Studies on a Protein kinase C-Phospholipase D-MAPKinase Pathway

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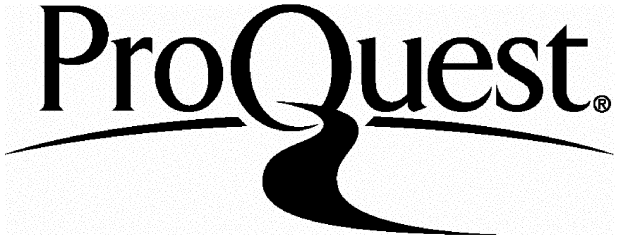

To my Mum, my Dad and my sister Carola

Ever tried? Ever failed?
No matter.
Try again.
Fail again.
Fail better.

Samuel Beckett

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It was a great pleasure and experience to work with all the members of Protein Phosphorylation Laboratory. Many many thanks to all the lab members (lots!) who have passed through the lab during my long stay. Particularly grateful to Richard W. and Tony H. who keep the lab running. My sincere thanks to Will H. and Frank C. for the scientific help in my first steps in the biochemistry world.
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Abstract

Activation of PKC has been postulated to play key roles in physiological cellular responses to a variety of signals. This includes various membrane-associated controls, such as exocytosis, receptor down-regulation and cross-talk among various signalling pathways. Evidence obtained with several cell systems suggests that sustained activation of PKC is needed for long-term cellular responses such as cell proliferation and differentiation. For example, the PKC activator, phorbol myristate acetate (PMA), will induce U937 cells to differentiate towards more mature macrophages.

The work in this thesis describes studies of a putative Phospholipase D-Protein kinase C-MAPKinase pathway that leads to differentiation. U937 human promonocytic leukemia cells were chosen for these studies, since a clear relationship between PKC and differentiation was established. This was known to be associated with microtubule reorganisation and granule release.

The studies here investigated specific molecular aspects of these events and the associated signals. It was established that sustained MAPK was itself sensitive to treatments designed to block PLD. This correlated with the release of granules and cell surface expression of β2 integrins. In parallel with this, PLD was found in fractionated membrane extracts alongside PKCβ1 and β2 integrins consistent with the notion that PKCβ1 is responsible for stimulating PLD1 in this compartment. The relationship between granule movement and MAPK activation led to an investigation into possible TPA-induced autocrine mechanisms. Partial inhibition of TPA-induced MAPK activation by marimastat and also blockade of endocytosis, led to the conclusion that an autocrine response contributed through the action of matrix-metalloproteinases to trigger a signal to MAPK downstream of an endocytic step. The implications of these conclusions are discussed throughout the thesis.

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Abbreviations

ADP	Adenosine 5'-diphosphate
aPKC	atypical PKC
APS	Ammonium persulfphate
ATP	Adenosine 5'-triphosphate
BIM	Bisindolylmaleimide-I
bp	base pairs
BSA	BovineSerum albumin
ButOH	Butan-1-ol
°C	degree Celsius
Ca^{2+}	Calcium ion
cDNA	complementary DNA
Ci	Curie
cPKC	classical PKC
cpm	counts per minute
DAG	Diacylglycerol
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphospate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra-acetic-acid
e.g.	for example
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene glycol-bis-tetra-acetic acid
ERK	Extracellular Regulated Kinase
EtOH	ethanol
FCS	Foetal Calf Serum
Fig.	Figure
g	gram
GTP	Guanosine 5'-triphosphate
GTPase	GTP hydrolase
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
HEPES	N-(2-hydroxyethyl)-piperazine-N'-(2-

	ethanesulphonic acid)
Ins(1,4,5)P3	Inositol (1,4,5) triphosphate
IPTG	Isopropyl-B-Dthiogalactopyranoside
kb	kilobase
kDa	Kilodalton
KLH	Keyhole limpet hemocyanin
l	litre
LPA	Lyso-phosphatidic acid
LPS	Lipopolysaccharide
LY 294002	2-(4-morpholinyl)-8-phenyl-4H-1 benzopyran-4-one
M	Molar
m	milli
mA	milliAmperes
μ	micro
MAPK	Mitogen Activated Protein Kinase
MARCKS	Myristoylated alanine-Rich C Kinase Substrate
MEK	MAPK/ERK kinase
n	nano
nPKC	novel PKC
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PH	Pleckstrin homology (domain)
PI 3-kinase	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMSF	Phenylmethylsulphonylfluoride
PRK	Protein kinase C-related kinase
PtdButOH	phosphatidylbutanol
PtdCho	phosphatidylcholine
PtdOH	phosphatidic acid

PtdEtOH	phosphatidylethanol
PtdIns	Phosphatidyinositol
PtdIns(3,4)P_2	Phosphatidylinositol (3,4) bisphosphate
PtdIns (3,4,5)P_3	Phosphatidylinositol (3,4,5) triphosphate
Rac	Related to A and C kinase
Rho	Ras homology protein
RNA	Ribonucleic acid
rpm	revolutions per minute
RTK	Receptor Tyrosine Kinase
SDS	Sodium dodecyl sulphate
SF9	*Spodoptera frugiperda*
SH2/3	Src homology 2/3 (domain)
TBS	Tris-buffered Saline
TEMED	N,N,N^1,N-tetramethylethylenediamine
TLC	Thin Layer Chromatography
TPA	12-O-tetradecanoylprolbol-13-acetate
v/v	volume/volume
w/v	weight/volume

Aminoacids were abbreviated according to the international one-letter- or three-letter code.

Chapter 1

Introduction

1.1 Cellular Signalling

The development of multicellular organisms represents a complex interplay of proliferation and differentiation events that proceed in a highly ordered manner. As traffic lights regulate movements of vehicles on roads by transmitting "stop" "get ready" and "go" signals to drivers, cells must respond to different signals. These transmission cascades of cellular signals from membrane to nucleus have to be exquisitely controlled in order for cellular growth to proceed in a regulated manner. Cells respond to extracellular signals with a specific set of mechanisms that regulate metabolic functions and gene expression. Between the signal and the gene, a system of different cellular components is assembled to guarantee a specific process of signal transduction.
The importance of these processes is reflected in the fact that the sequencing from the Human Genome Project has revealed that up to 20% of the 32000 human coding genes encode proteins involved in signal transduction, including transmembrane receptors, G-protein subunits and signal generating enzymes.

Extracellular signals in the form of ligands usually bind to extracellular domains of receptor molecules present in the plasma membrane of target cells. Activated receptors as such, or in association with so-called transducers, activate effector molecules either in a direct manner or via second messengers. The effectors, or further "downstream" targets of the effectors, finally modulate cell functions and gene expression at the transcriptional level. Certain target proteins are able to signal back on transducers and/or receptors to switch off the signal transduction; i.e. they trigger feedback inhibition. Organisation of signals transduction pathways in this manner allows the amplification and diversification of signals. Every layer of regulation is controlled both through signal input as well as localisation.

Many of these signalling events involve protein modification, in particular protein phosphorylation. Examples of this and of other modules operating in signalling cascades

will be described below, with particular reference to examples relevant to aspects of the thesis work described in subsequent chapters.

1.2 The role of protein phosphorylation

Protein phosphorylation is one of the principal methods of signal transduction in cells. Eukaryotes extensively use protein phosphorylation on serine/threonine and tyrosine residues to mediate signals from the cells surface into the cytosol and further to the nucleus. The phosphorylation of proteins describes the transfer of a γ-phosphate group derived from ATP (adenosine triphosphate) or sometimes GTP (guanosinetriphosphate) usually to the hydroxyl group of a serine, threonine or tyrosine group. This process is operated in the forward direction by protein kinases and in the reverse by protein phosphatases (Figure 1.1.). There are about 2000 protein kinases in mammals and about 1000 phosphatase subunits (Hunter, 1995), this gives evidence for the importance of this class of enzymes for higher organisms.

The phosphorylation of substrate proteins can:

-generate a conformational change of the substrate molecule which results in either its activation (e.g. phosphorylation of mitogen activated protein kinase MAPKinase on

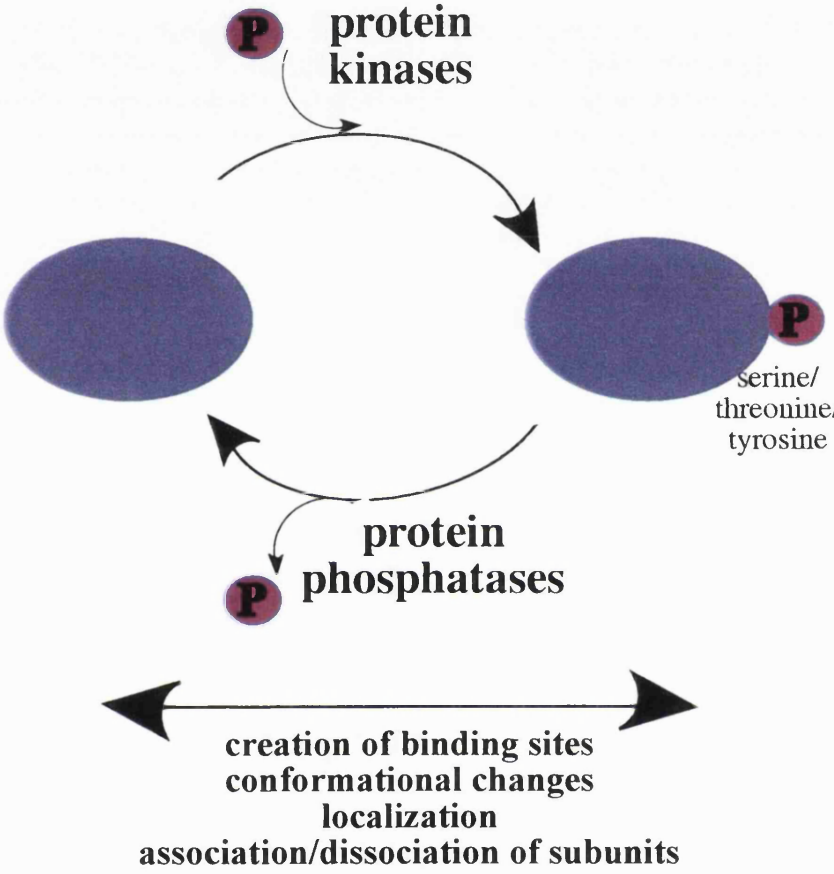


Figure 1.1.
The phosphorylation cycle
Phosphorylation by protein kinases of target molecules generally results in modification of serine,threonine and tyrosine amino acid residues. The other half of the cycle is carried out by protein phosphataseswhich remove the phosphate group, therefore returning the substrate to its unmodified state. The regulation of this cycle is finely balanced and the phosphorylation state of the target protein is dependent on the activities of both the kinases and phosphatases involved.

Thr183/Tyr 185(Anderson et al., 1990)) or inactivation (e.g. phosphorylation of src on Tyr527 (Bagrodia et al., 1991)).

-create binding sites for other proteins, e.g. phosphorylated tyrosine residues in EGFR which function as docking sites for proteins with for example src homology 2 domains (SH2 domains) like Grb-2 or phosphoinositol-3-kinase (Hu et al., 1992),(Batzer et al., 1994)

-can determine the localisation of a protein in a cell thereby regulating its access to potential substrate molecules (e.g. phosphorylated MAPKinase is able to enter the cell nucleus and phosphorylate the transcription factor Elk-1 (Batzer et al., 1994))

-can regulate association or dissociation of enzyme subunits which determine the location of a protein (e.g. phosphorylation of the inhibitory subunit of the transcription factor/inhibitor complex IkB/NFkB, such that on IkB degradation NFkB can translocate into the cell nucleus (Traenckner et al., 1995); the phosphorylation here is responsible for targeting IkB degradation).

Protein kinase cascades in signal transduction offer various advantages, allowing for signal amplification, as well as modulation through protein phosphatase activity. As the phosphorylation reaction is rapid and transient a high degree of amplification can be achieved, the more enzymes in the cascade, the higher the degree of amplification; such cascades also offer the opportunity for cross talk between different pathways, i.e. conditional controls (Seger, R.et al., 1995).

1.3.Receptors

Polypeptide growth factors, hormones and cytokines are essential factors in the regulation of cellular processes. The hydrophobicity of the cells membrane prevents their direct passage into the cell (for most), and the pathways they trigger are controlled by cell surface receptors. The activation of cell surface receptors transduces a signal leading to the direct

(via catalytic functions) and indirect (e.g. via second messenger systems) activation of intracellular signal transduction components.
Membrane receptors can be classified into distinct families based upon the ligands they recognise, the biological responses they induce and, more recently, according to their primary structures. A great variety of ligands bind and regulate the activity of the cell surface receptors, including organic molecules, lipids, carbohydrates, peptides and proteins.

1.3.1 Tyrosine kinase receptors

It has been over 20 years since protein tyrosine phosphorylation was discovered (Hunter and Sefton, 1980). One large family of cell surface receptors is endowed with this intrinsic protein kinase activity. These receptor tyrosine kinases (RTKs) catalyse transfer of phosphate of ATP to hydroxyl groups of tyrosines on target proteins (Hunter, 1998). RTKs are monomers in the cell membrane; they usually consist of single transmembrane domains separating the intracellular kinase domains from extracellular ligand binding domains. These RTKs can be classified into families according to their structural characteristics and include the cells surface receptors for many of the traditional growth factors, e.g. the PDGF, EGF and FGF receptor families (Heldin, 1995). Such receptors are often activated by ligand-induced dimerisation or oligomerization. The cytoplasmic domain contains a conserved protein tyrosine kinase core and additional regulatory sequences that are subjected to autophosphorylation and phosphorylation by heterologous protein kinases (See Figure 1.2)(Hubbard et al., 1998; Hunter, 1998)

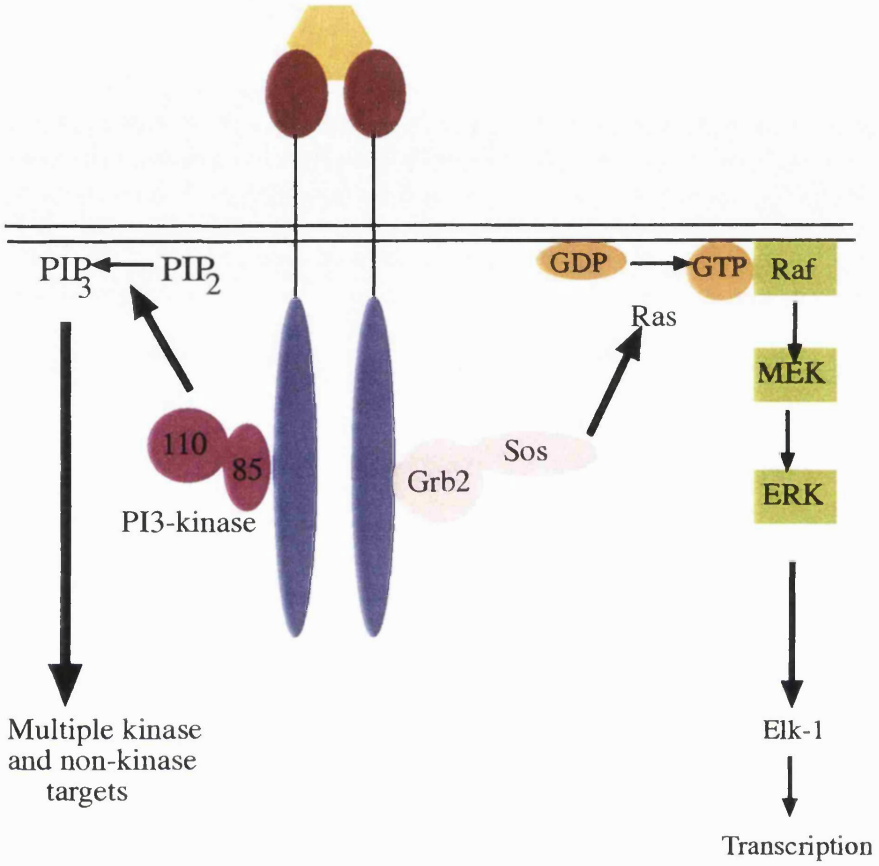


Figure 1.2.
Tyrosine kinase receptors
A schematic example of a growth factor receptor tyrosine kinase directly recruiting kinases and adaptor molecules through phosphotyrosine-SH2 domain interactions, typified by the PI3-K interaction via its p85 subunit and the Grb2-Sos interaction.

Receptor Tyrosine kinases and other cell surface receptors frequently activate serine/threonine kinases that convey the signal to targets in the cytoplasm and nucleus. This is an intrinsic function of the TGFβ receptors, which possess intrinsic serine/threonine protein kinase activity, but other receptors must take a more circuitous route to stimulate protein serine/threonine kinase activity. For example the Ras GTPase, activated by RTKs, binds the c-Raf protein kinase (a MAPK kinase kinase or MAPKKK), which consequently phosphorylates MEK (a MAPKK). MEK is a dual –specificity kinase that phosphorylates and activates ERK (a MAPK), which has multiple substrates involved in the regulation of cell growth and proliferation (Marshall, 1994)

1.3.2. Ser Thr kinase receptors

Almost without exception, the transmembrane serine-threonine kinases identified to date act as receptors for the transforming growth factor beta (TGFβ) family, a group of cytokines long known to regulate cell cycle progression, cell differentiation, immune surveillance, cell adhesion, extracellular matrix production and other crucial aspects of cell life in complex organisms (Massague, J., 2000). Aminoacid sequence similarities indicate that the TGFβ family comprises groups of isoforms that progressively diverge from bone morphogenetic protein 2(BMP2) and its counterpart in *Drosophila*, Decapentaplegic (Dpp). This protein family possesses seven cysteine characteristics of which six form 3 disulphide bonds in a threaded ring configuration called the cysteine knot. This structural motif generates a firm core that provides molecular stability. The bioactive forms are homodimers or heterodimers disulphide linked via the seventh cysteine of each monomer. Access of these factors to cell surface receptor is often regulated by complex interactions with various extracellular proteins, which for the TGFβ include the cleaved proregion of the biosynthetic precursor (Miyazono et al., 1993). On the basis of sequence similarities in the kinase domain, serine/threonine kinase receptors fall into two families-type I and type II receptors (See figure 1.3.a.). Molecular, biochemical and

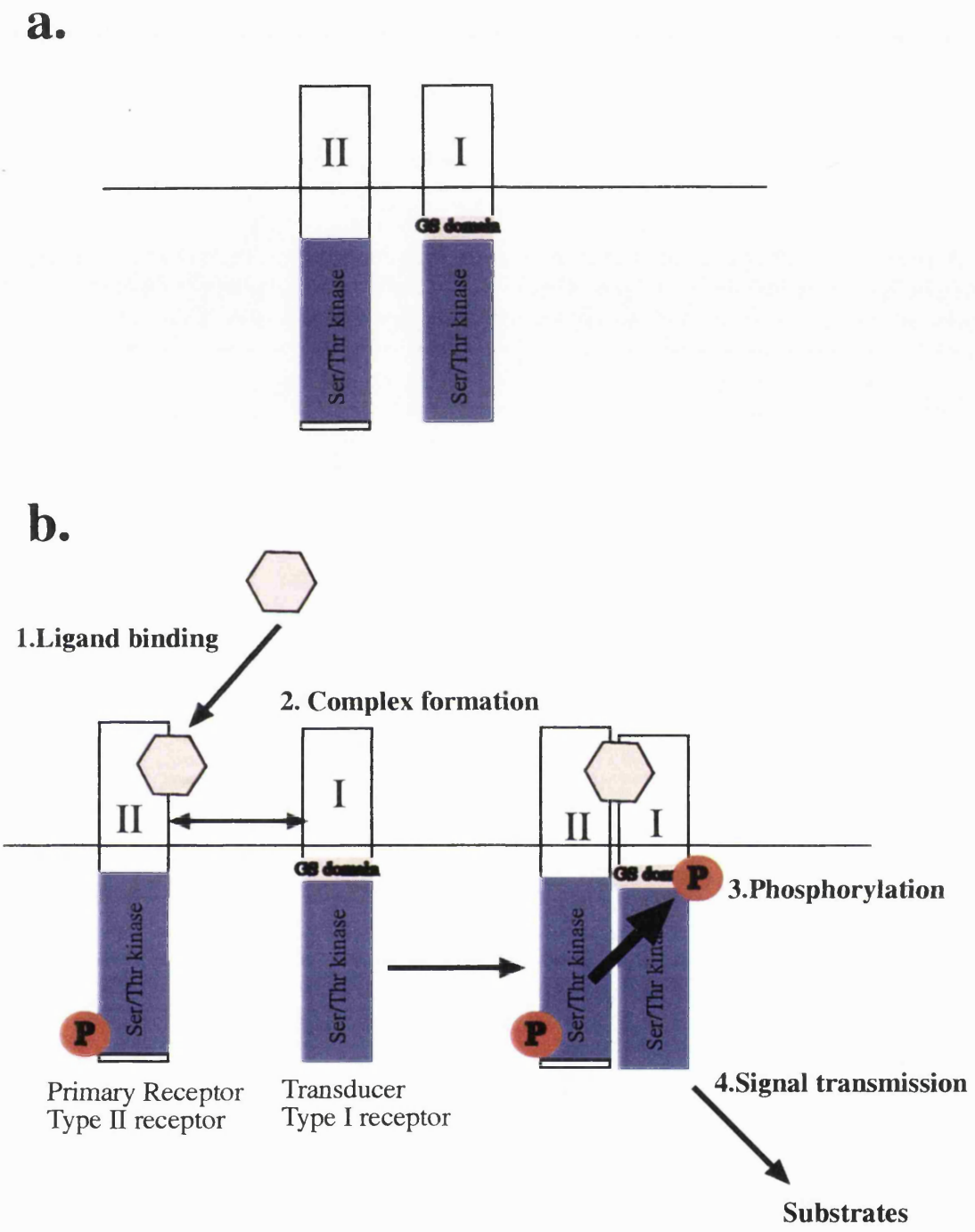


Figure 1.3.
Serine/threonine kinase receptors
a.The 2 families of Serine/Threonine kinase receptors
b.Mechanism of TGFβ activation.

genetic studies have demonstrated that TGFβ and related factors signal by interacting with 2 distinct transmembrane serine/threonine kinases, one from each family. Receptor II can bind ligand on its own, whereas receptor I cannot and an interaction of the two receptors is required for signalling (Laiho et al., 1991). The central event in TGFβ receptor activation is the transphosphorylation of TβR-I by TβR-II that occurs after formation of the ligand induced complex (Figure 1.3b.). Subsequently the signal is propagated. TβR I acts on downstream substrates (Wrana et al., 1994). Given these properties, the terms "primary receptor" and "signal transducer" seem more accurate than the old type II and type I receptor designations.

