting in poor detection with the antibody is supported by the observation that PI-TPβ is quite clearly detectable in the membranes of PC12 and RBL 3H3 cells, while PI-TPα which is expressed at comparable levels is not found in washed membranes. The preparation of membrane fraction using the cell cracker and sucrose density gradient centrifugation, while providing an efficient means of resolving many of the intracellular membranes into distinct fractions represents a long and protracted protocol involving a rigorous physical disruption of the cell coupled to a 16 hour centrifugation step. Since PI-TP is not an intrinsic membrane protein and by virtue of the hypothesised mechanisms by which it operates, it is suggested that any association with membranes is fairly weak and transient. This protracted handling of the material prior to examination

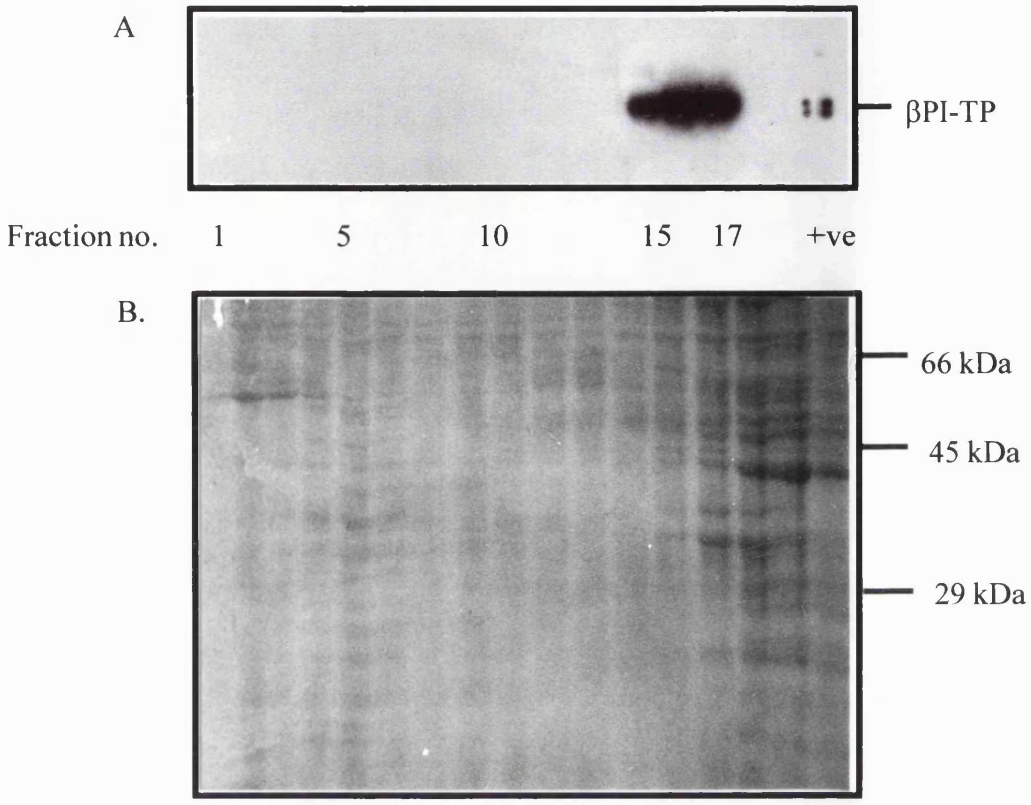


Fig 7.9 Western blotting of sucrose density gradient fractionated HL60 cells - probing for PI-TPβ in distinct membrane compartments.

$3.5x10^6$ cells were cultured in RPMI as per the normal protocol. The cells were thendisrupted using a ball bearing cell cracker. The disrupted cells were examined with a microscope, and were found to be disrupted in excess of 90%. The material was then resolved on a sucrose density gradient from 10.4% to 50% at $20x10^3$g for 16 hours at 4°C . This fractionation was prepared by Dr. J.Whatmore in this lab.

66µl samples of fractions from the gradient were treated with 3x sample buffer, and resolved by SDS PAGE. The gel was then Western blotted, and probed with monoclonal antibodies specific for PI-TPβ. As can be seen, a strong signal for PI-TP was observed corresponding to that portion of the gradient expected to contain cytosolic components but at no other points in the gradient (Fig. 7.9A.). This probably reflects the relatively weak, transient nature with which it is hypothesised that PI-TP interacts with membranes. Fig. 7.9 B shows a Coomassie stained gel of the material.

gives ample opportunity for the dissociation of the protein and its loss to the cytosolic phase. Another consideration in this analysis is that in the course of fractionating the various cellular membranes, the degree of dilution which the sample undergoes is considerably greater than in the studies utilising crude preparations of membranes.

7.4 Translocation of PI-TPβ to membranes and leakage experiments:

Having established the presence of PI-TPβ within membrane fractions of the cell it was decided to investigate whether stimulation of the cell using a variety of agents affected the distribution of the protein between membrane and cytosolic components.

7.4.1 Stimulation of HL60 cells with PMA

Work conducted in the laboratory of K.Wirtz had already demonstrated the fact that stimulation of the Swiss 3T3 cells with PMA led to elevated levels of phosphorylation of PI-TP and was associated with a redistribution of the protein into the Golgi complex. On the basis of these data it was decided to examine the influence of PMA on the kinetics with which PI-TPβ exited from SLO permeabilised cells.