1.3.3. G-protein coupled receptors

The identification in the mid 1970s of G proteins and their role in activating membrane bound adenylyl cyclase to synthesise cAMP in response to hormonal stimulation was a great step forward in understanding transmembrane signals transduction (Gilman, 1987). The study of G proteins revealed the principle that hydrolysis of protein-bound GTP could itself act as a signalling switch, and also brought us very near to the membrane receptor itself. The cloning of hormone receptors that are coupled to adenylyl cyclase as well as several neurotransmitter and drug receptors revealed that they all had a close relationship to rhodopsin, the seven transmembrane domain G protein-coupled light receptor (reviewed by Okada, T., 2001). This suggested that these serpentine G protein-coupled receptors would constitute a large family and indeed, with the recent addition of many odorant receptors, this has become by far the largest receptor family known, numbering over a thousand. Analysis with purified components indicated that the liganded receptors interact directly with the heterotrimeric G proteins leading to exchange of GTP for GDP bound to the α subunit, which dissociates from the β/γ subunits, allowing both the α GTP and β/γ complexes to signal to downstream effectors, such as adenylyl cyclase, by direct interaction. Signalling is turned off when the α subunit hydrolyses the bound GTP, either spontaneously or upon interaction with a GTPase activating protein (GAP), thus permitting the β/γ complex to rebind the GDP bound α subunit (Figure 1.4).

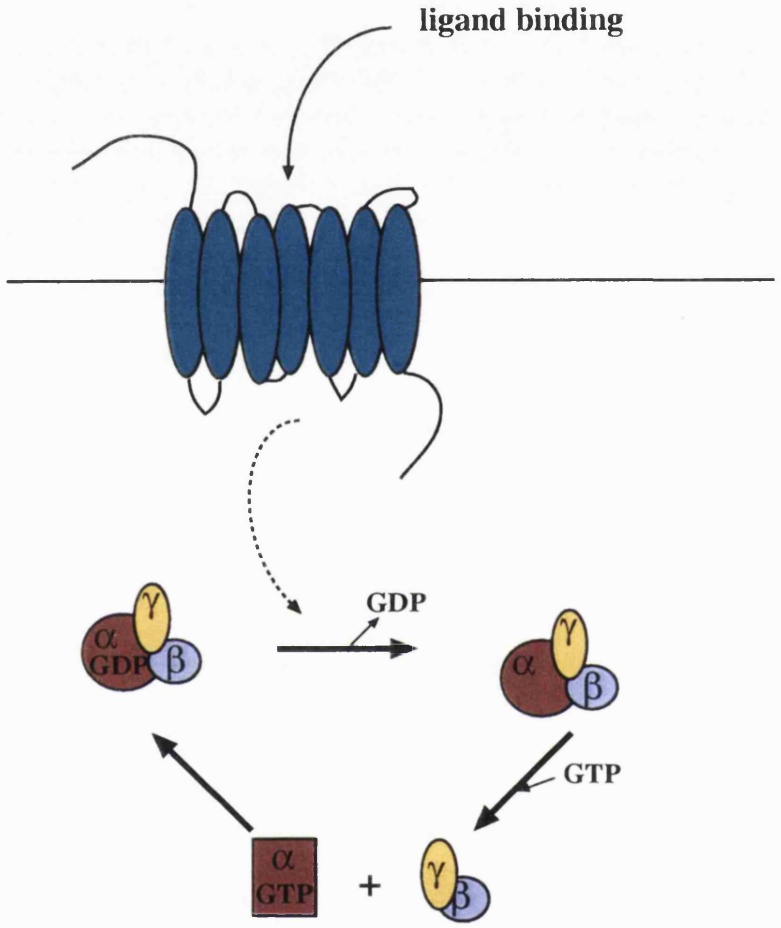


Figure 1.4.
The heterotrimeric G protein cycle.
A schematic showing the receptor triggered stimulation of G-protein nucleotide exchange and its reversal.

1.3.4. Non Receptor Tyr K

A prime example of receptors linked to non-receptor tyrosine kinases are the integrins (Kornberg L., 1992). These receptors bind to extracellular matrices - the glue that holds the body together. Integrins associate with or activate a number of non-receptor protein tyrosine kinases (PTKs), including focal adhesion kinase (FAK), c-Src, c-Abl and Syk, and it is thought that PTK activation initiates many of the downstream integrin signalling events (Frisch and Ruoslahti, 1997).

Integrins belong to a widely expressed family of cell-surface glycoproteins (Tamkun et al., 1986). These adhesion receptors bind specific ligands that are extracellular matrix proteins, soluble ligands, or counter-receptors on other cells. Integrins are heterodimeric transmembrane receptors that consist of an α subunit non-covalently associated with a β subunit (Ruoslahti, 1991). Each subunit has a large N-terminal extracellular domain, a hydrophobic transmembrane region, and a short C-terminal cytoplasmic domain. In vertebrates, at least seventeen different α and eight β chains have been cloned and shown to form at least twenty-four αβ (Hynes, 1992; Kumar, 1998; Ruoslahti, 1991)

β-chain	α-chain partners
β1	α (1-9,V)
β2	α (L,M,X)
β3	α (V,IIb)
β4	α6
β5	αV
β6	αV
β7	α (4,E)
β8	αV

Some redundancy in signalling does exist between the various types of receptor, but most integrins have specific functions.

When integrins bind their ligands in the extracellular matrix, they cluster together to generate focal adhesions at the cell membrane. These structures permit integrins to communicate into and out of the cell to regulate adhesion, spreading and migration (Burridge et al., 1988). The focal adhesion comprises a large number of structural and signalling molecules. Actin-binding proteins associate with the actin cytoskeleton at the focal adhesion, and include α-actinin, filamin, talin, paxillin, vinculin and tensin. Signalling proteins which colocalise with this structure include $p125^{FAK}$, $p130^{CAS}$, c-src, PI3-kinase, PKCs, ras, the tyrosine phosphatases LAR and PTP1B, the serine/threonine phosphatase PP1, and the dual specificity phosphatase PTEN (reviewed in (Craig and Johnson, 1996; Dedhar and Hannigan, 1996; Yamada and Miyamoto, 1995).

An early event following integrin engagement is the tyrosine phosphorylation of a number of proteins such as $p125^{FAK}$, $p130^{CAS}$, paxillin, tensin and src. Integrin receptors do not posses kinase activity and it is not clear how these proteins are tyrosine-phosphorylated. One possible mechanism is that p125FAK undergoes a conformational change and activation following association with a ligand-bound integrin receptor. Then p125FAK oligomerises and trans-autophosphorylates providing docking sites for the other SH2-containing proteins such as members of the Src family tyrosine kinases, which go on to phosphorylate/recruit other proteins like Shc, Grb2-mSOS, PI 3-kinase.

Integrins are divided into eight subfamilies, and the β2 integrins constitute one such group. The most striking feature of the β2 integrins is that they are expressed exclusively in leukocytes, which is why they are also referred to as "leukointegrins". They play a major role in leukocyte cell-cell and cell-matrix adhesion during inflammation and other immune responses. The β2 integrins are designated CD11/CD18, because they are composed of a common β chain, CD18 and one of four unique α chains, CD11a (αL subunit), CD11b (αM subunit), CD11c (αx subunit) and CD11d (αD subunit).

β2 integrins are signalling receptors, but they are also targets of and functionally affected by intracellular signals. There are usually two types of signalling by β integrins (and integrins in general): transmission of signals into the cell following binding of ligands or counter-receptors to the integrins (outside-in signalling) and regulation of the avidity and conformation of integrins by signals generated by other receptors within the cell (inside-out signalling).

1.4. Interactions in signal transmission

1.4.1.Complexes: Protein-protein interactions define specificity in signal transduction.

The localization of a signalling protein within a cell affects the molecules with which that protein can interact. Movement of a signalling protein to a different cellular location - translocation- may result in a change in its interacting partners and hence in its activity. Regulation of the localization of signalling proteins is a key aspect of many signal transduction pathways. The activation of phosphatidylinositol-3-OH kinase by receptor tyrosine kinases leads to the generation of a specific lipid, phosphatidylinositol-3.4.5-triphosphate, at the plasma membrane. The lipid binds to so called pleckstrin-homology domains in proteins such as that in PKB, a serine/threonine protein kinase. This causes PKB to be retained at the plasma membrane, where it comes into contact with another protein kinase (PDK1) that phosphorylates and activates it. Phosphorylated PKB can then move to other sites in the cell, including the nucleus, while maintaining its activity.

Control of localization is also important in the regulation of many transcription factors, which can be kept out of the nucleus, and hence away from their target genes, by regulated interactions with other proteins. For example, Forkhead transcription factors are inactivated when phosphorylated by PKB. Phosphorylation creates a binding site for 14-3-3 proteins, impairing the import of these transcription factors into the nucleus.

1.4.2. Protein domains in signal transmission. Protein signalling modules.

1.4.2.1.Protein-protein interactions and complexes

With 30,000 to 40,000 genes potentially expressed in the human genome, cells face the difficult task of assembling gene products into functional complexes and localizing them to appropriate sites. Of course, cells have developed a number of different strategies to deal with this problem, one of which is to spatially restrict proteins to their site of function thus improving the probability that they will interact with their proper partners. In particular, targeting of proteins to specific membrane-bound organelles has proven to be an effective cellular mechanism in maintaining the fidelity and efficiency of protein activities.

A variety of conserved domains have now been demonstrated to mediate interactions to related target sequences (see Figure 1.5). The discovery of SH2 domains as a means of recognising specific phosphorylated tyrosines in activated PTKs receptors and receptor

PTK targets was a breakthrough in understanding how activated PTKs propagate signals, since it revealed how the association of proteins could be induced by phosphorylation thus propagating a signal (Pawson and Gish, 1992). Indeed, this discovery illustrated a totally new function for protein phosphorylation, namely the regulation of protein-protein association.

SH2 and PTB domains specifically bind short stretches of sequence that contain phosphorylated tyrosine residues. The SH2 (src homology domain 2) domain was originally identified in the non-receptor tyrosine kinase src. These motifs have now been found in wide range of signalling molecules, including PLCγ, p120 RAS-GAP, SHP2, the p85 subunit of PI3-Kinase and the adaptor proteins such Nck and Grb2 (reviewed in (Pawson, 1995)). The high affinity binding of SH2 domains to target sequences is specified by the phosphotyrosine itself and up to three residues C-terminal to it. PTB (p-Tyr-binding) domains generally recognise up to 5 residues away on the N-terminal side of the P-Tyr, but only a subset of PTB domains (e.g. the Shc and IRS-1 PTB) bind to their target proteins in a phosphorylation-dependent manner.

domain	binds to	examples of proteins
PH	PI (3,4,5)P_3 PI (3,4)P_2 PI (4,5)P_2	btk PKB PLCδ1 dynamin
FYVE	PI(3)P	Fab, EEA1
PX	PI(3)P	$p40^{phox}$, $p47^{phox}$, PLD
C1	DAG	cPKC
C2	PtdSerine	nPKC
WW	proline rich region	Pin1
PDZ	5 hydrophobic residue motifs at C-terminus	MAGUK proteins
SH2	P-Tyr	src, Grb2, STAT
SH3	proline rich region	src, PI-3K
PTB	P-Tyr or Tyr	Shc, IRS 1

Figure 1.5.
Domains involved in protein/protein and protein/lipid interactions. See text for further details.

A number of SH2/SH3 adaptor proteins play critical roles in transducing signals from activated receptor protein tyrosine kinases to downstream signalling pathways. A good example is Grb2, which recruits the Ras GDP/GTP exchange factor Sos to the plasma membrane upon binding to activated receptor protein tyrosine kinases.
Other identified protein binding domains are WW domains which are functionally related to SH3 domains (these both bind poly-proline motifs) and PDZ domains which are often present as multiple repeats in signalling proteins. These latter domains typically bind C-terminal motifs of the type S/TXV.

1.4.2.2.Protein-lipid interactions

Another important step forward was the discovery that domains involved in membrane signalling can recognize molecules other than proteins. Research within the past decade has identified protein domains that specifically bind to phosphatidylinositol (PtdIns) phospholipids, collectively called phosphoinositides (PIs), as major determinants in localizing proteins to their sites of function. These PI-binding motifs, which include PH (Pleckstrin homology), FYVE (Fab1p/YOTP/Vac1/EEA1), ENTH (Epsin NH_2-terminal homology) and tubby domains, are found in proteins implicated in a diverse array of cellular processes, such as protein transport, exocytosis, endocytosis, actin cytoskeletal organisation, cell growth regulation, and control of gene expression, cytoskeletal organisation, cell growth regulation, and control of gene expression (Hurley and Meyer, 2001).
PH domains comprise a large family of more than hundred domains. These domains are 100-120 amino acid protein modules best known for their ability to bind phosphoinositides. While certain PH domains bind specifically to PtdIns(4,5)P_2, another subset of PH domains binds preferentially to the products of agonist-induced phosphoinosited-3-kinases (PI-3 kinase)(Czech, 2000). See Figure 1.6.
Recently, it was determined that Phox Homology (PX) domains (see Figure 1.7.), including those in two NADPH subunits, bind to PIs, identifying another family of effector proteins (reviewed in (Wishart et al., 2001)). It was initially identified as a conserved motif of 130 residues within the p40phos and p47phos subunits of the neutrophil NADPH oxidase superoxide generating complex (Ponting and Kerr, 1996). The PX motif can also be found in a wide variety of proteins involved in cell signalling

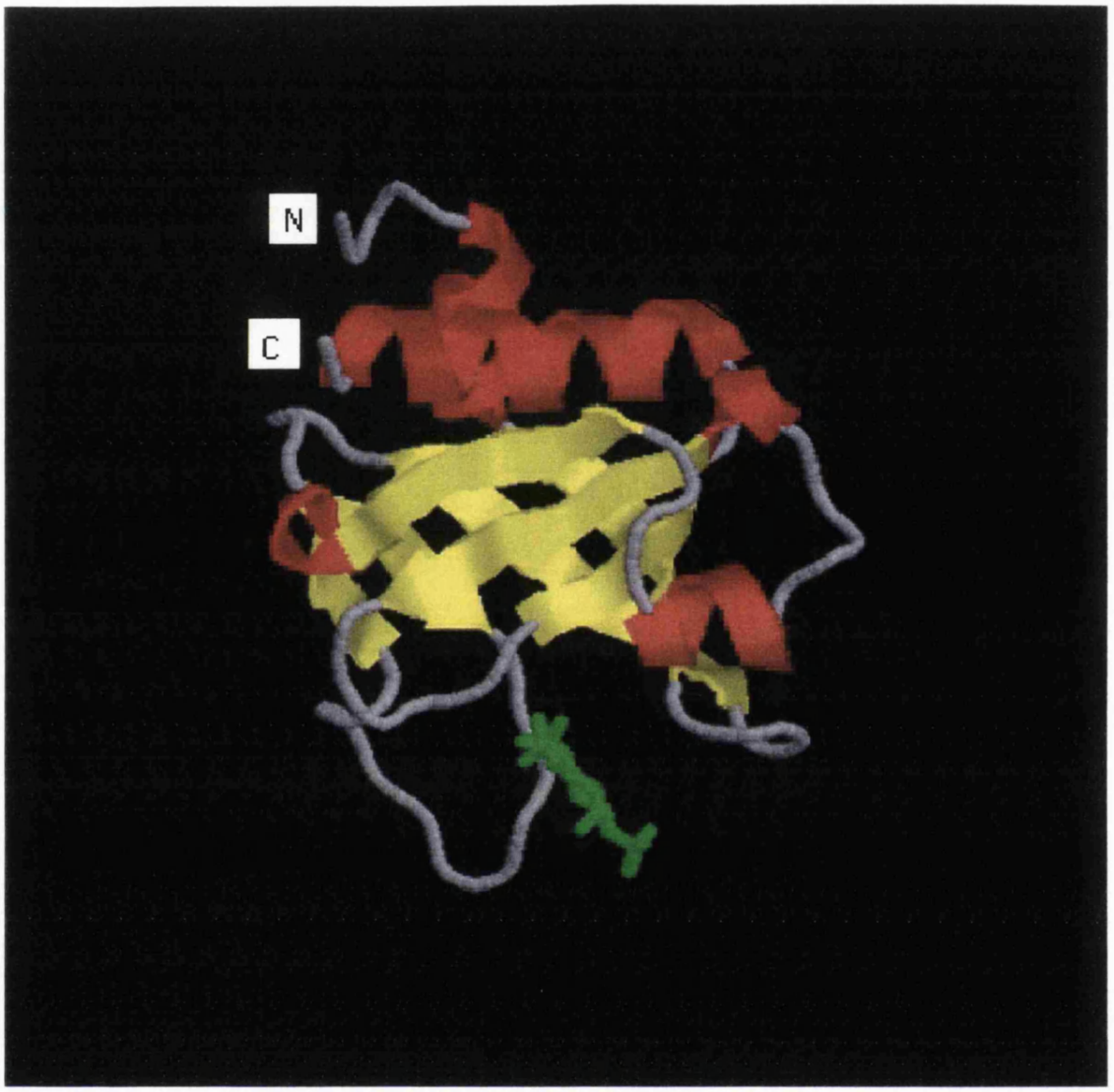


Figure 1.6
A representative PH domain
Structure of the PH domain from PLCδ1 bound to Ins(1,4,5)P_3,rendered with RasMol using the Protein Data Bank (PDB),entry (1MAI)
α helices are shown in red.
β sheets are shown in yellow. They form the sandwich approximately parallel to the plane of the page.
The bound ligand, Ins(1,4,5)P_3 is shown in a green stick representation.

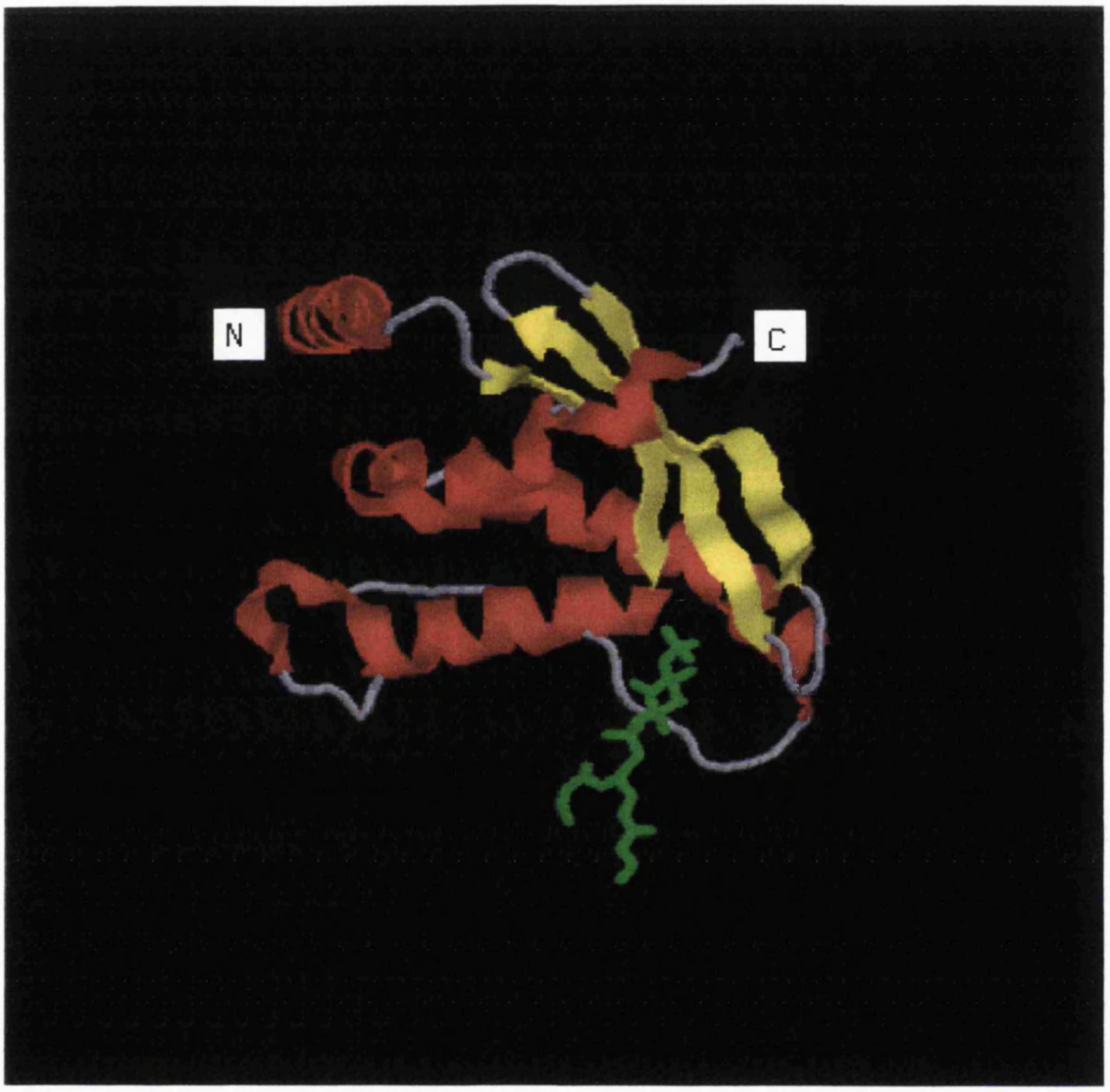


Figure 1.7.
Representative PX domain.
Structure of the PX domain from p40phox bound to PtdIns3P, rendered with RasMol using the Protein Data Bank (PDB), entry (1H6H)

α helix shown in red.
β sheets shown in yellow. They form the sandwich approximately parallel to the plane of the page.
The bound ligand, PtdIns3P, is shown in a green stick representation.

pathways (Phospholipase D1 and D2, PI-3 kinase and Spo14p), vesicular trafficking and yeast vacuolar morphology (human sorting nexins, Vamp7p) and control of yeast bud emergence and cell polarity (Bemp1/3p). Although the function of PX domains has remained unclear, it is possible that it plays a role in regulating the subcellular localization (membranes or vesicular structures) of their corresponding proteins.

1.4.2.3.Phosphoinositides

Phosphoinositide-based signalling dates back to 1953, where the basis was laid for identification of the second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, derived from phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-biphosphate. A wide range of cell-surface receptors, both G-protein coupled and receptor tyrosine kinases, regulate the activity of this family of phospholipases (PLC). In the 1980s an entire new family of phosphoinositides emerged and the presence of six hydroxyls on the inositol ring showed the true versatility of this minor class of lipids. Phosphorylation of the 3-hydroxyl by the family of 3-kinases provides us with PI(3)P, PtdIns(3,4)P2, and PtdIns(3,4,5)P3. The production of the higher phosphorylated forms is controlled by G proteins and receptor tyrosine kinase receptors(Leevers et al., 1999), whereas PtdIns(3)P is constitutively present and is involved in membrane trafficking events(Stack and Emr, 1994; Stack et al., 1995).

The Golgi body is the central waystation and distribution centre in eukaryotic cells for vesicle-mediated sorting and transport of proteins to and, sometimes, from the cell surface and other cellular compartments. There is increasing evidence in various experimental systems for essential roles of phosphoinositides in secretory transport and vesicle movement. A new and exciting idea is that phosphoinositides are probably not required for some indistinct reason, such as the biophysical properties of the membrane bilayer. Rather, phosphoinositides have emerged as key determinants for the molecular recognition of membranes by proteins. Phosphoinositides first mark distinct membrane surfaces. Second, the number of modular domains found to be capable of docking proteins to phosphoinositides is still on the rise. Third, the particular phosphoinositide species present in a membrane serves to recruit and nucleate assembly of the appropriate protein complexes to direct vesicle traffic properly.

Since its discovery in 1988 the activity of PI3-kinase has been associated with the actions of mitogenic growth factors. PI3K activity, tested in phosphotyrosine immunoprecipitates

from cells treated with growth factors, was detected in response to stimulation of cells by PDGF, insulin, IGF-1, EGF, and numerous cytokines that initiate signalling through receptor tyrosine kinases activities. Stimulation of PI3 K was also found in response to G protein-coupled receptor agonists and chemotactic agents. Subsequents studies have shown that the PI3-kinase activity associated with these events corresponds to the type I PI3-kinases. The stimulation of type I PI 3 kinases by this multiplicity of ligands results in specific cellular outcomes that depend in part on cellular content (eg glucose transport in adipose and respiratory burst in leukocytes). The type I enzymes include the α, β, δ forms which have associated regulatory subunits with SH2 domains, while the γ isoform has an associated p101 subunit that contributes to heterotrimeric G-protein control. Thus this class of effector has evolved in a manner that permits multiple receptor types to link to it.

PtdIns(3)P is the most abundant 3'-phosphoinositide in mammalian cells. The recent identification of the PtdIns(3)P-binding FYVE domain, which is conserved in a number of proteins, give us a new key to understanding the role of PtdIns(3)P in membrane trafficking. The FYVE finger was first identified as a double-zinc binding domain, contains eight cysteine residues involved in zinc coordination and initial studies with the early endosome autoantigen EEA1 (Mu et al., 1995) showed that mutations in the FYVE domain inhibit binding of EEA1 to endosome membranes. Later studies showed that the endosomal localization of EEA1 requires an intact FYVE finger as well as PI 3-kinase activity suggesting the possibility that the FYVE finger actually binds specifically to PtdIns(3)P. (Burd and Emr, 1998; Gaullier et al., 1998; Patki et al., 1998). FYVE fingers are identifiers for PtdIns(3)P-specific binding and hence promises to yield new insights into the role of PtdIns(3)P in membrane trafficking, by pointing out a number of potential PtdIns(3)P effectors.

1.5 Effectors

There are many effectors that act to transmit signals proximal to activated receptors. These include second messenger generators as well as the classes of proteins that these messengers act upon. In the case of phosphoinositides the signal generators include the PLCs and PI3kinase noted above and their responders include Protein Kinase C (see below), PDK1 and PKB.

Both PDK1 and PKB have a PH domain that binds PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. PDK1 was originally identified by two groups as a phosphatidylinositol(3,4,5) triphosphate-dependent kinase, which phosphorylates the activation loop of PKB (Alessi et

al., 1997a; Stephens et al., 1998). PDK is a 63kD protein with an amino-catalytic domain (related to the AGC superfamily of protein kinases) and a carboxy-terminal PtdIns(3,4,5)P_3-specific PH domain and ubiquitously expressed in human tissues (Vanhaesebroeck and Alessi, 2000). Activation of class I-type of PI 3-kinases can lead to a rise in PtdIns(3,4,5)P_3 in the plasma membrane. This increase promotes the PH domain-dependent recruitment of PDK1 to the membrane to phosphorylate its substrates, which includes members of the AGC superfamily of protein kinases, PKB,p70^{S6K} and p90 ^{RSK} (Alessi et al., 1997b; Dutil et al., 1998; Jensen et al., 1999; Le Good et al., 1998; Pullen et al., 1998; Stephens et al., 1998). PDK only phosphorylates the activation loop of PKB once the PH domain of PKB has bound to either PtdIns(3,4,5)P3 or PtdIns(3,4)P_2, and undergone the necessary conformational change (Franke et al., 1997; Stokoe et al., 1997).

Below I will discuss in more detail some of the effectors that mediate signalling processes within the cell, with particular emphasis on those which are relevant to this study (Chapters 3, 4, 5 and 6). The proteins reviewed are Phospholipase D , Protein Kinase C and MAPK cascade.

1.5.1.Phospholipase D

Phospholipase D was first discovered in plants, more precisely in cabbage leaves, in 1948 as a distinct phospholipid-specific phosphodiesterase activity that hydrolyses phosphatidylcholine (PC) to yield phosphatidic acid (PA) and choline.

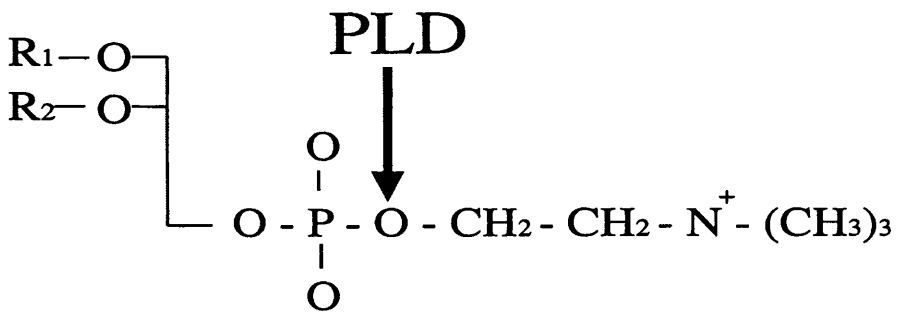
Phospholipase D is present in bacteria, fungi, plants (Wang et al., 1993) (Wang et al., 1994) and animals (Exton, 1997) . It is widely distributed in mammalian cells, where it is regulated by a variety of hormones, growth factors, and other extracellular signals. Activation of phosphatidylcholine-specific phospholipase D (PC-PLD) has been implicated as a critical step in numerous cellular pathways, including signal transduction, membrane trafficking, morphology and the regulation of mitosis (reviewed by Cockroft 2001).

Although PC-PLDs are phosphodiesterases that catalyse the hydrolysis of phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline, these activities can also catalyse a phosphatidyl transfer reaction in which a primary alcohol acts as nucleophile

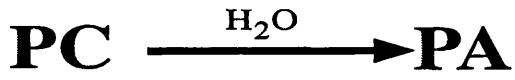
acceptor in place of H_2O. The resulting production of phosphatidyl alcohol represents a specific assay for PLD (see Fig.1.8)

The first eukaryotic PC-hydrolysing PLD to be purified and subsequently cloned was a plant PLD, opening the way for yeast and mammalian PLD genes that, together defined the PLD gene family. Generally eukaryotic PLDs proved to be sensitive enzymes that were hard to purify. Mammalian PLDs, in particular, have resisted many attempts at purification. Only one enzyme, the oleate-dependent PLD first discovered in brain membranes (Kafner, 1975), was purified to homogeneity from porcine lung and yielded a protein band of 190 kDa (Yamashita, 1994). The properties of oleate-activated PLD have been reviewed in (Liscovitch,1996).

The first reported cloned mammalian PLD (hPLD1a) (Hammond et al., 1995) has 1072 amino acids and a molecular mass of 124 kDa. It is specific for PC. A shorter splice variant of hPLD1a with 1034 amino acids (hPLD1b) (Hammond et al., 1997) (Katamaya et al., 1998), which has similar regulatory properties has been identified and, more recently, another PLD (PLD2) (Kodaki and Yamasita, 1997) which has 932 amino acids has been cloned from a mouse cDNA library (Colley et al., 1997) . PLDs from different species (*Saccharomyces, Ricinus* and *Streptomyces*) have several aminoacids sequence motifs (such as GSANIN and HKD) that are also conserved within the human enzymes and these presumably represent components of the catalytic region (see Fig.1.9)(Sung et al., 1997).



a.Hydrolysis



b.. Transphosphatidylation

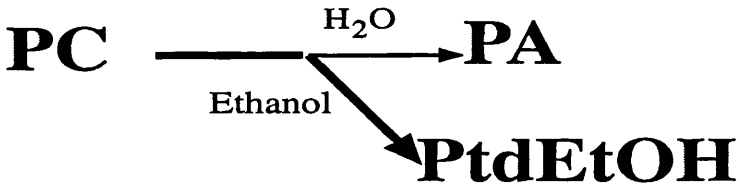


Figure 1.8.
Phospholipase D reactions.

PLD1 activity in vitro requires PIP_2 and can be activated independently by cPKC isotypes, ARFs and Rho family members; combinations of these effectors produce a synergistic response (Colley et al., 1997) indicating that these may well cooperate in vivo to regulate PLD. These properties of PLD1, in combination with earlier studies on PLD

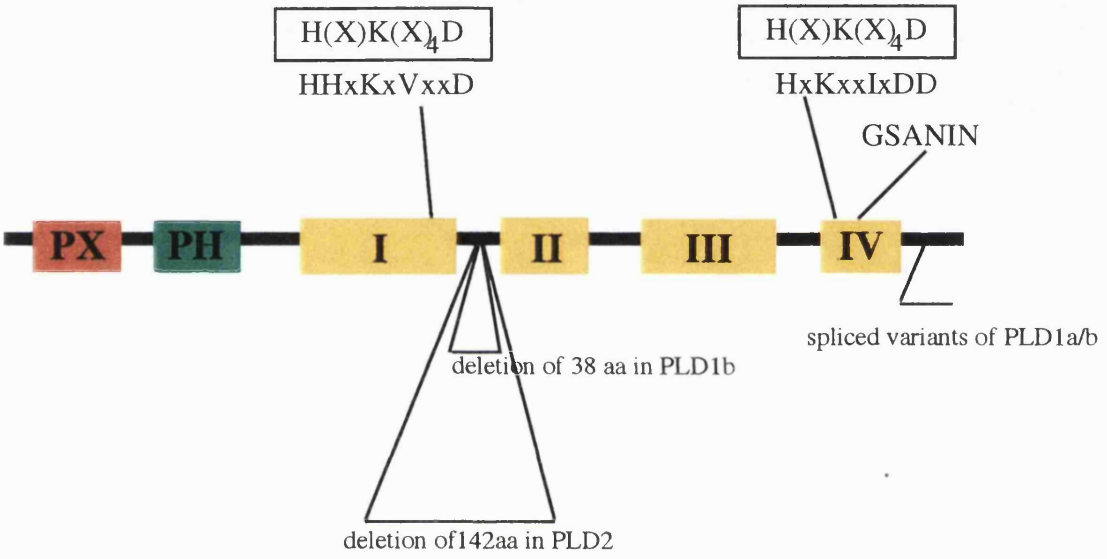


Figure 1.9.
Phospholipase D Domain Structure

activity, imply that PLD1 participates both in regulating secretion, in co-ordination with ARF and PKC α, and in propagating signal transduction responses, such as cytoskeletal reorganisation, through interaction with Rho A family members (Frohman, 1996).

Activators of PLD

The purification of molecules capable of activating PLD in vitro was facilitated enormously by the development of an assay that measures hydrolysis of PC on exogenous vesicles of defined lipid composition.(Brown et al., 1993). In addition the cloning and purification of recombinant PLD1 has validated and extended the list of activators and has provided specific reagents with which to follow PLD activity. Three protein factors and a lipid cofactor are important for activating directly PLD (Brown et al., 1993; Cockcroft et al., 1994; Hammond et al., 1997; Singer et al., 1996):

(1) members of the Arf subfamily of GTPases

(2)members of the Rho subfamily of GTPases

(3) phosphaditylinostol-(4,5)-bis-phosphate (PIP)2

ARF

Members of the mammalian Arf family activate PLD, both in the exogenous assay and in semi-intact cells where PLD has been reconstituted. The two members of the yeast Arf family can also activate PLD but less potently.

Others have shown that ARF (ADP ribosylation factor) (Brown et al., 1993) regulates the binding of the coat proteins COP I (Ostrom Liang et al., 1997) to budding ER and Golgi membranes. In several cell types ARF stimulates the abundant PLD activity found in Golgi-enriched membranes (and the stimulation is inhibited by brefeldin A, an inhibitor of ARF activation) (Ktistakis et al., 1995) and plasma membrane-associated PLD (Caumont et al., 1998) is stimulated by ARF1, -3, -5 and -6. The myristoylated ARFs are more effective than the non-myristoylated forms indicative of a requirement for interaction of membrane associated proteins. Myristoylation of Arf has been shown to be important for full activation of PLD.(Brown, 1995)

Rho

In various assays, all three representative members of the Rho subfamily (including RhoA, Rac1 and Cdc42) have been shown to activate PLD (reviewed by Cockroft 2001).
. Like Arf, lipid modification of RhoA (by isoprenylation) is important for PLD activation. Rho A was shown to interact with a C-terminal fragment of PLD1(comprising amino acids 712-1074) by a yeast-two hybrid approach as well as by an in vitro co-precipitation assay. None of the two Rho A binding motifs identified in other effector proteins could be identified within the C-terminal fragment of PLD1,indicating that PLD1-RhoA interaction motif is unique (Yamazaki et al., 1999).

PIP_2

The requirements of this lipid cofactor has been established in the exogenous substrate assay, where it was subsequently shown that phosphatidylinositol (4)-phosphate, or phosphatidylinositol, can substitute.(Liscovitch et al., 1994) (Brown et al., 1993)
Steed et al. have identified a PH domain in the N-terminal region of both PLD1b and PLD2 (Steed et al., 1998). Analysis of the amino acid sequences of both human isoforms revealed important putative Pleckstrin homology domains and identified additional members of the PLD gene family that help to delimit the catalytic domain. The presence of Pleckstrin homology domains in the PLDs resolves several contradictory observations regarding PLD regulation and the domain structure of the proteins.
The 303 N-terminal amino acids of hPLD1 are not involved in substrate binding or the interaction with PIP(2). Hoer et al. data indicated that the putative PH domain of hPLD1 is not responsible for the essential effects of PIP2 on PLD activitY (Hoer, A., 2000). Probably the PH domain function is to regulate PLD by mediating its interaction with phosphoinositide containing-membranes.

PKC

The involvement of PKC in the activation of PLD comes from pharmacological studies. Phorbol esters, potent activators of PKC activity, enhance production of phosphatidylalcohol in intact cells.(Liscovitch et al., 1994; Singer et al., 1997). It was subsequently shown that pure PKCα was capable of activating partially pure PLD activity or essentially pure PLD1(Hammond et al., 1997; Singer et al., 1996). Interestingly activation required neither the catalytic domain of PKC nor its kinase activity. Activation appears to be through a direct protein-protein interaction between PLD and the regulatory domain of PKC suggesting perhaps that some of the calphostin C effects on transport may be related to this pathway of PLD activation.

Phorbol esters can stimulate secretion and there is abundant evidence that PLD is regulated by PKC in most mammalian cells (Conricode et al., 1992) (Singer et al., 1996) largely arising from studies of the effects of phorbol esters (Exton, 1994; Lopez et al., 1995), PKC inhibitors, down-regulation of the enzyme and overexpression and deletion of specific PKC isoenzymes. Activation of PKC is also associated with translocation of the enzyme to cell membranes, and this relocalization is probably required for PKC activation of PLD, which is predominantly membrane associated. Studies on the effects of purified PKC on purified PLD have shown that the activation does not require phosphorylation (i.e. is ATP independent) and that for PKC α the regulatory domain is involved in the activation. This does not mean that an additional phosphorylating event could not take place *in vivo* for PLD regulation.
These points together would indicate that perhaps phosphatidylcholine specific PLD has a role in PKC regulated secretion.

Other PLD involments

MMPs play an important role in degrading basement membrane and facilitating invasion and metastasis of cancer cells. PLD seems to be involved in mediating MMP-2 release: PLD is activated by laminin, MMP-2 is release by exogenously added PA and laminin induced-MMP-2 release is inhibited by primary and not secondary alcohols (Liscovitch, 1995). PLD is also likely to mediate the synthesis and release of MMP-9 in response to phorbol-ester stimulation (Exton, 1999). It remains to be determined whether PLD is involved in this process as a signalling molecule (i.e. an upstream regulatory element) or as part of the protein-transport machinery. It has been reported that activation of PLD and production of PA modulate integrin-mediated adhesion in eosinophils (Verhoeven, 1999) . Hence, PLD may affect laminin induced MMPs release by inside-out signalling directed at cell-surface integrins.

A potential role of the lipid second messenger PA could be the regulation of translocation and subsequent activation of Raf-1 in vivo. The first PA-binding protein was Raf-1 kinase and it translocates to membranes after PLD2 is activated [Ghosh, 1996][Rizzo, 1999].Other PA binding proteins have been purified, some of them proteins involved in intracellular traffic. The C-terminal domain of Raf-1 kinase interacts strongly to PA, and treatment with 1% ethanol significantly reduce translocation of Raf-1 from cytosol to membrane after stimulation with TPA(Bell, 1996). PA addition reversed the inhibition effect of brefeldin A on Raf-1 translocation by insulin (Romero, 1999).
Mutations that disrupt Raf-PA interaction prevented recruitment of Raf-1 to membranes whereas disruption of Ras-Raf interaction did not affect agonist-dependent translocation,

indicating that this translocation is primarily mediated by a direct interaction with PA and is independent of association with Ras (Romero, 2000).
Also PLD1 and 2 have been suggested to play a role in regulating the cytoskeleton. They are assumed to act at multiple sites in vesicular transport. PLD activity is required for LPA-induced rearrangement of the actin cytoskeleton to form stress fibers (Cross, 1996). PLD might be involved in the cross-linking of actin filaments mediated by α-actinin (Exton, 2001).

1.5.2.Protein kinase C

1.5.2.1.Background

Protein kinase C (PKC) comprises a family of phospholipid dependent serine/threonine protein kinases that have important roles in cell proliferation, differentiation, development, secretion and probably tumour promotion (Stabel and Parker, 1991); (Nishizuka, 1992);(Blobe et al., 1996). The first member of this family was originally discovered by Nishizuka and co-workers in 1977, when it was characterised as a protein kinase from brain tissue which was activated by limited proteolysis and phosphorylated histone and protamine *in vitro*(Inoue et al., 1977);(Takai et al., 1977). Protein kinase C is the major cellular receptor protein for phorbol esters, substances known to interfere dramatically with proliferative and differentiation events and promoting oncogenic transformation of cells *in vivo* and *in vitro* (Castagna et al., 1982),(Niedel et al., 1983)(reviewed in (Nishizuka, 1984)). Phorbol esters mimic the natural PKC activator diacylglycerol (DAG) and are able to intercalate into the phospholipid bilayer for prolonged time periods, since they are metabolised very slowly, which explains the prolonged proliferative effects on cells. Many of the pleiotropic effects of phorbol esters have been attributed to the activation of PKC.