Earlier attempts to examine any effects on the behaviour of PI-TP using the polyclonal antibody failed to reveal differences in the kinetics of the leakage process. Given the later demonstration of the fact that this reagent very much favours the recognition of the α isoform of PI-TP (Demonstrated by Dr. N. Goldring in this lab - data not shown) it was considered possible that any retained PI-TPβ within the cell may have been below the threshold of detection. Previous work in this lab had demonstrated that by the end of the first five minutes of permeabilisation, all of the immunoreactive material detectable using the polyclonal antibodies had exited from the cell.

For the purposes of these experiments, $50x10^6$ HL60 cell were prepared as per the methods described in Chapter 2. The cells were resuspended in the usual permeabilisation buffer, to the appropriate volume, divided into two 2.5 ml aliquots

and allowed to stand for 15 minutes at 37°C to recover. At the end of this period, one portion of the cells was stimulated by pre-treatment with 10nM PMA for five minutes prior to permeabilisation. At the end of this period, 180µl cells were dispensed into individual Eppendorf tubes maintained on ice. 20µl of permeabilisation cocktail was then added and the assay transferred to a 37°C water bath. The samples were removed from the incubation at time points of t=0, 30s, 60s, 120s, 180s, 300s, 600s and 900s and the solid cellular material removed from the permeabilisation supernatant by a 5s pulse spin in a microfuge. The samples of supernatant and the cell pellet were then maintained on ice until the end of the assay, when 100µl of 3x concentrated SDS PAGE sample buffer was added to the supernatant, while the cell pellet was treated with 100µl of RIPA buffer for 15 minutes on ice with occasional mixing using a vortexer. At the end of the RIPA extraction, the insoluble material was pelleted by centrifugation at $10x10^3$g for 15 mins at 4°C, the solubilised material retained and treated with 50µl of concentrated sample buffer. The material prepared in this way was then resolved by SDS PAGE and blotted onto PVDF before being probed with PI-TPβ specific antibodies.

The results of these experiments are shown in Fig. 7.10. As can be seen, the accumulation of PI-TPβ in the supernatant is accompanied by a concomitant lose of PI-TP from the permeabilised cell. Results from stimulated cell pellets are shown adjacent to unstimulated cells and the supernatants from stimulated cells are shown in juxtaposition with those from the unstimulated sample. As can be seen, the accumulation of PI-TP in the supernatant in both sets of samples is similar and the loss of PI-TP from the permeabilised cell appears to follow identical time courses. Densitometric analysis of the blot using TINA software showed that in both cases approximately 20% of PI-TPβ was retained in the cells pellet.