1.5.2.2.Family members

The first PKCs to be molecularly identified (PKCα, PKCβ, PKCγ) were isolated from brain cDNA libraries by low stringency screens (Coussens et al., 1986; Parker et al., 1986). PKC isoforms are abundant in the brain and further screens yielded three additional PKCs: PKCδ, PKCε, PKCζ(Ono et al., 1987);(Ono et al., 1989) Screening of cDNA libraries from other tissues has led to the identification of the other PKC isoforms, known to date as PKCη (Osada et al., 1990). PKCθ (Osada et al., 1992), PKCι(Selbie et al., 1993)(of which PKCλ is the mouse homologue (Akimoto et al., 1994)) and the PKC related kinases, the PRKs(Mukai and Ono, 1994),(Palmer and Parker, 1995), PKD(Van Lint et al., 1995) and PKCμ (Johannes et al., 1994). Recently, two other relatives of these last enzymes have been identified, PKCν and PKD-2(see Figure 1.10). On the basis of their sequence and biochemical properties, these isotypes were divided into three groups: the conventional or classical PKCs (cPKC) isoforms α, βI, βII and γ that are activated in a calcium-dependent manner, the calcium independent novel PKCs (nPKC) δ, ε, η and θ and a third group for atypical PKCs (aPKC) ζ, ι or λ. The last group of isotypes is unresponsive to DAG, phorbol esters and calcium.

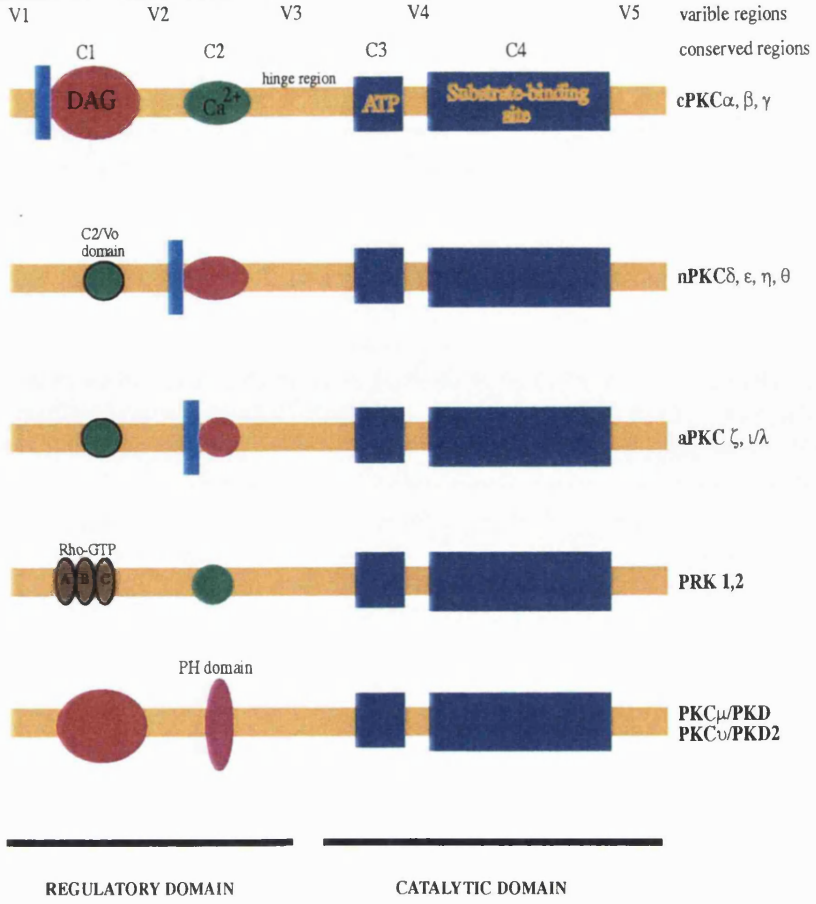


Figure 1.10.
The domain structure of the PKC superfamily.

The classical PKCs (cPKCs) are the best characterised, possibly due to having been identified first. This subgroup consist of isoforms PKCα, PKCβI, PKCβII and PKCγ. The β gene undergoes alternative splicing, resulting in two isoforms differing in their 50 C-terminal amino acid residues (Coussens et al., 1987) cPKCs are activated by calcium, phospholipids, phosphatidylserine (PS) and DAG (or phorbol esters experimentally). They are closely related isotypes sharing the basic structure of an N-terminal regulatory domain and a catalytic domain at the C-terminus (Coussens et al., 1986; Knopf et al., 1986; Parker et al., 1986). These domains can be further divided into conserved regions (C1-C4) which all have defined roles in the activation process of the molecules and variable regions (V1-V5). The functions of the four conserved regions of the PKC family are fairly well defined. The C1 region is juxtaposed to a pseudosubstrate site

which serves to hold the enzyme in an inactive conformation by preventing access to its substrate binding site (House and Kemp, 1987; Pears et al., 1990). Under conditions where PKC is not activated, the N-terminal pseudosubstrate site is tightly bound to the catalytic site in the C-terminal part of the protein. Pseudosubstrate peptides are efficient inhibitors of PKC in vivo and *in vitro* and likewise can be used as *in vitro* substrates if the central alanine residue in the substrate motif is replaced by a serine(Schaap et al., 1989).The C1 region binds phorbol esters and DAG within the two zinc finger motifs, comprising a tandem repeat motif of cysteine and histidine residues. Addition of cofactors like DAG, phorbol esters and calcium is believed to interrupt the interaction of the pseudosubstrate site with the catalytic domain, thus allowing substrate molecules to become phosphorylated by PKC. Mutation and deletion analysis has provided evidence that the C1 domain is the binding site for phorbol ester. Binding studies have shown also that DAG competes with phorbol dibutyrate (PDBu) for binding to PKC and the two molecules are therefore assumed to interact with PKC at the same site(Sharkey and Blumberg, 1985). PKC ζ, and ι/λ contain only one zinc-finger motif and these isotypes are unresponsive to phorbol esters and diacylglycerol. The C1 domain is absent from the PRKs, which are not responsive to phorbol esters.

The C2 region has been shown to mediate calcium and phospholipid binding and the calcium independent enzymes (n- and a-PKCs) lack a classic C2 domain (Shao et al., 1996). The C2 region of PKC has no similarity to any other known calcium-binding motif (e.g. an EF hand). Although nPKCs do not have a C2 domain and do not bind calcium, their amino terminus shows some homology to the C2 domain of the conventional PKCs and was implicated in membrane localisation of the protein. Similar C2 domains have been identified in numerous other proteins (reviewed in (Ponting and Parker, 1996))

The C3-V5 region has been identified in all PKC isotypes as the catalytic domain, which contains the motif of the ATP binding site and mediates substrate binding. The V3 region serves as a hinge region that can be cleaved by proteases to generate a PKC molecule that is no longer dependent on cofactor induced activation. The other variable regions V1, V2 and V4 which are highly conserved within each isotype across species most likely serve to distinguish the isotypes in terms of their substrates and their localisation in the cell.

1.5.2.3.How are the different PKCs activated?

When growth factor receptors are stimulated, PtdIns(4,5)P_2, (phosphatidylinositol 4,5-bisphosphate) is hydrolysed by PLC (phospholipase C) to generate DAG (diacylglycerol) and Ins(1,4,5)P_3 (inositol 1,4,5-trisphosphate). Ins(1,4,5)P_3 releases calcium-ions from intracellular stores which can then bind to the C2 region of PKC and lead to subsequent translocation of the enzyme to the plasma membrane, where it is activated by the binding of DAG and PtdSer to its C1 domain.

In this model phorbol ester would mimic the action of DAG and by its persistence in the cellular membrane lead to long-term activation of PKC. Once PKC is in the active conformation the enzyme becomes more sensitive to proteolytic degradation and dephosphorylation.

1.5.2.4.Diversity and localization or different isotypes

The presence of an entire family of at least thirteen PKC isotypes and the evolutionary conservation of the individual PKC isotypes across species suggests that the individual PKC isotypes must mediate some isotype specific role. A lot of effort has been invested to elucidate the role of each of them, but the lack of isotype specific activators/inhibitors or substrates has made this a very difficult task, especially for *in vivo.*

The human leukemic monoblast U937 cell line used in the studies presented in this thesis expresses PKC isozymes β1,β2, ε and ζ. Indirect immunocytofluoresence using affinity purified PKC-specific antibodies indicates that each of the endogenous PKC isozymes display a unique compartmentalisation within the intact cell. PKCβ1 is distributed between two identifiable pools: a cytoplasmic pool which redistributes to the plasma membrane upon activation with acute phorbol ester treatment, and a membrane-bound pool associated with intracellular vesicles containing β2 integrin adhesion molecules, CD11b and CD11c (Kiley, 1995). The vesicle associated PKCβ1 translocates with the secretory vesicles to the plasma membrane upon agonist-stimulated activation. PKCβ2 is associated with the microtubule cytoskeleton in resting cells. PKCε is associated with filamentous structures in resting cells and redistributes to the perinuclear region upon activation with phorbol esters. In differentiated U937 cells, PKCβ1 remains associated with vesicles translocating from the trans-Golgi region to the plasma membrane and PKCε is primarily associated with perinuclear and plasma membranes. PKCζ, which does not respond to phorbol ester treatment, is primarily cytosolic in undifferentiated cells and accumulates in the nucleus of

differentiated cells, blocked in G2 phase of the cell cycle. The data clearly demonstrate that individual PKCs localise to different subcellular compartments and promote the hypothesis that PKC subcellular localization is indicative of unique functions for individual PKC isozymes.

1.5.3.MAPK Signalling cascades

Mitogen-activated protein kinase (MAPK) cascades are among the most thoroughly studied of signal transduction systems and have been shown to participate in a diverse array of cellular programs, including cell differentiation, cell movement, cell division, and cell death.

MAPK cascades are evolutionary conserved being present in all eukaryotes and play a key role in gene expression as well as in cytoplasmic controls. They typically are organised in a three-kinase architecture consisting of a MAPK, a MAPK activator (MEK, MKK or MAPK kinase) and a MEK activator (MEK kinase(MEKK) or MAPK kinase kinase). Transmission of signals is achieved by sequential phosphorylation and activation of the components specific to a respective cascade.
The MAPK cascade initiates with the activation of a receptor tyrosine kinase in response to mitogens or neurotrophins. SH2 domain-containing proteins are recruited to the tyrosine phosphorylated receptors leading to the plasma membrane relocation of Grb2/SOS, which allows GTP exchange on ras, a small GTPase. The active GTP-ras further recruits the raf proto-oncogene which is stimulated. Raf phosphorylates and activates MEK (1 and 2) , which phosphorylates and activates Mitogen Activated Protein Kinases (ERK1 and ERK2). Following activation, ERKs can translocate to the nucleus where they phosphorylate and stimulate the function of proteins working as transcription factors. Thus, engagement of a ligand at the cell surface transduces a signal to the nucleus by way of several intermediary proteins, four of which are protein kinases.

In mammalian systems five distinguishable MAPK modules have been identified so far.. These include (see Figure 1.11) the extracellular signal-regulated kinases ERK1 and ERK2 in the ERK 1/2 cascade, which preferentially regulates cell growth and differentiation, as well as the c-Jun N-terminal kinase (JNK) and p38 MAPK cascades, which function mainly in stress responses like inflammation and apoptosis. Moreover, MAPK pathways control several developmental programs.

MAPKinase (p42 MAPK/ERK2) was originally discovered in cytosolic extracts of 3T3-LI adipocytes as an insulin-stimulated serine/threonine kinase which could employ microtubule-associated protein 2 (MAP-2) as a substrate (Ray and Sturgill,1987). Biochemical characterisation demonstrated that after challenging fibroblasts with mitogens like EGF, PDGF, NGF or phorbol esters an activating phosphorylation occurred within the dual phosphorylation motif T-E-Y (aa Thr183/Tyr 185). Homology screening discovered a closely related enzyme (90% homology), called p44 MAPK (or ERK1) which is almost identical to p42 MAPK in its biochemical. Cytoplasmic targets of p42/p44 phosphorylation include cytoskeletal elements (e.g. microtubule-associated protein 1, tau), kinases (e.g. ribosomal protein S6 kinase p90), upstream signalling elements (e.g. EGF receptor, mSOS, Raf-1 and MEK), downstream signal generators (e.g. phospholipase A2) and nuclear proteins (e.g. transcription factors Elk-1 and SAP-1, lamins (Seger and Krebs, 1995) The transcription factors Elk-1 and SAP-1 bind to a domain of the serum response element (SRE) in the c-fos promoter together with the serum response factor (SRF) to form the SRE ternary complex which is able to induce (Hill and Treisman, 1995)

The p42 and p44 MAPKinases designated as "ERKs" (Extracellular Regulated Kinases) are phosphorylated and activated by" MEKs" (MEK1/MEK2, MAPK/ERK-kinase).

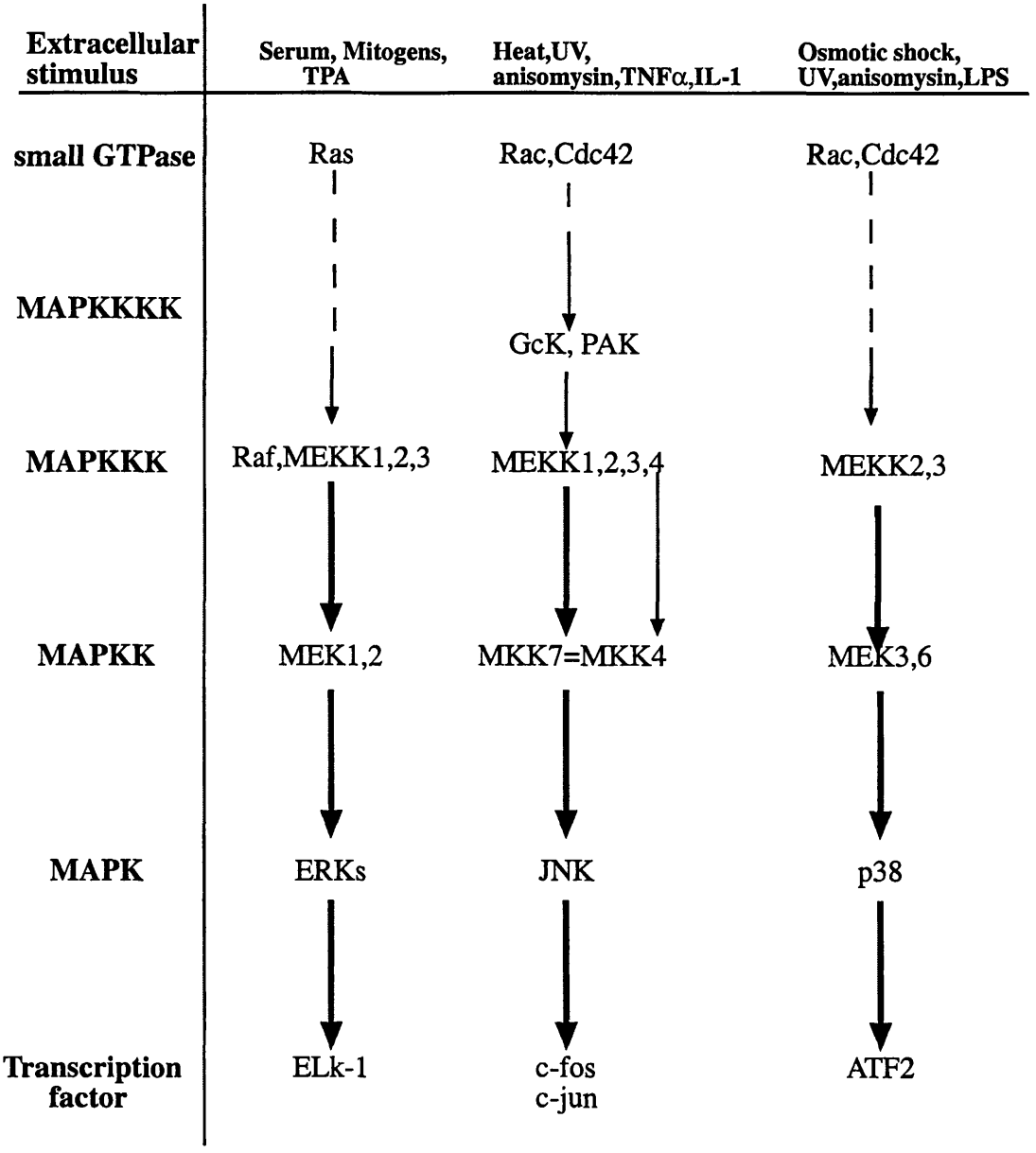


Figure 1.11.
Diagram of three mammalian MAPK pathways.
See text for details.

These enzymes function as "dual-specificity kinases", meaning that they phosphorylate their substrate on a Thr and a Tyr residue in the activation loop. Mansour and co-workers have demonstrated that MEKs can function as oncogenes *in vivo* by injecting a constitutively active form of MEK-1 into nude mice which results in tumour formation.

1.5.3.1.Physiological roles of the ERK/MAPK signalling cascade

Shortly after the discovery of p42 MAPK and its upstream activator MEK, it became evident that this signalling pathway plays a key role in the control of cellular proliferation and differentiation. Miltenberger and co-workers demonstrated that overexpression of an inhibitory c-Raf1 protein or the use of Raf antisense RNA decreased proliferation of NIH 3T3 fibroblast cells while overexpression of a constitutively active Raf enzyme accelerated cell division (Miltenberger et al., 1993). Overexpression of non-activatable forms of MEK-1 in NIH cells significantly reduced the rate of proliferation, which was correlated to a similar reduction in p42 MAPK activity. Constitutively active MEK-1 raised the basal MAPK activity and caused accelerated proliferation (Seger et al., 1994). Finally, a dominant negative form of MAPK or MAPK antisense cDNA were demonstrated to reduce the ability of fibroblasts to proliferate (Pages et al., 1993). These data provide compelling evidence for the idea that cell proliferation is controlled by the ERK/MAPK pathway.

Another physiological response that seems to be regulated through the ERK/MAPK signalling pathway is cellular differentiation. p42/44 MAPK have been implicated in processes such as neurite outgrowth of PC12 cells, T cell maturation and mast cell development (Alberola-Ila et al., 1995; Qui and Green, 1992; Tsai et al., 1993). PC12 cells represent an ideal system to study the effects of MAPKs on differentiation and proliferation. In these cells both EGF and NGF (nerve growth factor) activate the ERK/MAPK cascade, yet while EGF causes a short term activation which peaks after a few minutes and elicits proliferation, NGF causes long-term activation (several hours) and induces differentiation into neurons (Traverse et al., 1992) (Cowley et al., 1994; Qui and Green, 1992). These results suggest that the strength and duration of signals transmitted through the ERK/MAPK cascade are themselves sufficient to determine the response of cells to stimuli.

1.5.3.2.Other MAPK pathways in Mammalian Cells

Besides the ERK/MAPK pathway, which mainly mediates mitogen and hormone signals, mammalian cells have developed at least two other related sets of signalling modules that respond to various stimuli specially those which represent stress factors for cells.

JNK/SAPK pathway

The JNK/SAPK protein kinases were identified originally as activities that phosphorylate the transcription factor c-Jun within its amino-terminal activation domain in cells exposed to UV radiation or cycloheximide. C-Jun together with c-fos forms the AP1 complex which mediates transcriptional regulation of many immediate early genes containing the so-called "TPA responsive element sequences" (TRE). The protein kinase activity was designated c-Jun $\underline{N}H_2$-terminal $\underline{k}$inase: JNK. Cloning of this activity revealed the presence of a 46 kDa and a 55 kDa form of JNK. Because these kinases respond very strongly to a number of stress-induced stimuli, including translational inhibitors, inflammatory cytokines such as tumour necrosis factor (TNF-α) and interleukin 1(IL-1) or heat-shock, they were alternatively named stress-activated protein kinases (SAPK) when the murine genes were cloned. However they are not exclusively activated by stress induced stimuli but by mitogens such as e.g. EGF as well. The defining property for the JNK/SAPK proteins is that instead of the motif T-E-Y present in ERKs, they contain the sequence T-P-Y which must be phosphorylated on Thr and Tyr for activation.

The p38/RK pathway

Another signalling cascade sensitive to stress signals has been identified in mammals based on a homologous pathway in budding yeast. The ERK-like protein in this system is termed p38 or RK (Reactivating Kinase), which is closely related to the HOG1 gene product of *S. cerevisiae.* The activation domain of this protein contains the sequence T-G-Y. p38 is activated by classes of agonists that also activate SAPKs (e.g. UV light, TNF- α or IL-1) and osmotic shock or lipopolysaccarides (LPS).

1.6. Aims and Objectives.

The work in this thesis has attempted to address the TPA-dependent control of PLD and its role in cellular responses. This was initiated by work on identification of the mammalian PLDs in RBL cells and subsequently on the connection between PKC activation, PLD and ERK activation in the U937 cell model system.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and Radiochemicals

NEN	[^{3} H] Myristic Acid
	En^3 Hance spray
	[^{14}C] Phosphatidic Acid
National Diagnostics	Acrilagel (ultrapure), Bisacrylagel (ultrapure)
BDH Laboratory Supplies	Ethanol, Methanol, Tween-20,
	NNN'N Tetramethylethylenediamide,
	2,2,4 Trimethylpentene, chloroform
Calbiochem	Aprotinin, Leupeptin, Colchicine, Vinblastine
Amersham International	Hyperfilm, ECL western blotting kits, donkey anti-rabbit IgG and anti-mouse IgG coupled to horseradish peroxidase, rainbow markers
Schleicher and Schuell	Nitrocellulose membrane
Gibco BRL	Ultrapure sucrose
DAKO	
Jackson Laboratories	Cy3-conjugated donkey antiRabbit IgG(H+L)
	Cy3-conjugated donkey antiRabbit IgG(H+L)
Whatman International	3MM paper
	K6 Silica Gel 60 plates
Falcon	sterile plastic-ware for cell culture
Roche	Complete protease inhibitors
EMBL workshop	stainless steel ball homogenizer

All reagents used for cell culture of eukaryotic cells were obtained from Gibco BRL. Organic solvents were purchased from BDH and other chemicals were supplied by Sigma-Aldrich Company Ltd.

2.1.2.Buffers and solutions

Sample buffer 4X	250mM Tris-HCL pH 6.8, 8% w/v SDS, 20% v/v glycerol, 0.1 M DTT, 0.001% bromophenol blue
Urea buffer	8M urea 1% SDS 100mM Tris (unbuffered) 150mM NaCl 50mM EDTA 1% β-mercaptoethanol
TBS	saline solution 10mM Tris pH 7.5
PBS	8g/l NaCl, 0.25 g/l KCl, 1.43 g/l Na_2HPO_4, 0.25 g/l KH_2PO_4
SDS Running buffer 10X	0.250M Tris-Hcl 0.192M Glycine 0.1%(w/v) SDS
Wet Transfer buffer	26mM Tris-HCl 192mM Glycine 20% Methanol
HB buffer	250mM sucrose 10mM Hepes-KOH pH 7.2 1mM EDTA 1mM MgOAc

HBBS buffer
15mM Hepes
2.8mM NaHCO3
1.5mM CaCl2
1mM MgCl2
0.06 mM MgSO4
at pH 7.4 at 37°C
140 mM NaCl
5mM KCl

TLC plates activation solution
1% oxalic acid
40/60 methanol/H_2O
125mM EDTA

Coomassie-Brilliant Blue buffer
0.1%w/v Coomassie-Brilliant blue
10% acetic acid
50%methanol

RPMI 2% was obtained from ICRF cell services.

Primary antibodies used:

Name	**Origin**	**Description**
PKC β1	Peter Parker's Lab	rabbit polyclonal
CD18 (β2 integrin)	Peter Parker's Lab	rabbit polyclonal
CD11b(integrinαMsubunit)	Peter Parker's Lab	rabbit polyclonal
CD11b(integrinαM subunit)	ICRF 44	mouse monoclonal
PLD1	Dennis Shields Lab (NY)	rabbit monoclonal
PLD	Santa Cruz	goat polyclonal
PLD 1a	Sylvain Bougoin Lab (Canada)	rabbit polyclonal

PLD1	Biosource	rabbit polyclonal
α-tubulin	Santa Cruz	mouse monoclonal
Phospho-p44/42 MAPK	New England Biolabs	rabbit polyclonal
p44/42 MAPK	New England Biolabs	rabbit polyclonal

Cell types used:

Mammalian

Cell type	**Origin**	**Brief description**
U937	Peter Parker's Lab	Derived from malignant cells obtained from the pleural effusion of a 37 years old caucasian male with acute histiocytic lymphoma
Clone 2U937	Peter Parker's Lab	TPA-resistant cell line
Hela	ICRF Cell Services	epithelioid carcinoma of the cervix
Hela OHIO	ICRF Cell Services	human cervix carcinoma derivative of parent hela line
PC12	ICRF Cell services	rat adrenal pheochromocytoma

Antibody production peptides

Name	Peptide Sequence
CD11b COOH	N. KDMMSEGGPPGAEPQ C.
CD18 COOH	N. CLFKSATTTVMNPKFAES C.
PLD1 dephosPKC site	N. TKRHTFRRQN-$CONH_2$ C.
PLD1 phosPKC site	N. TKRHT(-P)FRRQN C.

2.2.Methods

2.2.1Mammalian Cell Growth and Maintenance

U937 cells were routinely grown in RPMI 2% with 10%(v/v) heat inactivated foetal bovine serum. Normal maintenance was at 37°C in a humidified atmosphere of 5% CO_2. Generally cells were passaged when they reached 5.10 5 cells/ml concentration by splitting them in new media (1/5, as required).

2.2.2Preparation of Cell Extracts

Whole cell extracts for analysis by western blot were prepared pelleting 1.10^6 cells in a microfuge at 10000rpm for 1 min, removing the supernatant and adding Laemmli sample buffer, without or with 8M urea (for PLD samples) before they were heated to 95° C for 10 min and then sonicated (3 burst at power setting of 20 microns) and separated by polyacrylamide gel electrophoresis (see 1.2.3) .The samples in urea SB were not boiled, only sonicated.

2.2.3.Polyacrylamide Gel Electrophoresis

Proteins were separated according to molecular weight by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), essentially as described by Laemmli, using Hoeffer Sturdier gel apparatus. To analyse the proteins10% and 8% acrylamide running gels and 6% and 5% stacking gels were used. Rainbow markers (from Amersham) were run in parallel as molecular weight standards. After electrophoresis the gels were further processed either by Western blotting (see 1.2.4) or stained with Commassie Brilliant Blue.

2.2.4Western blotting

Proteins separated by SDS-PAGE as outlined (1.2.1) were transferred to nitrocellulose using BioRad wet transfer tanks for 90 minutes at 4°C (constant current). The effectiveness of the transfer was assessed by ponceau staining of the nitrocellulose membrane. The membranes were blocked by incubation in either PBS or TBS 0.1%Tween-20 (v/v) with either 5% skimmed milk powder or 3% albumin for 1 hour at room temperature. After a brief wash in PBS/TBS the membrane was incubated with the primary antisera as described in the text. Several washing steps were followed by an hour incubation with the appropriate second antibody coupled to horseradish peroxidase. Extensive washes were carried out prior to developing the blot using ECL reagents according to the manufacturer's instructions.

2.2.5. Coomassie staining of SDS-polyacrylamide gels

The gels were incubated with coomassie-Brilliant Blue buffer (see Buffers) for 30 minutes and then destained in Destain buffer(see Buffers) overnight.

2.2.5 Fractionation

Cells were treated at a concentration of 5.10^5cell/ml. They were centrifuged at 4°C and were resuspended in cold TBS buffer with Protease inhibitors, after 2 washes the were resuspended in HB buffer before passing through a needle to separate possible cellular aggregates. Then using a stainless steel ball homogenizer (18 μm clearance) the cells were disrupted. Post nuclear supernatant were prepared by centrifugation in a Sorvall Techno Spin at 3200rpm for 8 min at 4°C. This was separated through a linear 14 ml velocity sucrose gradient prepared from 0.3M and 1.2 M sucrose in a Beckman SW40 rotor for 15 min at 25000rpm. 1ml fractions were pooled and the appropriate ones were further separated overnight on a 7.7 ml equilibrium sucrose step gradient (1.1ml of 1.6M, 1.4M, 1.2M, 1.0M, 0.8M, 0.6M, 0.4M sucrose) in a Beckman SW40 for an overnight

spin(average 14 hours). 1ml fractions were collected and an aliquot of them in SB were separated overnight by SDS-PAGE.

2.2.6 Measurement of PLD activity

2.2.6.1 Cell labelling

Radioactively labelled phospholipids (^{3}H myristic acid), supplied in toluene, were dried *in vacuo* and resuspended in 50µl of DMSO before being added to media for addition to the cells. Routinely, PLD activity was assessed *in vivo* after prelabelling the cells with 2 µCi/ml [^{3}H]-myristic acid for 6 hours.

2.2.6.2 In vivo PLD assays

PLD activity was monitored by measuring the production of [^{3}H] phosphatidylethanol. Ethanol was added to media to a final concentration of 2.5% (phospholipase D will transphosphatidylate ethanol to produce phosphatidylethanol) during 10 min prior treatment.

Cells were fractionated as 1.2.5. and assays were terminated by phospholipid extraction before separation by TLC, with phosphatidic acid standards on silica plates.

2.2.6.3 Lipid extraction

Fractions from subcellular fractionation (see 2.2.5) were added to give a ratio of 1:1:0.9 of methanol/chloroform/H_2O. Samples were vortex and spin at 3000rpm for 5 minutes to separate the aqueous and organic layers. The organic phase was placed in a new tube and a back extraction of the aqueous phase was made. After that the organic phases were evaporated under vacuum in a centrifugal evaporator. Lipids were redissolved in 50 µl of 2:1 chloroform/methanol (v/v).

2.2.6.4 TLC plate activation

Thin Layer Chromatography (TLC) plates (20cmx20cm, Silica 60, Whatmann) were "activated' after soaking in TLC plate activation solution (see 2.1.2) for 1 minute and baking in an oven at 125°C for 1 hour.

2.2.6.5 Thin Layer Chromatography

Samples for separation, prepared in $CHCl_3$: MeOH (2:1, v/v), were drop loaded onto pre marked positions on TLC plates in 2ξ25µl volumes and then allowed to air dry. TLC was performed in tanks that were preequilibrated for 1 hour in the 75ml of upper phase of a mixture of 2,2,4-trimethylpentane: ethylacetate:acetic acid:H2O (2:13:3:10, v/v), to assist equilibration 2 pieces of 19cmx19cm Whatmann 3MM paper were added to the tanks. After tank equilibration, the TLC plates were added and chromatography was performed until the solvent reached the top of the plates.

2.2.6.6 Visualisation and quantitation of ^3H phospholipids

After chromatography TLC plates were air dried for 30 minutes and 3 layers of En^{3}Hance liquid scintillation fluid was applied to the plates (as according to manufactures instructions). Plates were then air dried before being placed in a autoradiography cassette with autoradiographic film for at least 18hours at -7O°C to allow visualisation of ^3H by fluorography. Films were developed.

Using the film image, regions corresponding to various phospholipids, as determined by standards, were scraped into vials and after resuspension in scintilation fluid the radioactivity was determined by liquid scintillation counting in a Beckman LS6000IC.

2.2.7 Antibody Generation and Purification

Peptides corresponding to the required sites (see table) were designed and synthesised by Peptide Synthesis Lab at ICRF. These peptides were coupled to Keyhole limpet

hemocyanin with 25% glutaraldehyde and used in the immunisation of rabbits. The resulted immune-serum was checked by western blot for specificity and competition with the peptide was tested. Some of them were purified by competition affinity chromatography.

2.2.8 Affinity chromatography

For purification of antibodies 2mg of peptide were coupled to 2.5 ml of Sterogene Actigel ALD according to the manufacturer's instructions. After several washing steps the peptide-coupled Actigel was stored in the wash solution (PBS, 0.1%(v/v) Tween 20, 0.02%(w/v) sodium azide) at 4 °C. The immune serum was made up to 40 ml with PBS, 0.1% Tween 20, 0.02% sodium azide, 20mM NaF, 10mM β-glycerophosphate, 1 mM EDTA,and incubated at room temperature for 1 hour. After clearing by centrifugation (10000 rpm for 10 min) the serum was applied to a column which had been pre-packed with the peptide coupled Actigel. With a flow rate of 1ml/min the column was washed with 40ml wash solution, 15ml was solution plus 0.5M NaCl and then back into low salt. Elution was in 100mM Glycine pH2.0 that was collected in 0.5 ml fractions and immediately neutralised with 50 µl 1M Tris-HCl (pH 8.8). The elution profile was analysed using Bradford reagent and peak fractions were pooled, treated with protease inhibitors cocktail mix and stored at 4°C or –70° C.

2.2.9 Immunofluorescence

U937 cells grow in a suspension therefore we had to spin down the cells after their treatment before carry on with the cell staining.

Coverslips were treated with poly-L-lysine for an hour at room temperature and then let to dry out. Cells were treated and washed in ice cold PBS buffer to spin them down at 800rpm into the poly-L-lysine-coated coverslips. Then fixation with 3% paraformaldehyde for 10 minutes and permeabilisation and blocking with saponin and BSA was carried out. Cells were incubated with first and second antibodies for an hour prior to be mounted in Mowiol and images viewed by confocal microscopy

2.2.10 Zymography

The method of quantitative gelatinolytic zymography was used for the detection and quantitation of MMPs. The protocol is based on a published and validated method which uses an improved single step staining/destaining procedure (Leber,1997). Conditioned media from cultures were collected, centrifuged at 4000 g for 20 minutes and the concentrated if necessary. Laemmli's buffer without reducing agent was added to media. Samples were separated by 10% SDS-PAGE containing 1 mg/ml gelatine.

Transfer the gel into 2.5% Triton X-100 for 1 hour at room temperature with gentle shaking, rinse gel twice in collagenase buffer and incubate O/N (16 hours) at 37°C.

Stain the gel 1 hour (to achieve better background staining, the staining process should be extended to several hours) in 30% methanol/10% glacial acetic acid solution containing 1.5% (w/v) Coomassie blue and destained in the same solution in the absence of dye. Unstained areas correspond to zones of MMP proteolytic activities.

2.2.11Polymerase chain reactions (PCR) and agarose gel electrophoresis

The reactions were performed using 50 µg of DNA template, 100pmoles of each of the primers, 5 µl of 10X Taq buffer, 0.25 mM of each of the four dNTP's and sterile water to a total volume of 50 µl. Finally, 2.5 units (1 µl) of Taq polymerase was to the reaction mix and a drop of oil drop of oil put on top. The tubes were placed in the thermal cycler and the reaction cycled as follow:

A basic program would be a 5 minutes, 95°C, followed by 30 cycles of a 95°C 45-65°C annealing step and an extension step at 72-74°C. The times and temperatures of both the annealing and extension steps varied relative to the primers and the templates used.

The reaction was stopped with 5 µl of 10X DNA gel-loading buffer, and separated on a 0.9% agarose gel in 1 X TAE buffer.

Chapter 3

Studies on PLD in RBL cells

3.1.Introduction

Mast cells and basophils express high affinity IgE receptor (FcεRI). Upon antigen (Ag) stimulation, cross-linking of receptor IgE complexes induces release of chemical mediators such histamine, serotonin, leukotrienes and prostaglandins. Rat basophilic leukemia (RBL) cells share many of the properties of the mucosal mast cells and are widely used as a model for the study of FcεRI mediated signalling events. Crosslinking of the receptors generally through the addition of multivalent antigen that binds to the IgE, triggers the receptors and leads to the activation of several tyrosine kinases as well as phospholipases C (PLC), A2 and D (reviewed in (Beaven and Baumgartner, 1996)).

Phosphoinositide–specific phospholipase C(PI-PLC) hydrolyses phosphaditylinositol 4,5-bisphosphate (Ptdns(4,5)P_2) yielding two second messengers: inositol 1,4,5 triphospate (IP_3) and 1,2-diacylglycerol(DAG) (Berridge and Irvine,1984). In addition, phospholipase D (PLD) is also know to play an important role in the generation of second messengers, phosphaditic acid (PtdOH) and DAG in RBL and peritoneal mast cells. Hydrolysis of phosphatidylcholine by PLD yields PtdOH and choline. PtdOH is further converted to DAG by phosphatidate phosphohydrolase. PLD can be activated in response to either antigen, phorbol ester or calcium ionophore in RBL cells.
This activation of various phospholipases generates an increase in cytosolic Ca^{2+} an increase in membrane-associated PKC activity, phosphorylation of myosin at sites phosphorylated by PKC and discharge of secretory granules by a Ca^{2+} dependent process of exocytosis. Many of these signals appear to be important for cellular degranulation of inflammatory mediators such as histamine or serotonin. All of these activities are correlated with an increase in F-actin, which in other studies is shown to be dependent on the activation of PKC. It is reported that RBL cells contain α and β isozymes of PKC which translocate to the particulate fraction when the cells are stimulated with antigen and are degraded when cells are exposed to phorbol ester. Furthermore previous studies have shown that in permeabilized RBL cells secretion can be stimulated by GTPγS alone or with phorbol ester TPA.

The evidence on the involvement of PLD and PKC in secretion in RBL cells indicated that this may be a good model for unravelling the relationship between these two signalling elements and other proximal events. Thus whilst there was clear and precise evidence on the PKC components that might contribute to the events under study, there was no indication of the potential multiplicity of PLDs that might do so. The initial studies directed at the RBL system were designed to define the complement of PLD proteins present.

3.2.Results

3.2.1 Protein kinase C presence in RBL cells

To confirm the pattern of PKC expression in RBL cells, cell extracts were blotted with different PKC antibodies .The α, β1, β2, δ, ε and ζ isoforms were detected in RBL cells (Figure.3.1). Where multiple immunoreactive proteins were observed, assignment of the correct band was achieved by competition with peptides for each antibody (as indicated in Figure.3.1). In the case of PKC β2 the competition with the peptide does not work as well as with the other antibodies since this peptide is very "sticky" due to its poor solubility and binds to many other proteins and the nitrocellulose membrane, but nevertheless the PKCβ2 band is competed.

In preliminary studies, some of these antibodies, only the ones which show high specificity, were used for immunohistochemistry and all of them except α showed a vesicular pattern. Anti PKC-α gives cytoskeleton staining.

3.2.2 Identification of PLD isozymes present in RBL cells

We were interested in knowing how many and which PLD isozymes were present in RBL cells in order to know what was their function in this model of secretion. To answer this question two different approaches were taken: a cDNA screen and a protein screen.

3.2.2.1. DNA screen

At the time that this project was started only the *S.cerevisiae* and the *R.comunis* PLDs were known. Sequence alignments were performed and degenerate oligomers designed to conserved regions within PC-PLDs from these two species (see Table 3.1.).

The name given to the primers coincides with conserved domains in different isoforms

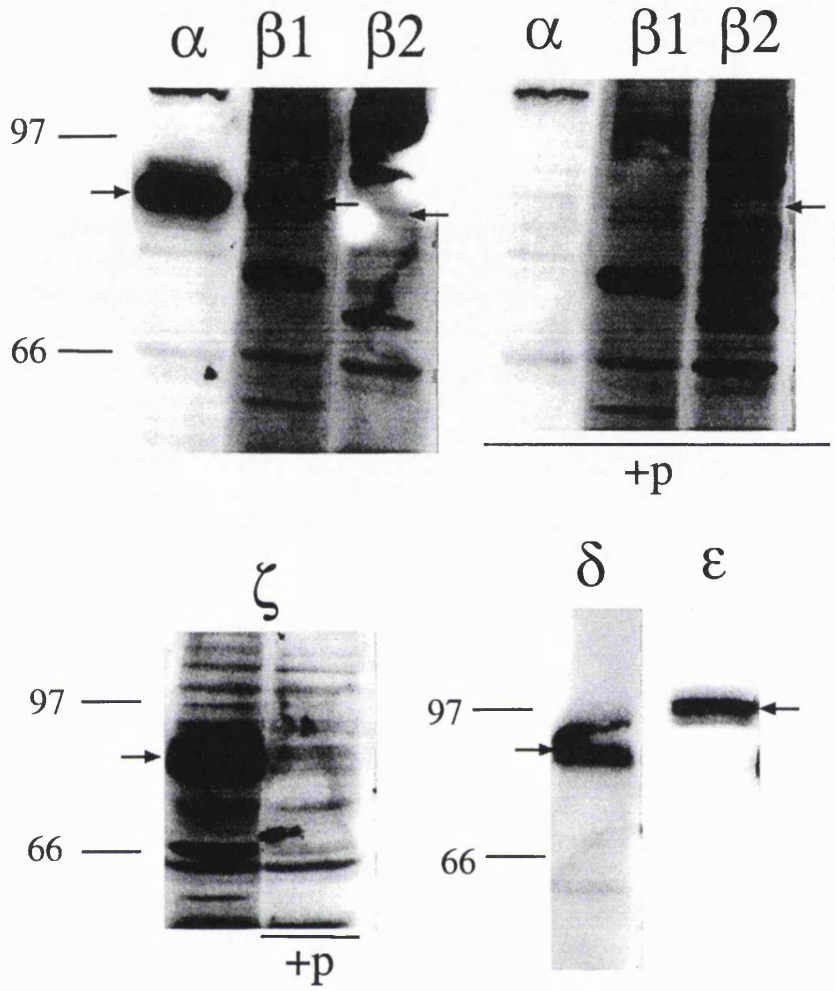


Figure 3.1.
PKCs present in RBL cells
Western blot of RBL cell extracts. $1x10^6$ cells were harvested into 4X SDS sample buffer and were loaded into a 10 % SDS-PAGE. After Western transfer to nitrocellulose membranes, the samples were probed for the different PKC antibodies shown in the presence(+p) or absence of the antigenic peptide. This is representative of two similar experiments performed.

and species of members of the PLD family (see Figure. 3.2). Using these oligomers we attempted to amplify DNA from a cDNA rat library.