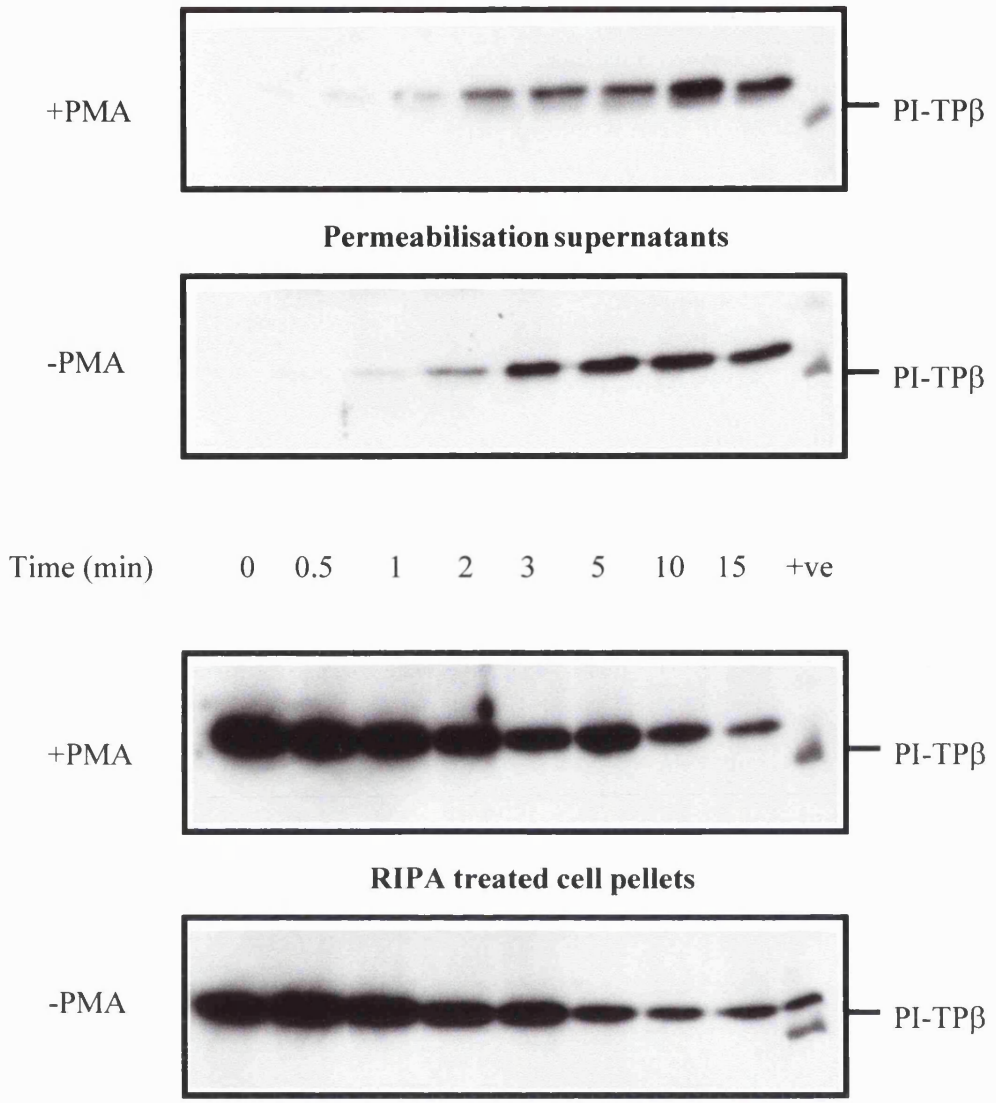


Fig 7.10 Effect of PMA on kinetics of leakage of PI-TPβ from SLO permeabilised HL60 cells.

HL60 cells were cultured, washed,and resuspended in a PIPES based saline buffer, as described in the protocol for PLC reconstitution in Chapter 2. Prior to permeabilisation the cells were incubated at37°C in the presence, or absence of 10nM PMA. 180µl of the cells were then dispensed into tubes containing 20µl of SLO reagent cocktail maintained in ice. The cells were then transferred to a 37°C water bath, and at time points shown, pelleted by pulse spin in a microfuge. The supernatant was then removed, and the residual cell pellet treated with RIPA buffer before being centrifuging at $10x10^3$g for 15 minutes. The RIPA supernantant was then blotted in parallel with the permeabilisation supernatant. RIPA extract lanes represent 1.2 x 10^6 cells. Permeabilisation supernatants represent 0.6 x 10^6 cells.

7.4.2 Effect of 5μM GTPγS on the kinetics of PI-TPβ leakage

$50x10^6$ cells were prepared according to the standard culture protocol described in Chapter 2. The cells were then washed and resuspended in 5ml of 20mM PIPES based buffer as described in Chapter 2 for PLC reconstitution of PLC. Permeabilisation cocktail was then prepared and divided into two aliquots. One of these had GTPγS added such that the final concentration of GTPγS in the system was 5μM. 450μl of the cell suspension was then placed in tubes maintained on ice. 50μl of the permeabilisation cocktail was then quickly added to each tube and the experiment transferred to a 37°C water bath. Samples were removed from the permeabilisation at time points t=0 , 0.5, 1, 2 ,3 , 5, 10 and 15 minutes and the cells immediately pelleted by pulse centrifugation in a microfuge. The cell residues were then extracted using 100μl of RIPA buffer on ice for 15 mins, while the supernatant was treated with 100μl of 3xSDS PAGE sample buffer. Insoluble material was then removed from the RIPA treated cell pellets by centrifugation at $10x10^3$ rpm in a chilled centrifuge for 15 minutes. The supernatant was removed and treated with 3xSDS PAGE buffer. Both sets of samples were then resolved and Western blotted, before being probed with a mixture of monoclonal antibodies characterised as being specific for PI-TPβ.

The results are illustrated in Fig 7.11. Samples are paired in the figure, with unstimulated cell being shown in parallel with their stimulated counterparts. As can be seen, there appears to be some effect of GTPγS on the kinetics with which PI-TPβ exits from the cell. In the presence of GTPγS, loss of the protein from the cell appears to be more rapid and complete by the end of the time course than in the case of the unstimulated cells. Densitometric analysis of the resultant Western blot shows that in the case of the unstimulated cells, after 15 minutes of permeabilisation approximately 30% of PI-TPβ is still present in the cell as compared with 18% in the GTPγS stimulated sample. This was performed by scanning the Western blots using a flat bed scanner and measuring the optical density of the bands using TINA software. These observations are preliminary results, but the apparent observed effect merits further investigation.

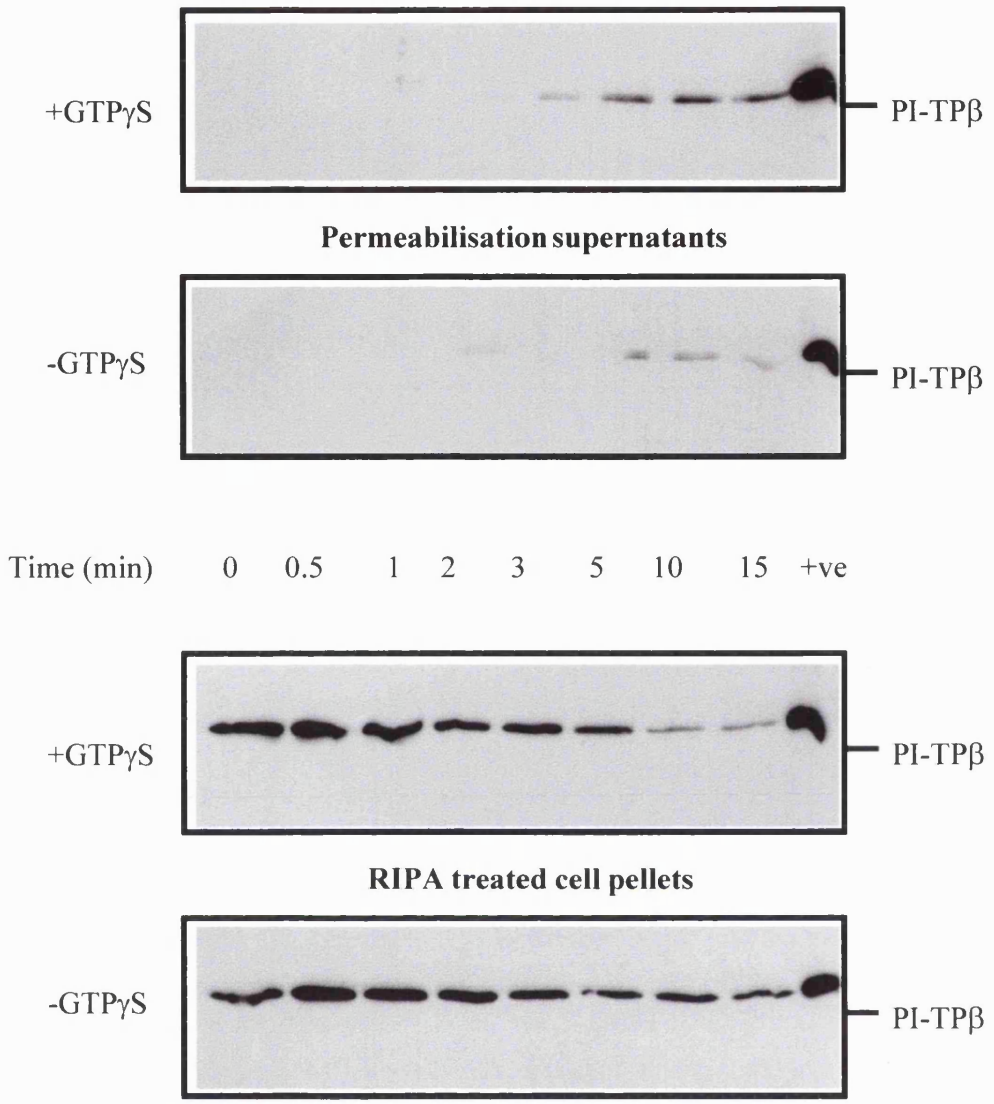


Fig 7.11 Effect of GTPγS on kinetics of leakage of PI-TPβ from SLO permeabilised HL60 cells.

HL60 cells were cultured, washed,and resuspended in a PIPES based saline buffer, as described in the protocol for PLC reconstitution in Chapter 2. 180µl of the cells were then dispensed into tubes containing 20µl of SLO reagent cocktail maintained in ice, in the presence , and absence of 5µM GTPγS. The cells were then tansferred to a 37°C water bath, and at time points shown, pelleted by pulse spin in a microfuge. The supernatant was then removed, and the residual cell pellet treated with RIPA buffer before being centrifuged at $10x10^3$g for 15 minutes. The RIPA supernantant was then blotted in parallel with the permeabilisation supernatant. RIPA samples represent $1.2x10^6$ cells, and permeabilisation supernatants $0.6x10^6$ cells.

7.5 Discussion:

With the development of the β specific antibody panel, many of the problems previously encountered were explained when it became apparent that HL60 cells and neutrophils did not reflect the situation in the cytosol prepared from bovine, or rat bran tissue, or any of the other cell types examined i.e. they were unusual in that the PI-TPα was represented at considerably lower levels than the β isoform.

The PI-TPβ specific antibodies facilitated the discovery that physiological levels of PI-TP found within the cell were comparable to those added back to cytosol depleted cells in the earlier work of this lab which led to the identification of PI-TP as the PLC reconstituting factor. It has been demonstrated that both the α and β isoforms are capable of reconstituting PLC activity in SLO permeabilised HL60 cells with equal efficiency. It will now perhaps prove more feasible to study the function of the β isoform of PI-TP in these cells by making use of the immunoprecipitating ability of the panel of antibodies raised against PI-TPβ.

One important thing to note from the experiments using PMA and GTPγS stimulated cells to examine the kinetics of PI-TPβ loss is the contrast between the observation made here, that after 15 minutes of permeabilisation, PI-TPβ is still clearly visible in the cell pellet and that using the polyclonal antibody which only poorly recognises PI-TPβ, all of the protein has leaked from the cell after 5 minutes, as illustrated in Chapter 4. This suggests that even in the case of cells which have been permeabilised for time periods beyond this, there are still likely to be traces of PI-TPβ in the cell. Hence the observed basal response of permeabilised cells in the absence of exogenously added PI-TP never really takes place in the absence of this protein. Further, this is in close agreement with other observations by Snoek et al that the β isoform of PI-TP is specifically associated with intracellular membranes (202, 204).

The observation that GTPγS leads to an increase in the rate at which PI-TPβ is lost from the permeabilised cell is of interest. It is proposed that this situation could arise as a result of increased turnover of inositol lipids in response to the stimulus. This may necessitate an increase in the supply of PI from the site of synthesis in the E.R. and lead to increased cycling of PI-TPβ between the membrane and cytosolic

phases. In the latter state, PI-TPβ would be free to leak from the permeabilised cell. Table 7.1 below shows the relative percentage of PI-TPβ remaining within permeabilised, RIPA buffer extracted cells after 15 minutes of permeabilisation under the stimuli discussed. This was calculated on the basis of densitometric measurement performed on the electronically scanned images. It should be borne in mind that at earlier time points in the permeabilisations, high signal intensities were observed compared with those attained at t=15mins. It is not known whether the response of the film to this range of signal intensity is linear - consequently at t=0 mins the image may be more nearly saturated than at later time points. Thus the quoted percentage values may represent an overestimate of the protein remaining.