Table 3.1

primers	sequences
DLC FOR	TTT/C ITI GGI GGI IT/CI GA
DLC REV	C/GC/AI AA/GA/G TCI A/GII CCI CC
WW FOR	TGG TGG T/CTI T/AG/CI CCI GA
WHD REV	TCA/G TGC CAI G/CG/CT/G A/TC/TI CG/TI GG
QAIL FOR	CAT/C TA/TT/C ATT/C TAT/C ATT/C GA
QAIL REV	T/CTC A/GAT A/GTA A/GTA A/GAT G/AT/AA A/GTG
GSANIN REV	A/GTT A/GAT A/GTT IGC IG/CT/A A/GCC A/GAT

The nomenclature used reflects the conserved amino acids. The location of them are in Figure 3.2.

PCR was performed as describe in Chapter 2 and the products run in 0.9% agarose gels. Despite varying the buffer conditions for PCR over a range of temperatures, none of the products obtained were the right size. We concluded that this approach was not going to be useful.

At that time we (and others) identified sequences within the Genbank EST databases that corresponded to rat PC-PLDs. Using alignments with the 2 known PLDs (yeast and castor bean) and the new mammalian EST clone, specific oligomers were designed to common PLD domains (see Figure.3.3).The aim was to amplify homologous PLD isoforms from rat cDNA. This time the amplification worked. The appropriately sized amplified cDNA were cut from the gel (see Figure 3.4) and purified with the Gel extract PCR prep DNA purification resin protocol. Ligations were set up and positives colonies picked. After doing mini-preps clones were digested and checked for positive inserts. Cloned PCR products were run out on agarose gels, purified and manually sequenced (See Figure 3.5)

Three of the isolated clones were found to be PLD sequences (Table 3.2)

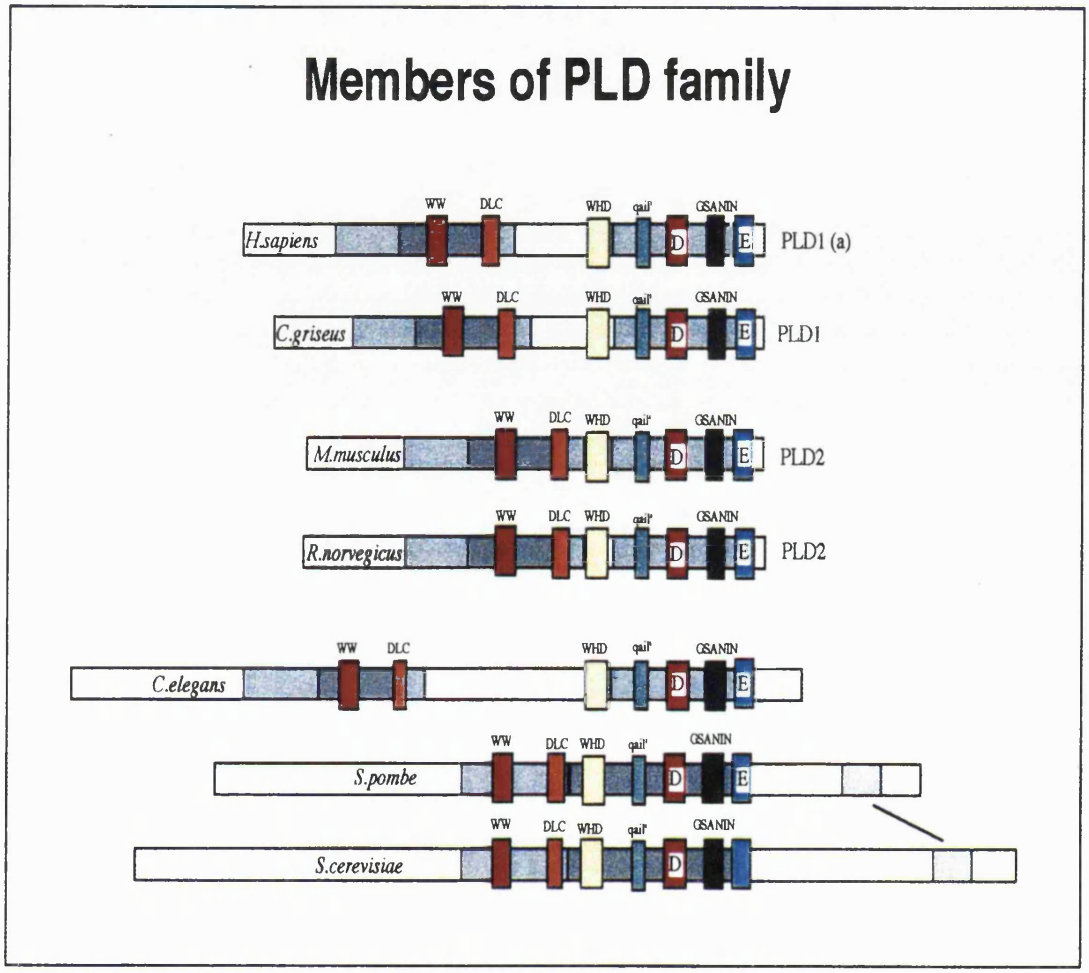
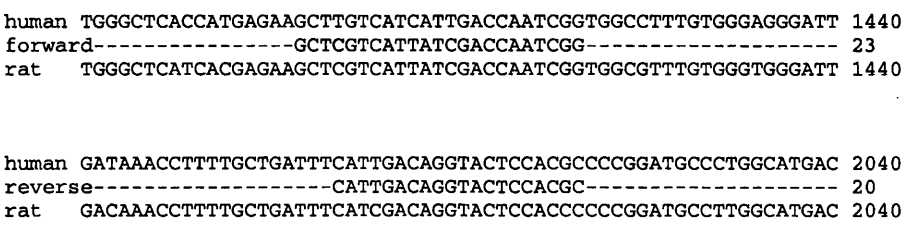


Figure 3.2
Scheme of domains conserved in different PLD family members from different species.
The figure indicates some members of the currently known family, showing that in principle the domains chosen should provide PCR products. The design of degenerate oligomers were based on S.cerevisiae motifs. The name of the different primers come from the aminoacid motifs conserved in different domains (for example, WW, DLC, WHD, GSANIN).



Positions 1438-1440 correspond to amino acid 480 in the protein

Positions 2038-2040 correspond to amino acid 680 in the protein

Figure 3.3.
Amplification of PLD1
Specific primers were designed for PCR. The two oligonucleotides are indicated as forward and reverse ie, coding and anticoding. The amino acid positions for these are also indicated.

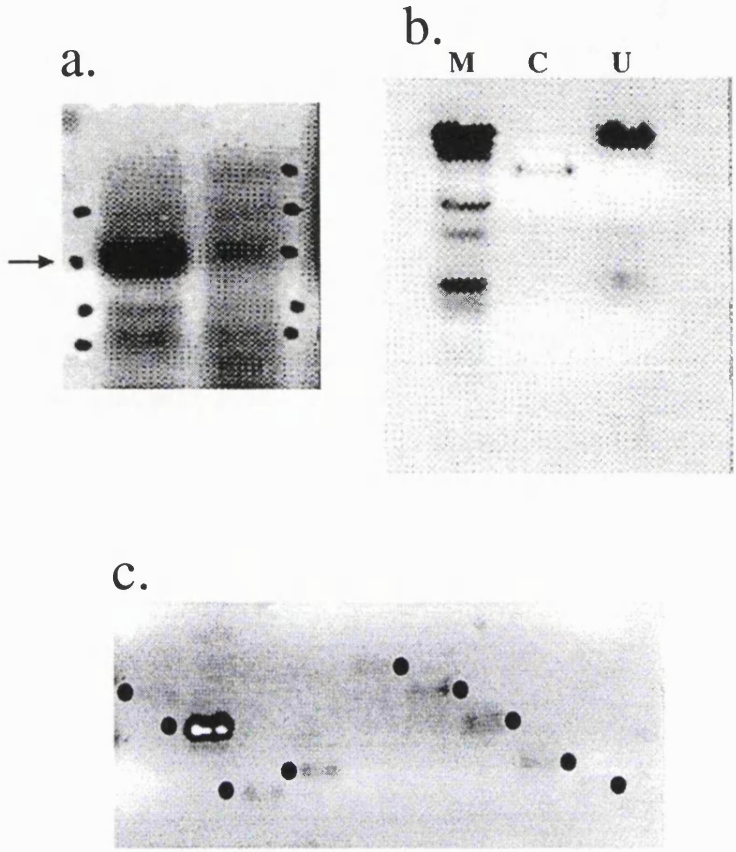


Figure 3.4.
PCR products from rat cDNA amplification
a-different PCR products obtained with specific oligos (dots). The arrow denotes 600pb fragment.
b-purification of pBluescript vector, M-markers, C-cut,U-uncut.
c-purification of fragments ready to be ligated to vector and transformation into bacteria. Bands are indicated by dots.

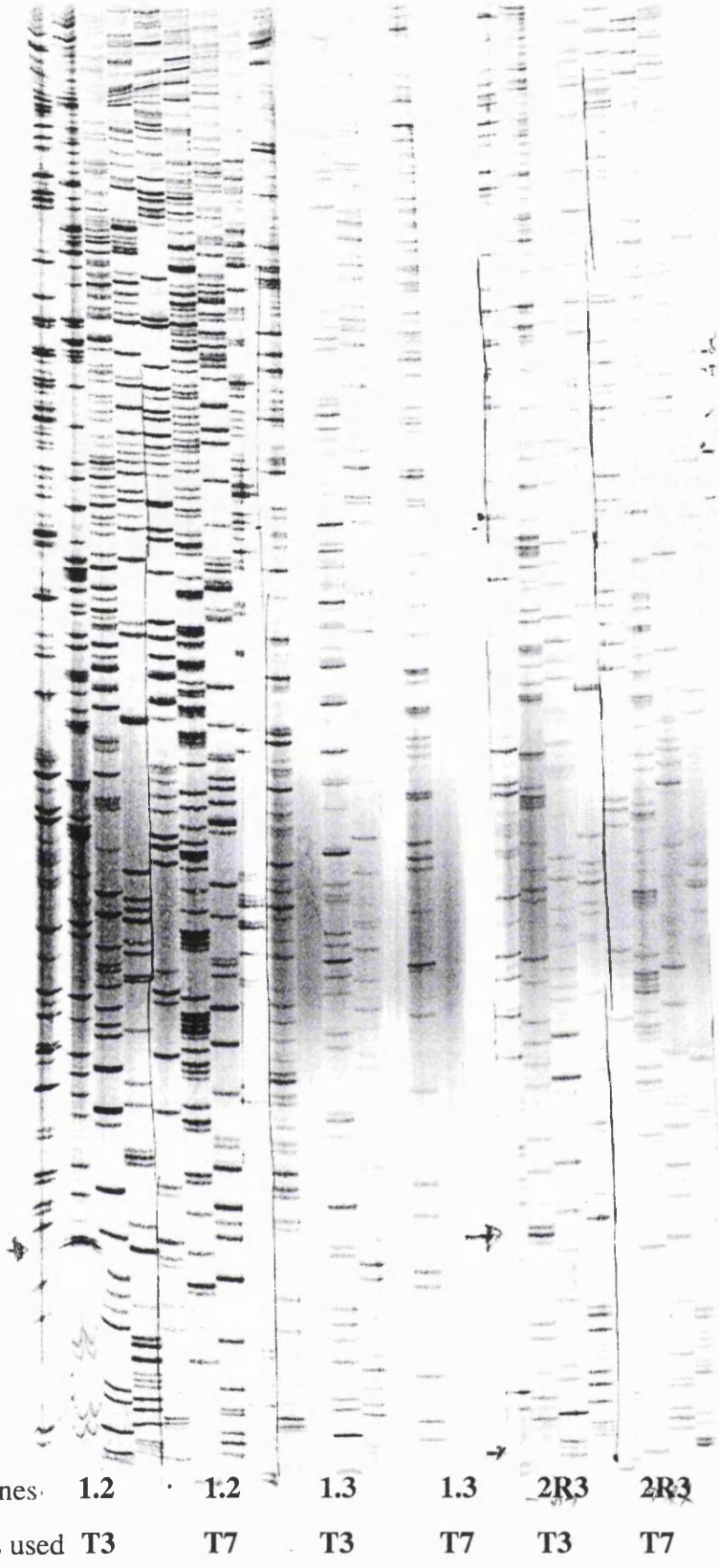


Figure 3.5
Manual sequencing of clones 1.2, 2R3 and 1.3.
Clones isolated as described in the text were subjected to sequence analysis (see Methods). All these three clones were identified as PLD sequences. Clone 1.2 was used as a probe for screening the cDNA RBL library (see text).

Table 3.2: Predicted Protein sequences from PCR clones.

clone	aa sequence	Length (base pairs, aminoacids)	Identity
1.2	LVII...DRYST	624 bp, 207 aa	PLD1a
2R3	LVII...DRYST	624 bp, 207 aa	PLD1a
1.3	LVII...DRYST	509 bp,169 aa	PLD1b

Using specific primers, the fragments amplified and cloned demonstrated that both PLD1a and PLD1b transcripts are expressed in RBL cells.

The fragment for clone 1.2 (624bp) was used as a probe in a Lambda zap rat library screen (See Figure 3.6). The procedures employed for this screen are documented in Figure 3.7. Following this scheme 20 clones were identified and isolated through 3 rounds of screening. Once the titre was low enough and the hybridisation with probe was positive, single positive colonies were picked up and subjected to maxipreps. After some manual and automatic sequencing, it was found that none of the positives were PLD related clones. See Table 3.3.

Next page, **Table 3.3:Closest matches for the sequences determined**

Inserts from individual clones were sequenced as described in Chapter 2. Then sequences were compared with the Genembl nucleotide database using a BLAST search engine. This was carried out in June 1998 before completion of the human genome and hence some of the closest matches relate to non-human DNA sequences despite use of a human cDNA library.

Sequences identified	Length
Rattus norvegicus chromosome 6 clone RP31-263K14 strain Brown Norway, Complete sequence. core =470 bits (237), Expect = e-130 Identities =381/416 (91%), Gaps = 13/416 (3%) X5	Length 611bp
Homo sapiens BAC clone RP11-759A7 from 2, complete sequence. Score =258 bits (130), Expect = 1e-66 Identities = 162/173 (93%)	Length 502bp
Homo sapiens BAC clone RP11-759A7 from 2, complete sequence. Score =258 bits (130), Expect = 1e-66 Identities = 162/173 (93%)	Length 382bp
Homo sapiens chromosome 1 clone RP11-365O16, complete sequence. Score = 36.2 bits (18), Expect = 7.4 Identities = 18/18 (100%)	Length 278 bp
Mus musculus 10, 11 days embryo cDNA, RIKEN full-length enriched library, clone:2810001M05, full insert sequence. Score =753 bits (380), Expect = 0.0 Identities = 562/623 (90%), Gaps = 6/623 (0%) X2	Length 656 bp
Mus musculus 18 days embryo cDNA, RIKEN full-length enriched library, clone:1110005D17, full insert sequence. Score = 61.9 bits (31), Expect = 1e-07 Identities = 50/56 (89%), Gaps = 1/56 (1%)	Length 425 bp
Homo sapiens sorting nexin 7 (SNX7) mRNA, complete cds. Score =264 bits (133), Expect = 2e-68 Identities = 172/185 (92%)	Length 239 bp
Homo sapiens sorting nexin 7 (SNX7) mRNA, complete cds. Score =476 bits (240), Expect = e-132 Identities = 438/500 (87%), Gaps = 4/500 (0%) Homo sapiens, Similar to hypothetical protein FLJ10283, clone IMAGE:3885345, mRNA. Score = 56.0 bits (28), Expect = 8e-06 Identities = 50/58 (86%)	Length 599 bp
Human DNA sequence from clone RP11-415H23 on chromosome 9 Score =597 bits (301), Expect = e-168 Identities = 490/549 (89%), Gaps = 8/549 (1%)	Length 565 bp
Meriones unguiculatus TP53 mRNA for p53, complete cds. Score =50.1 bits (25), Expect = 5e-04 Identities = 25/25 (100%)	Length 130 bp
Mus musculus, clone MGC:25668 IMAGE:4489112, mRNA, complete cds. Score =686 bits (346), Expect = 0.0 Identities = 593/669 (88%), Gaps = 8/669 (1%)	Length 686 bp
Homo sapiens 12 BAC RP11-315E17 (Roswell Park Cancer Institute Human BAC Library) complete sequence. Score = 44.1 bits (22), Expect = 0.032 Identities = 28/30 (93%)	Length 267 bp
Homo sapiens chromosome 7 clone RP11-533K11, complete sequence. Score = 97.6 bits (49), Expect = 2e-18 Identities = 121/145 (83%), Gaps = 1/145 (0%)	Length 179 bp
Human mRNA for KIAA0105 gene, complete cds Score =464 bits (234), Expect = e-128 Identities = 406/460 (88%), Gaps = 6/460 (1%) Homo sapiens chromosome 4 clone RP11-796L2, complete sequence.. Score = 42.1 bits (21), Expect = 0.12 Identities = 27/29 (93%)	Length 504 bp
Human DNA sequence from clone RP11-415H23 on chromosome 9 Sequence 128 from Patent WO0177389. Homo sapiens genomic DNA, chromosome 21q, section 51/105.	

```
pir:T46635 phospholipase D (EC 3.1.4.4) 1a - rat
           Length = 1075

  Score =  420 bits (1080), Expect = e-117
  Identities = 206/207 (99%), Positives = 206/207 (99%)
  Frame = +1

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          LVIIDQSVAFVGGIDLAYGRWDDNEHRLTDVGSVKRVTSGQSLGSLTAASVESMESLSLK
Sbjct:467 LVIIDQSVAFVGGIDLAYGRWDDNEHRLTDVGSVKRVTSGQSLGSLTAASVESMESLSLK 526


Query: 181  DKHQSHKNEPVLKSVDDTDMKLKGIGKSRKFSKFSLYRQLHRRNLHNSDSISSVDSPSSY 360
            DKHQSHKNEPVLKSVDDTDMKLKGIGKSRKFSKFSLYRQLHRRNLHNSDSISSVDS
SSY
Sbjct: 527  DKHQSHKNEPVLKSVDDTDMKLKGIGKSRKFSKFSLYRQLHRRNLHNSDSISSVDSASSY 586

Query: 361 FNHYRSHQNLIHGIKPHLKLFRPSSESEQGLTRHSADTGSIRSVQTGVGELHGETRFWHG  540
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Query: 541  KDYCNFVFKDWVQLDKPFADFIDRYST 621
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Sbjct: 647  KDYCNFVFKDWVQLDKPFADFIDRYST 673

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actgggttcaactggacaaacctttgctgatttcattgacaggtactccacgc
```

Figure 3.6.
DNA and aminoacid sequence of Clone 1.2.
The figure shows the alignments of the sequenced of clone 1.2 (Query) and PLD1a (Sbjct). This clone was used as a probe for a cDNA library screen (see text).

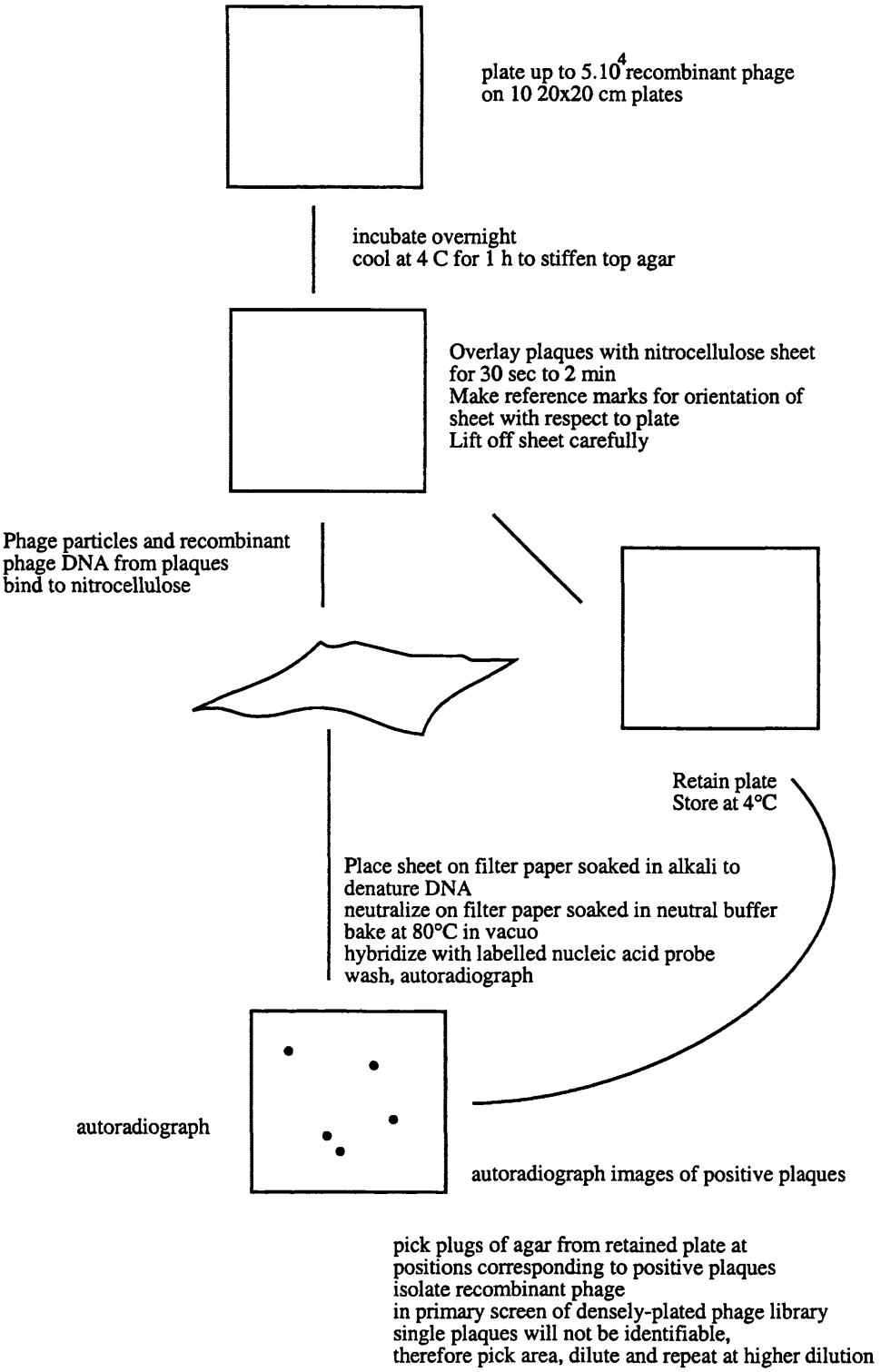


Figure 3.7.
Plaque-lift procedure and detection of positive clones by colony hybridisation.
3 rounds of cloning were employed in the screening.Thirty positives were isolated and sequenced.