Stimulus	Percentage of PI-TPβ remaining at t=15mins
Cells permeabilised in the absence of GTPγS	32.6%
Cells permeabilised in the presence of 5μM GTPγS	18%
Cells permeabilised after 5 mins. incubation in the absence of PMA	23%
Cells permeabilised after 5 minutes preincubation with 10nM PMA	21.5%

Table 7.1 Estimated percentages of PI-TPβ remaining in permeabilised cells under stimulation with GTPγS, or PMA.

It has been found that preparations of membranes exhibit very low levels of PLC activity upon stimulation with GTPγS when compared with those produced using permeabilised HL60 cells. It is perhaps significant that sucrose density fractionated HL60 membranes appeared to have no PI-TPβ associated with them, while permeabilised cells had quite readily detectable amounts of PI-TPβ even after

protracted permeabilisation. It must however be borne in mind that in the course of fractionation of the cellular membranes on a sucrose density gradient samples undergo a significantly greater degree of dilution than in the cruder freeze thawed, or sonicated material.

Another point of particular interest is the estimate of concentration of PI-TP in the cytosol of neutrophils and HL60 cells. The values determined using the methods described here are of the same order of magnitude anticipated from earlier work on PLC reconstitution and confirm that the quantities of protein added back to cytosol depleted cells during the initial studies on PI-TP are physiologically relevant.

Chapter 8

Discussion

The initial aim of this project was to examine the Phospholipase C isozymes present in neutrophils and HL60 cells. With this in view, various immunological reagents were deployed in order to identify those isoforms present. The isoforms which were unequivocally identified were the β2 and β3, G-protein regulated PLCs. Examination of the kinetics with which these proteins leaked from SLO permeabilised HL60 cells revealed discrepancies between this effect and the decay of GTPγS stimulation of PLC activity, implying that the decay was attributable to the loss of some other soluble cytosolic factor from the cell. Much of the work described in this thesis concerns the development of the reagents necessary for the study of this lost factor, later identified as being the mammalian PI-TP.

Further studies examining membrane fragments generated by freeze thawing, or sonication of cells also revealed that the PLCs β2 and 3 appeared to be associated with the membrane. These findings correlate with the state of affairs observed by Downes and Vaziri in turkey erythrocytes. They observed that a PLC activity analogous to, but immunologically distinct from those observed in mammalian systems, was present in the cytosol in considerable excess to that required to meet the needs of the cell under physiological levels of stimulation and that some of this activity appeared to be bound in close juxtaposition to the membrane on cytoskeletal components (18).

Simultaneous investigations being conducted in this lab into the nature of the soluble factor already proposed as being responsible for the decay of PLC signalling had led to the identification of the Phosphatidylinositol Transfer Protein as this factor (2). In order to further study this protein a polyclonal rabbit antiserum was developed against a PI-TPα/GST fusion protein. Using this antibody it was demonstrated that PI-TP leakage from SLO permeabilised HL60 cells followed kinetics which were very similar to those for the decay of GTPγS responsiveness. Examination of the effect of various stimuli on the cell, including GTPγS and PMA treatment did not appear to influence the kinetics of leakage. This study was however complicated by the

apparently very low levels of the protein present in the cytoplasm - in order to obtain a clear signal for the protein in Western blots of permeabilisation supernatants it was necessary to precipitate total protein using TCA and employ protracted exposures using ECL Western blots. This was not reflected by the situation with the PLCs β which were found in HL60 cells - no precipitation step was required for the visualisation of PLCβ2 and 3 in permeabilisation supernatants - as signalling proteins it was anticipated that they should be present as a relatively low percentage of total cellular protein. This appeared to suggest that the PI-TP may be present in HL60 at lower concentrations than were initially anticipated on the basis of reconstitution observed per μg of purified PI-TP added back to permeabilised cells in which the GTPγS response had been allowed to decay.

It was further demonstrated that PI-TP was present in human neutrophil cytoplasm. Utilising the reagents then available however, the protein was not detected in association with membrane components of the cell.

Attempts were made to further capitalise on this reagent by attempting to immunoprecipitate PI-TP. This however proved impossible with the reagents then to hand. Similarly, attempts to quantify the protein using the reagents available were not possible, as the sera required affinity purification before use and while obviously containing high affinity antibodies directed against PI-TP, were not at sufficiently high concentrations to make their application as a capture phase for an immunoassay practicable. Constraints were also placed upon the availability of PI-TP purified from brain tissue because of the labour intensiveness of the purification protocol. While a distinct signal was obtained from both neutrophil and HL60 cell cytosol, using the polyclonal reagent, this was never sufficiently intense for an accurate determination of quantities to be possible by quantitative chemiluminescent Western blotting.