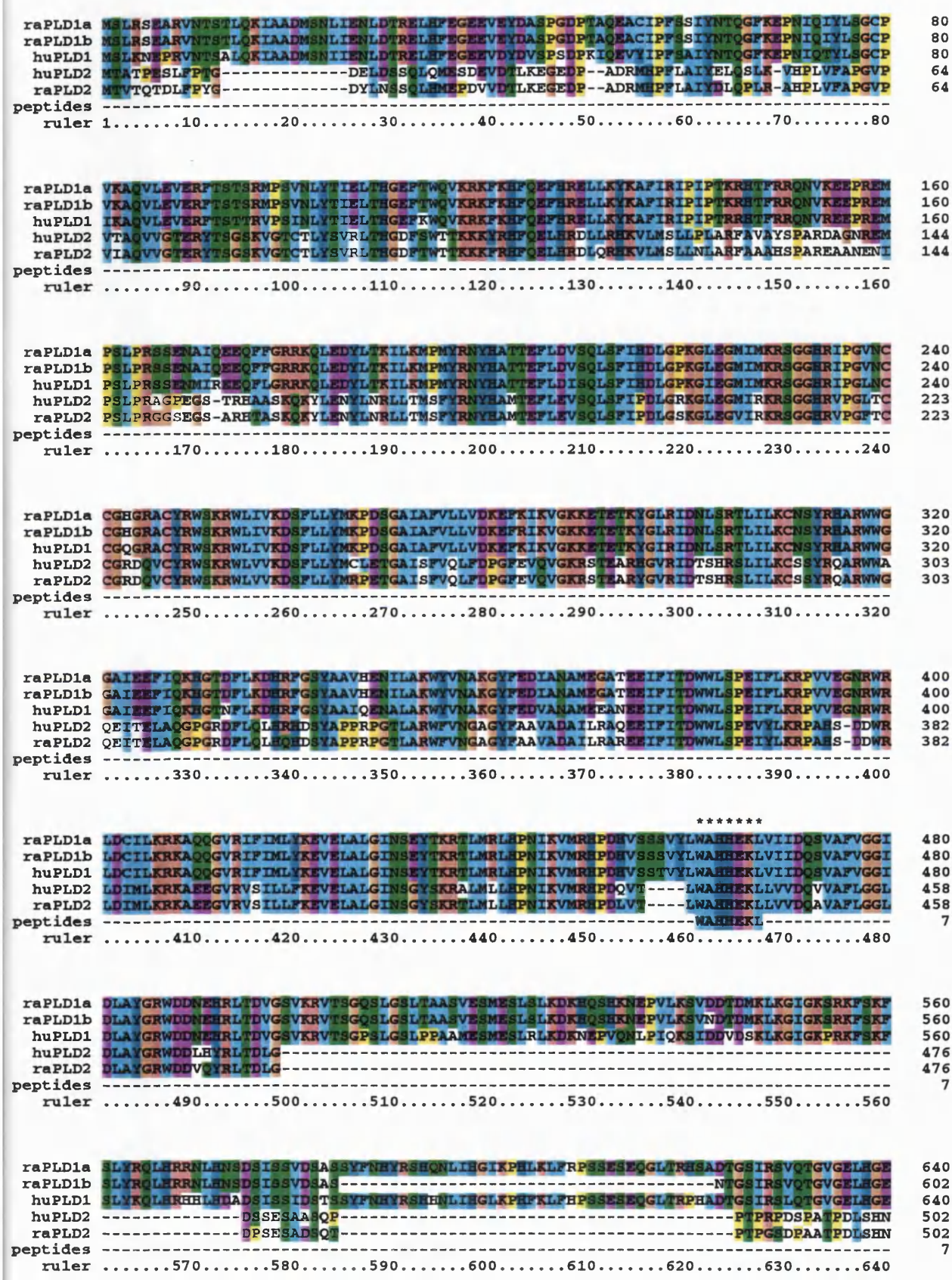
3.2.2.2. Protein screen

The generic approach to protein expression was based upon a screen for protein(s) including the conserved PLD domains. To achieve this, several antisera were generated to specific peptide fragments of human PLD proteins. To define whether these sera recognised putative PLD proteins RBL cell extracts were examined by western blot.

Antibodies were made against different regions of PLD sequences as indicated in Figure 3.8.

-GSANIN antibody: this is a conserved motif in the C-terminal region of all the species of PLD
-PLD2 antibody : against a PLD2 specific sequence (EHIFRCLPSNATRSLR)
-WAHHEKL antibody: aminoacids localized in the first highly conserved HKD motif (WAHHEKL)

Most of the work involving the protein screen was done with the GSANIN antibody. The GSANIN antibody was made with a mix of 3 peptides (IGSANINQRS, IGSANINERS, IGSANINDRS)(1:1:1). These 3 peptides correspond to 3 different PLD groups, plant, yeast and animals respectively and have been designed as a mixture because of the variability in the first of the last 3 aa. These have been used to immunise rabbits and the bleedouts purified by affinity chromatography on the peptide mixture (Figure 3.9). The 129 antibody seemed to provide the highest titre of immunoreaction with the greatest specificity and this serum was employed in future analysis (129*).
The 129 antibody was tested by western blotting against the baculovirus PLD1 and PLD2 expressed proteins which at this stage had been fully cloned (Hammond et al., 1997)(Figure 3.10). RBL extracts denatured in 8M urea buffer show 3 bands that disappear when competed with GSANIN peptides, therefore they appear specific (Figure 3.11). One of them, the 95 kD antigen ,appears to be in other cell lines, such as COS-7, 293 and Jurkat cells (Figure 3.12)


raPLD1a
raPLD1b
huPLD1
huPLD2
raPLD2
peptides
ruler

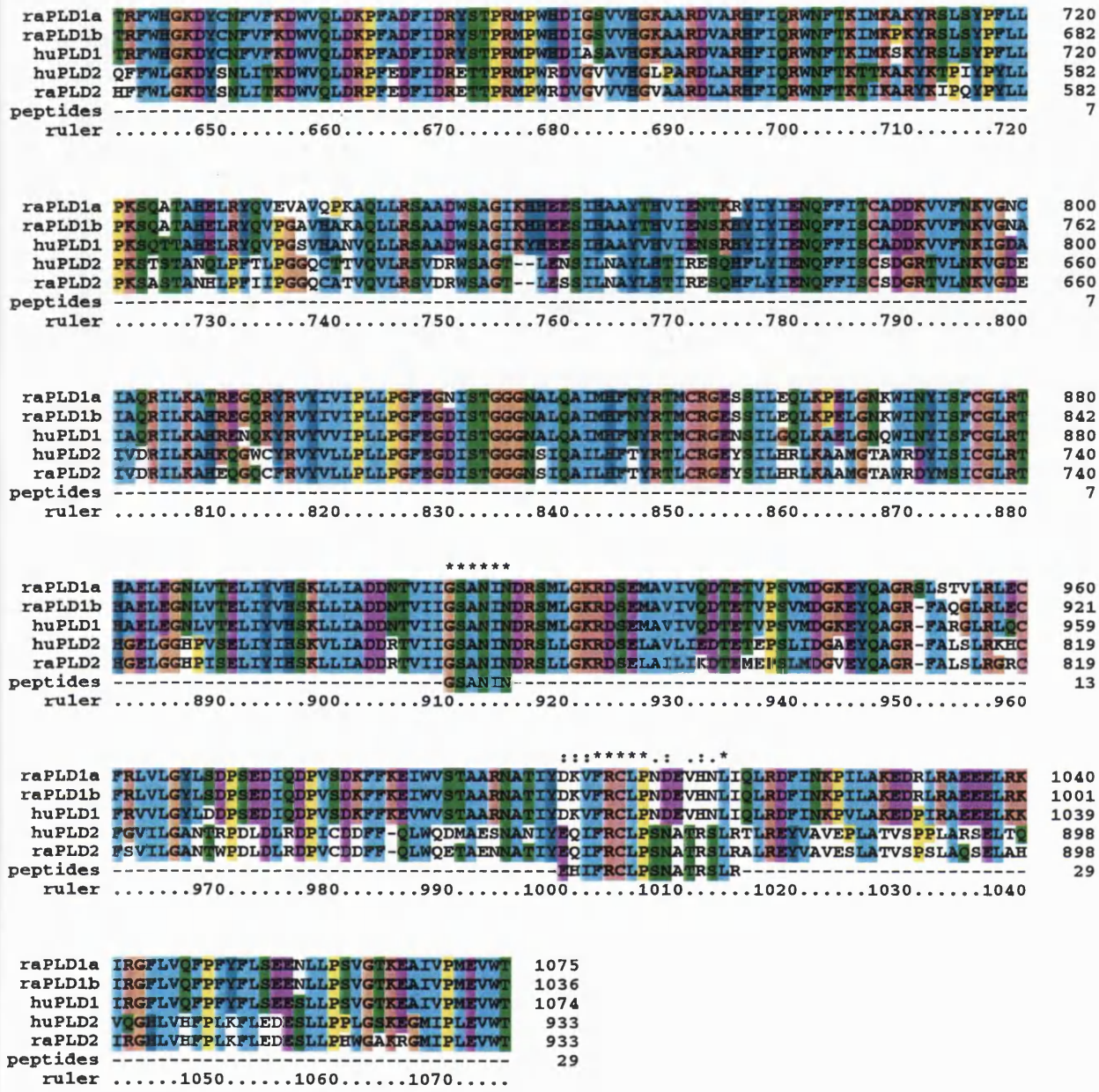


Figure 3.8.
Alignments of rat and human PLDs with the peptides used to generate the anitisera against specific conserved regions. The conserved regions are indicated by the asterisks over the aligned sequences.

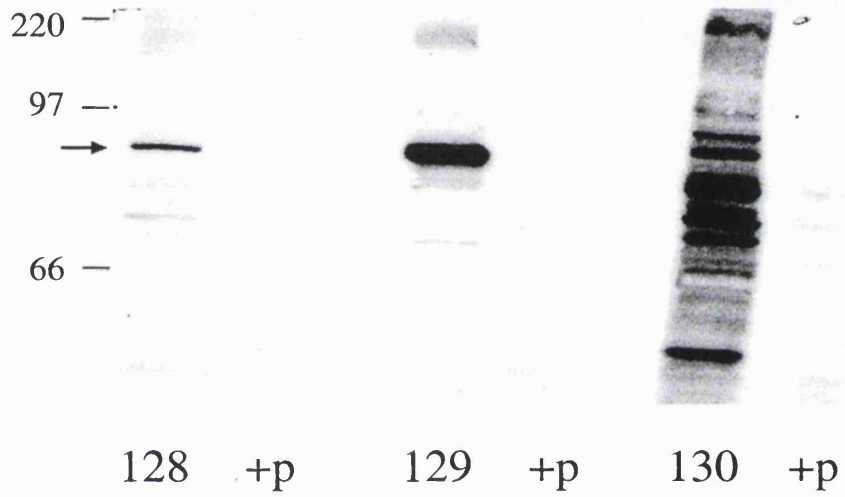


Figure 3.9.
Western blot of RBL cell extracts showing the immunoreactivity of 3 independent GSANIN antibodies.
1x10 RBL cells were harvested and loaded in 8M urea buffer prior running in a 10%SDS-PAGE gel. The numbers correspond to 3 different immunised rabbits. A crossreacting 95kD band is shown by an arrow.

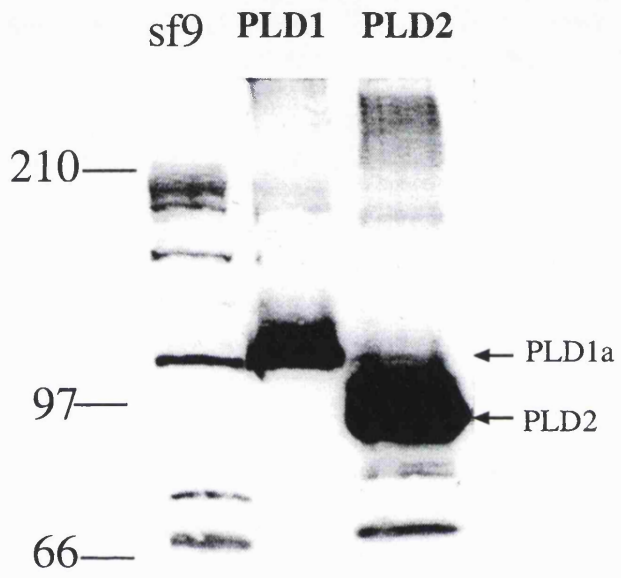


Figure 3.10.
GSANIN antibody 129* recognises PLD1 and PLD2 baculovirus expressed proteins.
Baculovirus expressed PLD protein in sf9 cells was run on a 10% SDS-PAGE gel in 8M urea buffer. A sample of uninfected sf9 cell extract was run as a control. Viruses were kindly provided by Professor M.J. Wakelam, Birmingham.

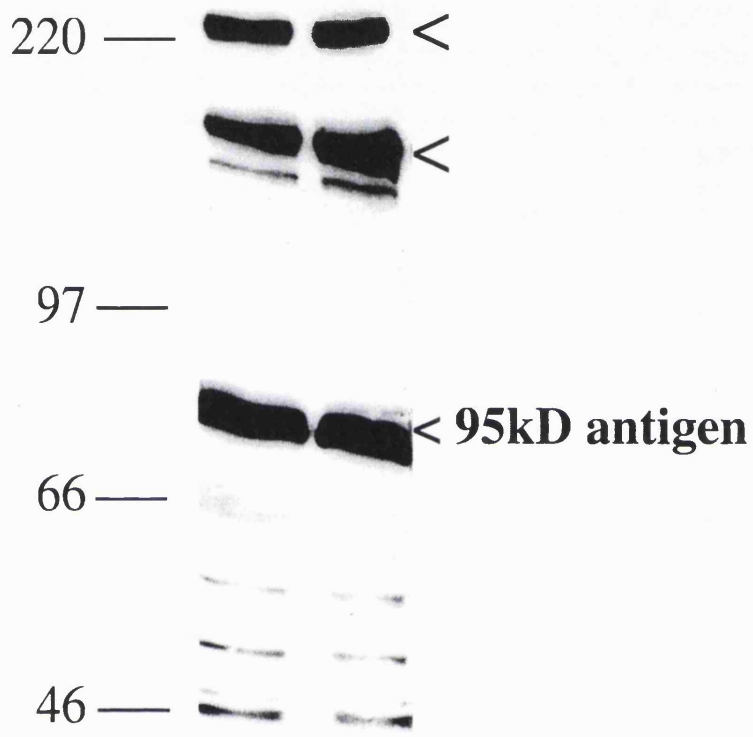


Figure 3.11.
RBL cell extracts in 8M urea buffer.
$1x10^6$ cells RBL cells were harvested and loaded in 8M urea buffer prior running in 10%SDS-PAGE gel. The figure shows 3 specific proteins(see arrow heads)that are recognised by the GSANIN(129) antibody. Duplicate samples were run to check reproducibility.

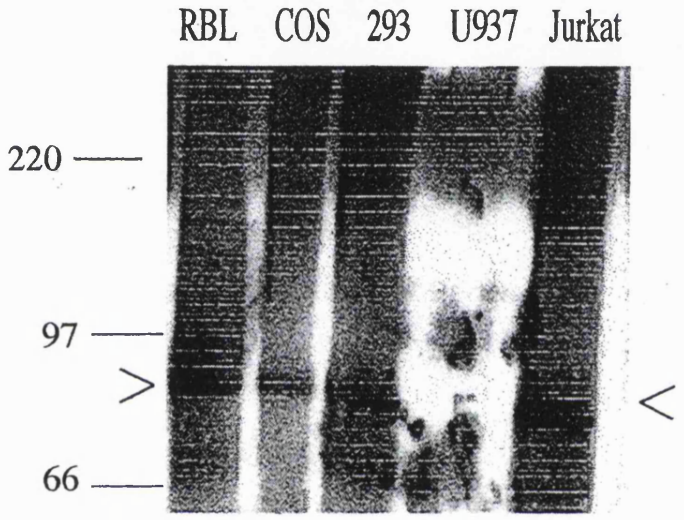


Figure 3.12
Different cell lines express the 95 kD antigen.
1x10^{6}cell were scraped into harvest buffer and put in to 4X SDS sample buffer containg 8 M urea. Western blot using the 129 GSANIN antibody reveals the 95kD band, (arrow).

To attempt to identify the 95 kD antigen as a candidate PLD we sought to purify the protein for microsequencing.

By immunoprecipitation from an RBL cell lysate with the GSANIN antibody bound to protein A beads, a 95 kD protein was precipitated and recognised by western blotting (Figure 3.13). This was not detected by silver stain or coomassie blue which

implies that there is not enough material immunoprecipitated for direct sequence analysis.

We went on to establish that the 95 kD protein was compartimentalized in the membrane fraction (pellet fraction in Figure 3.14). This time Jurkat cells were chosen for their higher concentration of protein. We intended to exploit this for enrichment to pursue a purification to determine whether or not the 95 kD is PLD-related. The aim was to sequence this protein to know if it was a novel PLD.

Despite various attempts to purify this protein from cells we were unable to produce sufficient material for sequence analysis.

In parallel and to aid in the protein analysis approach we designed an antibody to a second conserved PLD sequence, WAHHEKL(check location of peptide in PLD sequence in Fig.3.8). This antibody was tested against baculovirus PLD1 and PLD2 proteins. Surprisingly only PLD1 can be recognised by the WAHK antibody (Figure 3.15). Initial experiments in crude extracts also identified a 95 kD protein that stains specifically with the WAHHEKL antibody however a number of additional bands were recognised in a non-specific manner. The poor immunoreactivity of these sera precluded their use in the analysis of PLD expression.

3.2.2.3. Purification of the 95 kD protein from brain tissue.

Since the 95 kD antigen could be detected in RBL cells and other cell lines tested, could be competed with peptide and was immunoprecipitated, some further studies were carried out to determine if this protein was PLD related. One thousand rat brains were used to make brain extracts to employ them in a PKC-kinase screen (Ziegler et al., 1999). We made use of this and monitored the 95kD protein in the initial stages of this

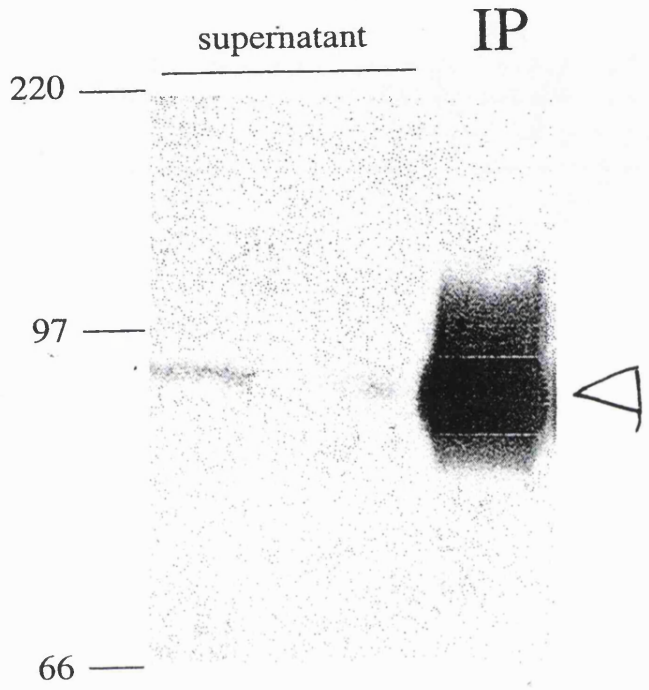


Figure 3.13.
Western blot showing immunoprecipitated material (95 kD protein)
The 129* GSANIN antibody was used to immunoprecipitate the 95 kD band from RBL cell extracts.The immunocomplex was harvested on protein A. The figure shows a western blot of protein A eluted material(IP)and duplicate lanes of the unbound supernatant material (supernatant). The 95kD band is indicated by an arrow.