Because of the various problems associated with the use of the rabbit sera, it was decided to raise a panel of monoclonal antibodies against protein purified from bovine brain tissue. To this end, a more rapid partial purification protocol was developed utilising DE52 anion exchange chromatography and gel filtration. This proved adequate for the production of material for immunisations and screening assays. Characterisation of the five members of the antibody panel revealed that four

of them were specific for PI-TPα, while the fifth member of the panel was an IgM specific for the small G-protein Arf which co-purified with PI-TP.

It was deemed desirable to raise antibodies against the β isoform of PI-TP. Shortly before this time, recombinant forms of both the α and β PI-TPs came on stream and it became possible to utilise this material for the purposes of immunisations into a panel of mice.

The recombinant materials were initially generated as C-terminally His tagged proteins to facilitate their purification using Ni^{2+} resin. Of particular interest was the observation that the α specific monoclonal antibodies 5F12 and 1A4 failed to recognise these proteins, while working well in Western blots of native material. Subsequently developed N-terminally tagged protein was recognised efficiently by both of the antibodies. From this it was concluded that the antibodies were specific for the carboxy terminus. This characterisation was further enabled by the development of a panel of C-terminal deletion mutants in this lab by Shuntaro Hara. These truncation mutants, designated Δ5, 10 and 20, were examined using Western blotting. The deletion of the first five residues from the protein completely abrogated recognition of the protein and from this it was concluded that the antibody was specific for a region encompassed by the carboxy terminal decapeptide.

This antibody was also found to strongly immunoprecipitate PI-TPα from brain cytosols and also from both neutrophil and HL60 cytosols., in the presence of low concentration of the denaturing detergent SDS. Making use of this property it, was attempted to immunoprecipitate PI-TP and associated proteins from HL60 and neutrophil cytosol - this however did not prove viable and yielded very inconsistent results, with no other proteins being consistently brought down with the precipitated PI-TP. Similarly, attempts to study the phosphorylation of PI-TPα in HL60 cells and neutrophils, while demonstrating that phosphorylation appeared to be taking place in response to stimulation of cells with PMA and fMLP, yielded relatively small quantities of labelled protein, such that phosphoamino acid analysis was not possible.

With the arrival of recombinant proteins and the development of specific monoclonal antibodies, the tools were in place to quantify the two isoforms. Early

quantifications of PI-TP in HL60 cells using the 5F12 PI-TPα specific monoclonal antibody had revealed that the protein was present in apparently surprisingly low amounts within the cytosol of these cells. Comparison of these results with those obtained using the polyclonal antibody which recognises both isoforms suggested that there was the additional presence of the β-isoform in these cells. Attempts to determine the presence of PI-TP at the cell membrane failed to detect significant levels of the protein.

The development of the second panel of monoclonal antibodies against the β isoform permitted the specific quantification of PI-TPβ. It was with some surprise that the preponderance of this protein in neutrophils and HL60 cells was determined. Quantitatively, this isoform is by far the most important of the two proteins and represents a very significant proportion of the cellular total protein, being represented by at least an order of magnitude more than the α isoform. This accounts for the poor results obtained in immunoprecipitation experiments conducted on HL60 cells and neutrophils despite the demonstration that almost quantitative immunoprecipitation of PI-TPα from rat brain cytosol was possible using the monoclonal antibody 5F12.

The situation in PC12 and RBL cells was also investigated. It was revealed that in these cells, the α-isoform was expressed at a significantly higher percentage of total protein than was found to be the case in HL60 cells and that the levels of both proteins were roughly comparable. This possibly reflects subtle functional differences between the two proteins. Both PC12 and RBL cells are derived from cells involved in regulated exocytosis - in the case of PC12 cells, adrenal chromaffin cells and in the case of RBL cells, mast cells. It has been demonstrated by Hay et al using PC12 cells that a PI-TP activity is involved in priming of cells for exocytosis (4). Ohashi et al have similarly identified a function for PI-TP in the formation of secretory vesicles (205).

With the benefit of hindsight it can be appreciated that the polyclonal antibody preparation used for the initial probing and quantification yielded results skewed in favour of the α-isoform. This reagent was raised utilising an PI-TPα/ GST fusion protein and purified originally utilising similar material - once recombinant PI-TPα came on stream, this was used for the production of an affinity column for antibody

purification. While a number of epitopes are obviously shared between α and β PI-TP, a significant portion of the immunoreactivity of the sera was directed against epitopes unique to the α isoform. Purification of these antibodies using the recombinant PI-TPα will have purified all of these activities. Perhaps an alternative procedure for future preparation of this reagent would be to use the recombinant β isoform in an affinity column, which would produce a reagent containing antibodies directed against epitopes shared by both isoforms and hence producing a more uniform response to both proteins, making the preparation a more useful panspecific tool. Further chromatography on the eluent utilising PI-TPα should also provide a polyclonal antibody specific for the α isoform.

It is perhaps ironic that the α-isoform of PI-TP was identified first as the PLC reconstituting factor released upon permeabilisation of the cell (2). This can be traced back to the original purification protocol and the DE52 chromatography used as the first chromatographic step - fractions were selected from this material on the basis of ability to reconstitute PLC activity in permeabilised HL60 cells. PI-TPβ behaves in a manner similar to the α-isoform, but elutes from DE52 and Heparin sepharose at slightly elevated salt levels compared to the other isoform. Consequently the reconstitution attributable to the presence of this protein was missed as a consequence of inhibition of PLC activity by the elevated level of NaCl.

It is equally ironic that HL60 cells and neutrophils were the main targets of study - the original rabbit antibodies and the first panel of monoclonal antibodies were produced against a protein which is expressed at almost vanishingly small levels in the cells under scrutiny. It is however anticipated that these reagents will be of value in investigating the function of PI-TPα in systems in which this protein is more heavily represented and that the β isoform specific reagents will be similarly useful in unravelling the function of this protein in HL60/neutrophils and other cells. The strong immunoprecipitating ability of these antibodies both in the presence and absence of SDS will hopefully now mean the possible identification of other proteins with which PI-TPβ may interact, as well as a determination of the extent to which phosphorylation of this protein occurs in vivo, the site, or sites of this modification and the functional significance of these events.

Utilising the polyclonal antibodies available and the α specific monoclonal antibodies, no interaction between this protein and cellular membranes was clearly demonstrable - upon permeabilisation of the cell with SLO for periods of time beyond a few minutes, all of PI-TPα was found to have left the cell. One striking difference in the behaviour of the PI-TPβ which has already been observed is the extent to which the protein remains bound to the insoluble material remaining after freeze/thaw fracture of cells. This bound PI-TPβ can be liberated by treatment with RIPA buffer, which solubilises membranes and strips proteins. This observation was originally made using RBL and PC12 cells. This is in agreement with immunofluorescent data derived by de Vries et al, which demonstrated that PI-TPβ is preferentially retained in permeabilised cells and that the two isoforms localise to different compartments within the cell when microinjected (202, 204). To further characterise the distribution of PI-TP within HL60 cells, material prepared using disrupted cells on a sucrose density gradient was probed with the β and α specific antibodies. Surprisingly, no PI-TP was detected in fractions other than those corresponding to cytosol, which contrasts with the earlier findings of Snoek et al, that PI-TPβ was specifically localised to the Golgi. It is possible that the interaction of the protein with membranes is such that it does not survive passage through the sucrose density gradient. The sonication procedure was repeated with HL60 cells and an essentially identical result was obtained, with PI-TPβ being demonstrably present in the membrane fraction, as well as the cytosolic component. Because of the very low levels with which PI-TPα was found to be expressed and the demonstration that this isoform of PI-TP is cytosolic in location, the distribution of this protein in HL60 cells and neutrophils was not investigated (168).

The above observation has important implications for the operation of the permeabilised HL60 system used for the detection of PLC reconstitution and possibly the function of other systems which are influenced by the polyphosphoinositides, such as PLD and secretion. Even after prolonged permeabilisation with SLO, HL60 cells are still capable of generating a robust PLC response by comparison with membrane preparations. This can now possibly be linked to the presence of considerable amounts of PI-TPβ remaining resident within the permeabilised cell, coupling the sites of PLC hydrolysis at the plasma membrane or nucleus with the sites of PI-synthesis at

the endoplasmic reticulum. It has already been demonstrated that both isoforms of the protein and yeast homologues are capable of reconstituting PLC activity in this system (152). Thus the possibility exists for the detection of activities which are sensitive to PI/PIP/PIP_2 levels displaying differential decay in cells permeabilised with SLO. Thus those functions which are serviced by PI-TPβ may be expected to be resistant to the decay observed in the case of PLC signalling via the PLCβ isozymes.

The contribution that residual PI-TPβ makes to these processes may conceivably be identified by utilising members of the PI-TPβ specific antibody panel, possibly as Fab(') fragments to facilitate entry to the cell interior via the membrane pores produced by SLO treatment. Comparison of the response generated by stimulation of the cell either in the presence, or absence of inhibitory antibody fragments would provide evidence for the contribution, or otherwise of residual transfer activity to the various cellular process under scrutiny. Hopes for the success of such an approach have been provided by the application of the PI-TPα specific antibody panel to the reconstitution of PLC signalling using recombinant protein. It was however suggested that the PI-TP/antibody complex produced by preincubation of the protein with specific antibody may be large enough for entry to the cell to be hindered. Hence future work may include the application of antibody fragments rather than the intact protein.

One of the principle questions that it was hoped would be answered using the antibodies developed in this programme was the relevance of the quantities of PI-TP added back to permeabilised cells in earlier studies which initially identified PI-TPα as the PLC reconstituting factor. Initial attempts at quantification using either the polyclonal antibody, or the monoclonal antibody 5F12 yielded values which were of slightly lower magnitude than initially predicted. The polyclonal antibody did however suggest that PI-TPβ may be present at quite significant levels. With the application of the PI-TPβ specific antibodies to this question it has become apparent that the quantities of PI-TP added back to the system in earlier studies are indeed physiologically relevant in terms of reconstitution of PLC signalling. It is hoped that the function of other systems with respect to PI-TP may now be studied using these tools.

Recent work emerging from the lab of C.P.Downes (206) has called into question the model in which PI-TP acts as a cofactor in the operation of the kinases functioning in the synthesis of PIP and PIP_2. In kinetic studies on the function of the PLC signalling system and synthesis of PIP and PIP2 it has been demonstrated that there is not a straightforward, first order kinetic relationship between PI-TP and the synthesis of the polyphosphoinositides used in the signalling system and that the PLC signal generated upon stimulation of the turkey erythrocyte ghosts used in the assay displayed first order kinetics with respect to the levels of PI in the system. Thus it has been suggested that PI-TP serves to carry PI from the site of synthesis at the endoplasmic reticulum and deposit it into a suitably PI-depleted membrane. Such a situation could be envisaged as existing in the cell membrane, where activation of the kinases and PLC activity would cause a chemical gradient within the cell with respect to PI.

This represents probably the simplest model for the way in which PI-TP performs PLC reconstitution and in the absence of evidence for a more complicated alternative mechanism would be the most appealing according to the principle of Ocum's razor. It has however been suggested that PI-TP serves a more complicated role than is initially appreciated on the basis of in vitro PI-transfer and that this lipid transfer is not the end of the story - PI-TP may well be carrying out other functions within the cell. This has been borne out by a number of lines of evidence.

A. Kauffman-Zeh et al were able to demonstrate an association between EGF receptor, PI 4-kinase and PI-TP on the basis of immunoprecipitation of the receptor complex from A471 cells (171). In addition, PI-TP has been found to be associated with a PI-specific PI 3-kinase analogous to the vps 34 gene product in yeast. The gene encoding this protein is one of a group of genes which effect vesicular trafficking and protein sorting in yeast. When recombinant PI-TP was added to a complex of the PI 3-kinase and its 150 kDa regulatory adapter, p150, a considerable increase in PI 3-kinase activity was observed (94). More recently studies examining the function of truncation mutants, and proteolytically cleaved PI-TPα have been able to divorce PI-transfer activity from reconstitution. This work was conducted by Dr. S. Hara, and S.E. Prosser in this lab (191, 207).

Certainly in terms of transfer of PI and PC, both of the PI-TP isoforms display very similar properties. PI-TPβ is also capable of transferring sphingomyelin to some degree. However, the physical behaviour of the two proteins remains very similar. It is suggested that if the function of PI-TP were simply to transfer PI about the cell according to the random diffusion of PI bound transfer protein, then a single PI-TP isoform would be adequate for the purpose. The observation that the two isoforms are not universally distributed within all cells, as illustrated by the relative dearth of the α isoform in neutrophils and HL60 cells suggests further that there is a functional difference between the two isoforms. With the development of immunoprecipitating monoclonal antibodies specific for β PI-TP it is now hoped that coimmunoprecipitation may be feasible to begin to identify those proteins with which PI-TPβ may be interacting. As explained in preceding sections, it has become clear why the initial attempts to investigate PI-TPα function in this manner failed in HL60 cells. In addition now that the presence of significant quantities of the α isoform of the protein have been shown to be present in other cultured cell lines, the behaviour of this protein can perhaps now be investigated in this way with more success.

In conclusion, the phosphatidylinositol transfer proteins are potentially of the utmost importance to cellular homeostasis because of the increasingly appreciated importance of the inositol phospholipids in a variety of processes. Whether or not these proteins directly interact with the lipid kinases at the plasma membrane, the importance of a PI-transfer activity of some description to the PLC signalling system has now been firmly demonstrated. Among the questions remaining to be answered must be included whether or not the activity of these proteins is regulated in any way to provide for a vectorial function, as well as the significance of the two different isoforms and their apparent differential distribution both within the cell and between cells of different origins. It is hoped that in the near future the immunological reagents developed in the course of this project will find application in the answering of these questions.

Future prospects:

With the identification of the two PI-TP isoforms and the generation of specific immunoreagents for these proteins it is hoped that the function of the two proteins may be further investigated in terms of differential function. As mentioned in Chapter 5, the PI-TPα specific antibodies are being applied in immunofluorescent microscopic studies in a variety of cell types, including RBL 3H3 and mast cells. The PI-TPβ antibodies are currently in the process of being assessed for their possible application in this method.

The phosphorylation state of PI-TPβ remains to be addressed - as can be seen from the amino acid sequence in Chapter 1, this protein also possesses five putative phosphorylation site for PKC. Initial information from the lab of K.W.A. Wirtz suggests that this protein is not phosphorylated in Swiss 3T3 fibroblasts (Oral communication). The situation in neutrophils and HL60 cells remains to be assessed, particularly in the light of the fact that PI-TPα is very much less well expressed in these cells by comparison to other cell lines.

While quantification of the protein using Western blotting and densitometry is adequate to arrive at a 'ball park' figure for the concentration of these proteins in cells, the development of a reliable, quantitative ELISA system would yield more accurate data and facilitate the extension of these studies to a variety of cells and tissues - the method used here for quantification of the protein is both cumbersome and time consuming. With the development of a large panel of pan-specific monoclonal antibodies, the potential for finding a suitable combination of antibodies to constitute a capture phase in an ELISA system is obviously enhanced. The isotype specific antibodies already characterised could then be used for specific quantification of the captured proteins.

mAbs 5F12 and 1B11 (PI-TPα and β specific respectively) have been demonstrated as being specific for the terminal regions of PI-TP - 1B11 is probably, like 5F12, specific for the C-terminus. These antibodies, or Fab fragments generated from them may represent potential probes to investigate the function of the N-terminus of these proteins.

It is particularly encouraging that of those PI-TPβ-specific antibodies which immunoprecipitate rPI-TPβ in the absence of SDS, three of them also recognise native PI-TP from brain, neutrophil and HL60 cytosols in Western blots. Thus it may be anticipated that these antibodies may prove particularly useful for investigating PI-TPβ/protein interactions by co-immunoprecipitation.

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