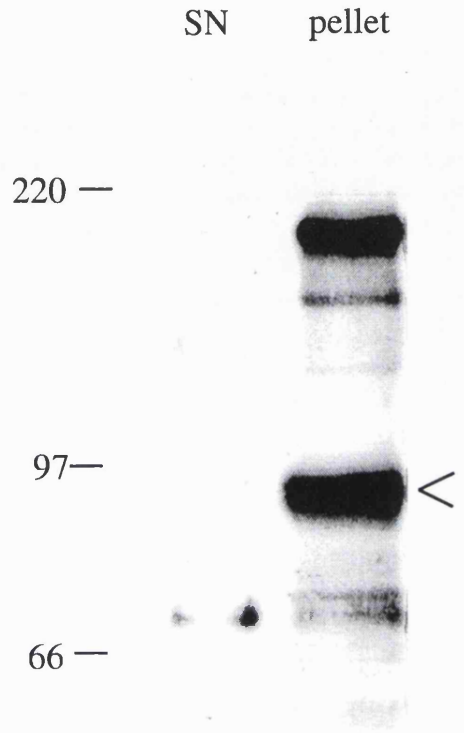


Figure 3.14.
Cellular fractionation of Jurkat cells
A membrane fraction was obtained as described in Chapter 2. The membrane associated proteins(pellet) and cytosolic proteins (SN) were subject to western blot with the 129* antiserum. The 95kD band is shown by an arrow.

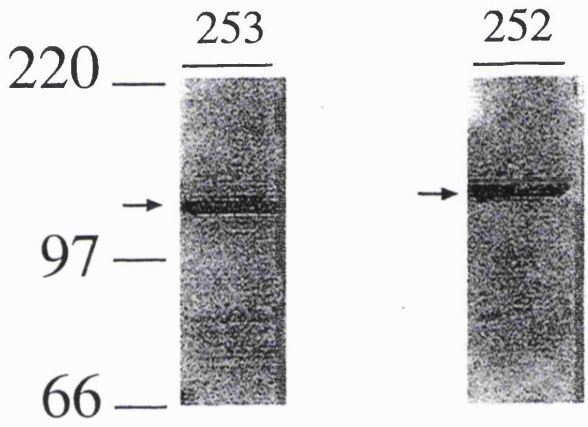


Figure 3.15.
PLD1b baculovirus expressed proteins
Western blot with 2 WAHK antibodies(252,253 correspond to 2 different immunized rabbits). The PLD1 isoform is recognised by both, as indicated by the arrows. Molecular weight markers (kD) are shown on the left.

preparation and took fractions for further purification. This brain extract was purified by sequential chromatography on MonoQ, MonoS and MiniQ columns(see Chapter 2).
In the first mono Q column bound proteins were eluted with a linear gradient of 0-1M NaCl. The fractions collected were blotted with the GSANIN 129* antibody to check if the 95kD protein was present there as shown in Figure 3.16. To be sure that the 95 kD protein was still specific for the GSANIN antibody a competition with the peptide was performed as shown in Figure 3.17
For subsequent purification, Fraction 9 was diluted and run on a mono S column using a 0-2 M elution with NaCl. Fractions were collected and blotted again with GSANIN 129* (Figure 3. 18). Fraction 11 from this last mono S column was further purified with a miniQ(SMART) column and blotted again with GSANIN 129* and stained with coomassie blue (Figure3.19). Relevant bands from fraction number 4 were cut from the acrylamide and sent for Mass Spectrometry analysis.

The results from Mass Spec showed that none of these proteins were related to PLD isoforms
The results were:
a.MMR1 mouse: possible GTP-binding protein MMR1-Mus musculus (mouse)
b.Eukaryotic Translation initiation factor 4B (EIF-4B)- Homo sapiens (Human)
c.Methyl-CPG-binding protein 2 (MECP-2 protein)- Rattus norvegicus (Rat)

The matches observed based upon the tryptic fingerprints are tabulated. No further matches have been identified in more recent public database searches. As shown, some identified proteins are not from the rat and furthermore do not match the apparent molecular weights of the proteins cut from the gel. It is concluded that the mass fingerprinting has failed to identify the proteins. This is most likely due to the poor coverage of the public sequence databases for the rat (and less so for the related mouse) genome since these sequences are not typical of contaminating proteins (e.g. human keratin, sheep keratin [from clothing], trypsin).

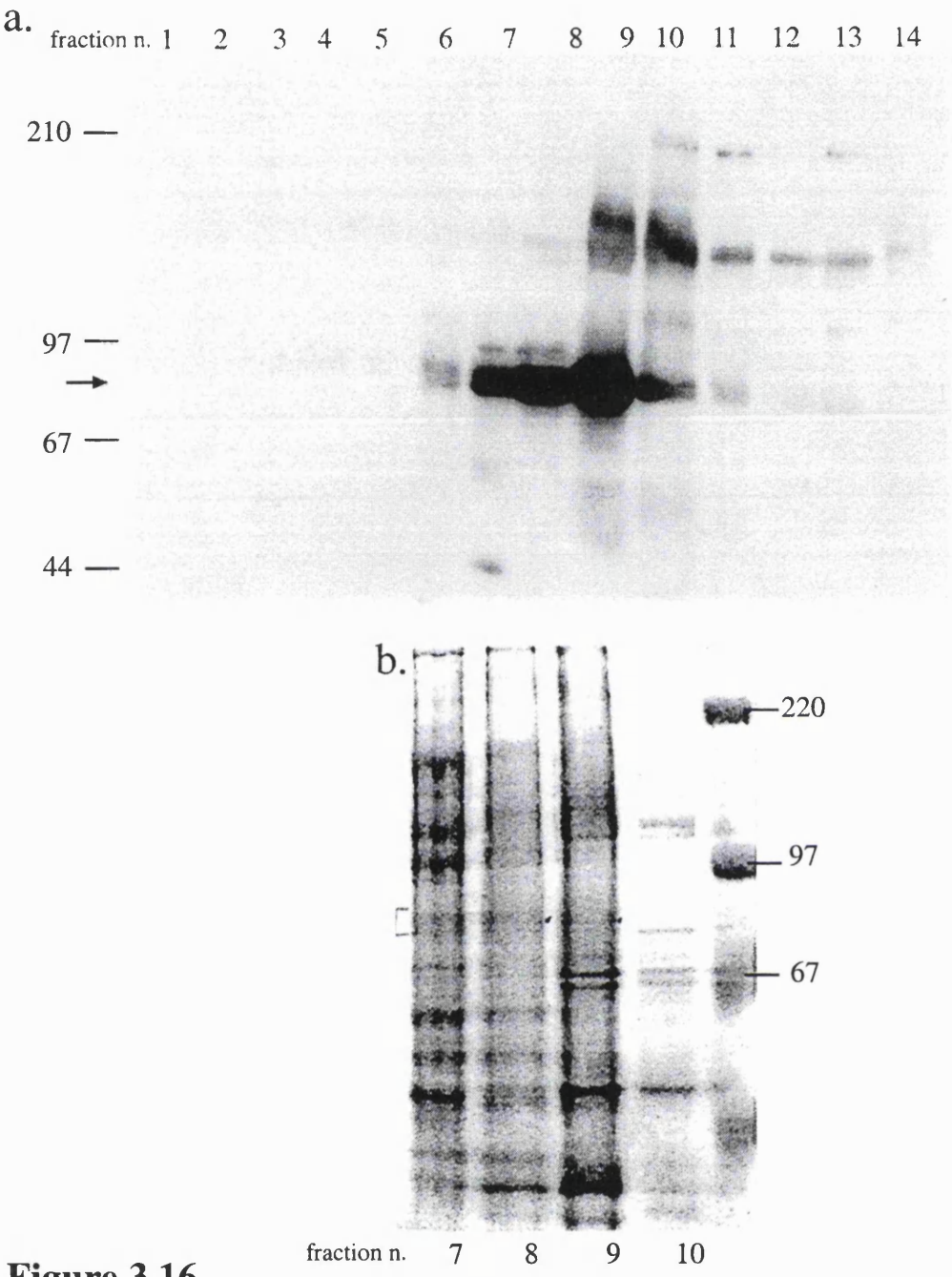


Figure 3.16.
HiLoadQ column fractionation of rat brain extract.
A rat brain extract was prepared in 20mM Tris pH7.5, 2mMEDTA, 1mMDTT by homogenisation and centrifugation at 100,000g for 1h. The soluble material was loaded onto a 50ml HiLoadQ column and washed with 5 column volumes. The bound proteins were eluted with a linear NaCl gradient from 0 to 1.0M. Fractions of 10ml were collected and samples taken for analysis on a 10%SDS-PAGE gel. **a.** After transfer they were blotted with the GSANIN antibody. The 95kD band is shown by the arrow.(Fraction 9 wastaken for further purification). **b.**Comaasie blue stained gel showing the state of purification.

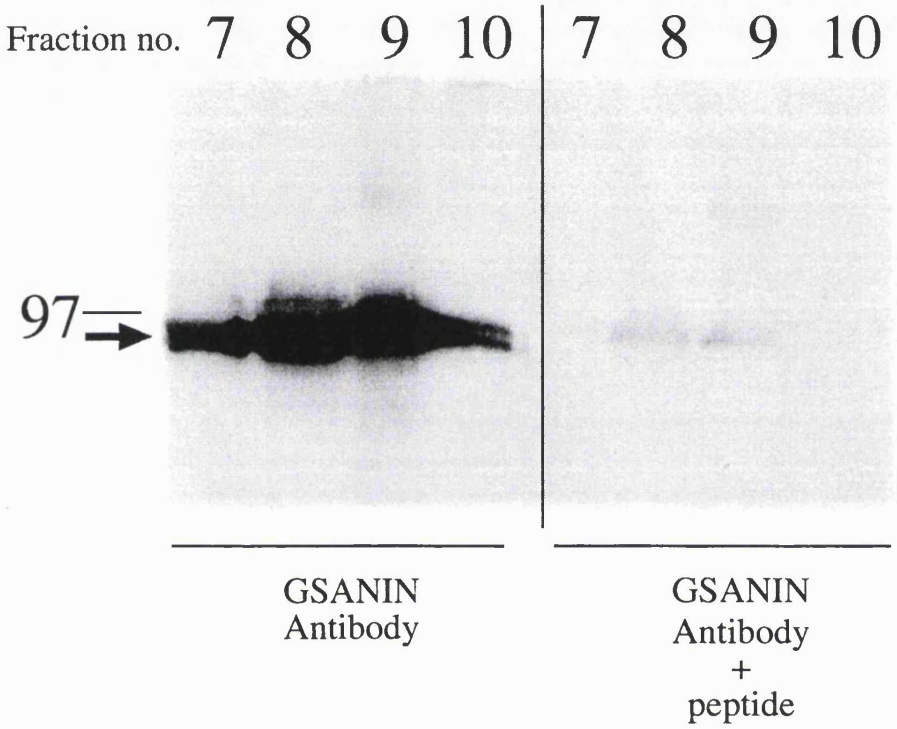


Figure 3.17.
Peptide competition of the 95 kD antigen recognised by GSANIN 129 antibody after monoQ fractionation.
Samples separate by MonoQ fractionation were loaded in a 10% SDS-PAGE gel and blotted with the 129 GSANIN antibody in the presence or absence of the GSANIN peptide. The 95K protein is indicated by the arrow.

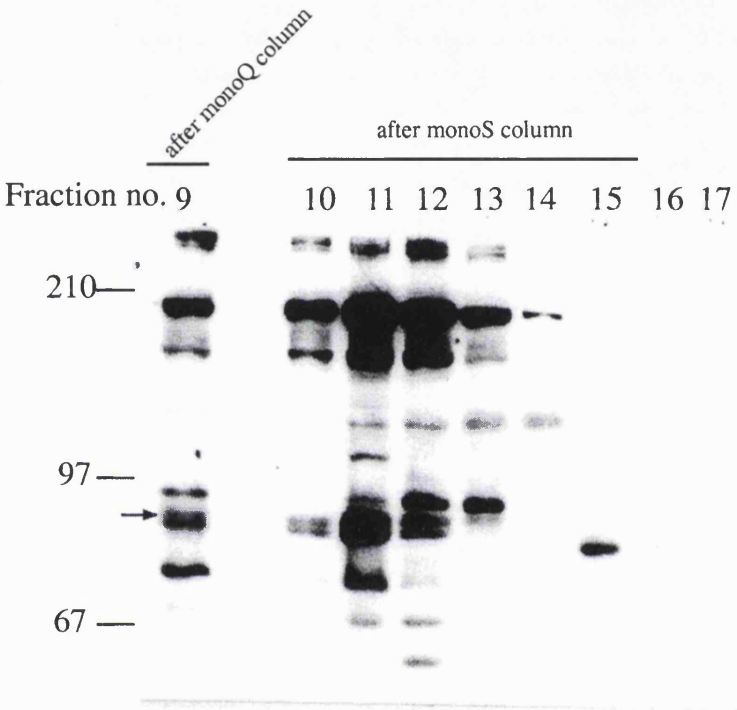


Figure 3.18.
Fractions collected after passing fraction 9 through a monoS column.
The peak from the MonoQ fractionation, fraction 9, was diluted 4-fold with 20mM MES buffer pH6.5, 2 mM EDTA, 1mM DTT and loaded onto a Mono S column (1ml). The column was washed with 5 column volumes and then eluted with a linear gradient (20ml) of NaCl from 0.0 to 1.0M. Fractions of 1ml were collected. Fractions were run in a 10%SDS-PAGE gel and blotted with GSANIN antibody. Fraction 11 was chosen for fractionation on a miniQ (SMART) column.

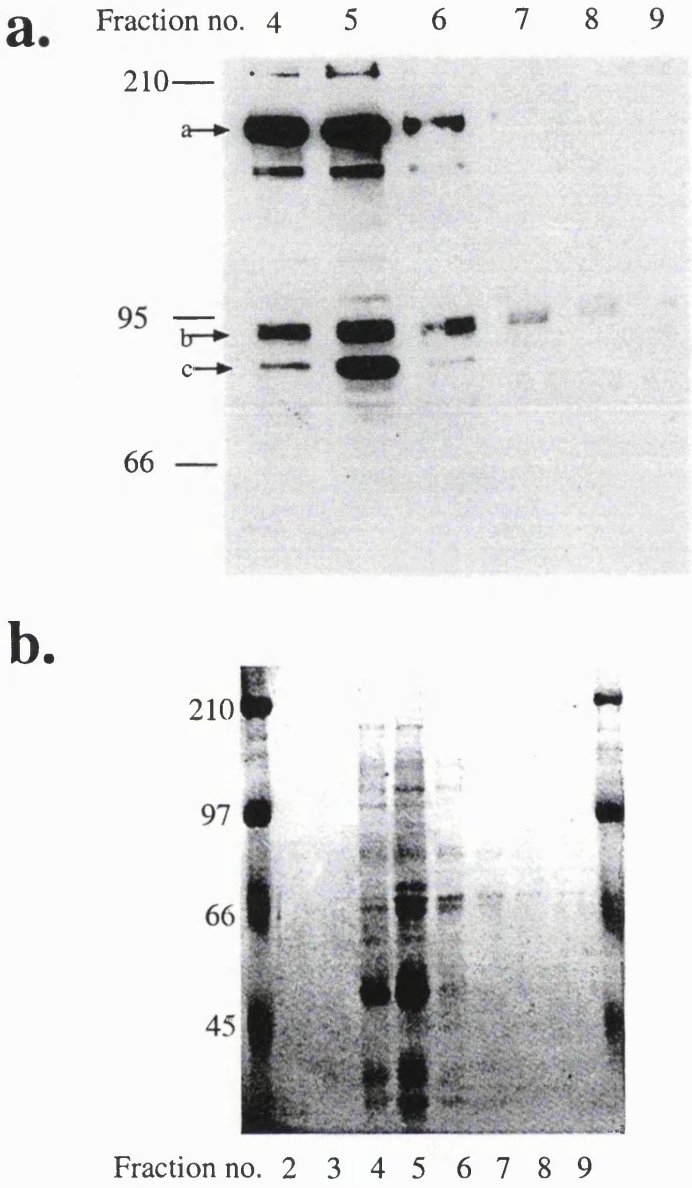


Figure 3.19.
Fractions collected after mini Q (SMART) column.
a.Western blot with GSANIN antibody.
Fracion 5 was chosen for cutting specific bands (indicated by arrows) and sent for mass spec analysis**.**
b.Commasie blue stained gel showing the state of purification.

3.3 Summary

RBL cells were chosen as a model to investigate the role of PLD and PKC in secretion. The first step was to identify which PKCs and which PLDs were present in these cells.

Using specific antibodies available in the laboratory (specifically designed to pick up different isoforms of PKC), it was shown by western blot that 6 PKC isoforms were present in these cells. This confirms the previous analysis by Beaven and colleagues (Ozawa et al., 1993).

Four different strategies were taken to identify (novel related) PLDs in RBL cells: amplification of a cDNA library by PCR using degenerate oligomers, amplification of a cDNA library by PCR using specific oligomers, screening of a phage library with a specific PLD probe and western blots using antibodies designed against conserved regions in order to pick up immunoreactive proteins.

By PCR, endogenous PLD1a and PLD 1b were detected using specific oligomers in RBL cells It appeared that the degenerate oligomers were not able to identify these and other sequences. To further characterise PLD expression a PLD1 cDNA fragment was used in a library screen. Surprisingly no sequence related to PLDs (including PLD1a and PLD1b) was found using this library screening approach. One of the reasons could be that not enough endogenous PLD mRNA is present in these cells and is therefore underrepresented in this library. The stringency used in the hybridisation of the probe is also one of the factors that could have influenced the range of proteins picked up by this method, however, having established the presence of PLD1a/b in these cells we would have expected to have detected these.

The "protein" strategy was used in order to identify new proteins with conserved domains in the PLD family. New antibodies specific to different conserved regions of different PLDs were designed and raised in rabbits. The idea of isolation through activity was not pursued due to the limited success of other groups and Dr.McKinnon from this laboratory. Baculovirus expressed PLDs, but not endogenous PLD1a/b proteins, could be detected by western blot in RBL cells with the designed antibodies. Probably the amount of endogenous PLD in these cells limits detection of the PLD1a/b proteins present.

One of the highest titre antibodies that showed specificity for the baculovirus expressed PLD1/2 proteins , shows specificity for a novel 95 kD protein. Some attempts to purify this protein were carried out from cell cultures without success. In addition the protein was purified on a larger scale from rat brains. This also proved to be unsuccessful despite

extensive purification. It was notable that during purification the 95 kD antigen may have become proteolysed as judged by the increasing immunoreactivity of bands smaller than 95 kD. Nevertheless, sequence analysis of the antigens observed at the final mini Q step were uninformative. Thus we were unable to make progress on identifying this 95kD protein as a novel PLD with conserved GSANIN motif.

In this chapter we have shown that PLD1 a and PLD1 b are present in RBL cells. At the time this information was new since nobody has studied endogenous PLD in these cells. The attempt to clone new PLDs was unsuccessful. It remains possible that other PLDs are present in these cells, having different roles in secretion/cell signalling. For example, we know now that there are 3 PLD genes present in the human genome(see Chapter 7). Nevertheless, at that time it was useful to know that the two recently cloned PLD1 splice variants were present in RBL cells. Progress from other groups(Brown et al., 1998) had shown that exogenously expressed PLD1b(and a) was found in secretory granules and lysosomes and moved with vesicles independent of activity. The confirmation of the presence of PLD1a/b suggested that the distribution of the expressed proteins may indeed reflect their physiological behaviour.

There was no advantage in pursuing our studies in RBL cells. The level of transfection in RBL cells was poor, limiting the studies we wanted to carry out to understand the PKC-PLD link. As discussed in next chapter, we decided to switch to a cell line where defective (somatic/selected) mutants were available. This model was considered better in trying to solve the PLD-PKC paradigm.

Chapter 4

U937: a PLD-ERK link?

4.1 Introduction

U937 cells originated from the pleural fluid of a patient with diffuse histiocytic lymphoma. This cell line has been an important model for the disciplines of cancer, haematology and immunology. As sources of leukemic cells, they have been used for the study of neoplasia and therapeutics (Cabanas et al., 1988; Martin et al., 2000; Suzuki et al., 2001). As sources of haematopoietic cells, they have been used for biochemical and biological analysis of regulation and differentiation in myelopoiesis. When stimulated with immunomodulatory factors, the cell line develops properties of host-defence effector cells (Heidenreich et al., 1996) They are also a source of cytokines that affect other cell types (Palacios et al., 1982)

U937 cells differentiate exclusively toward macrophage-like cells, and among the agents that can trigger this differentiation are the phorbol ester class of PKC agonists. Prolonged incubation (24-72 hours) with phorbol esters induces these non-adherent promonocytic cells to differentiate into adherent macrophage like cells. Associated with this response, phorbol esters induce growth arrest, substratum adherence and β2 integrin expression in these cells (Harris and Ralph, 1985).

Differentiation is a complex multistep process and response to phorbol esters may include different classes of 'receptor' as well as different members within classes. In U937 cells there is evidence that individual PKCs isozymes have specific roles in regulating this response. U937 cells express PKC isozymes β1 ,β2, δ, ε and ζ. Indirect immunocytofluoresence using affinity purified PKC-specific antibodies indicates that each of the endogenous PKC isozymes display a unique compartmentalisation within the intact cell. PKCβ1 is distributed between two identifiable pools: a cytoplasmic pool which redistributes to the plasma membrane upon activation with acute phorbol ester treatment and a membrane-bound pool associated with intracellular vesicles containing β2 integrin adhesion molecules CD11b and CD11c. The vesicle associated PKCβ1 translocates with the secretory granules to the plasma membrane upon agonist stimulated activation. PKC β2 is associated with the microtubule cytoskeleton in resting cells (Kiley and Parker, 1995). See Figure 4.1.

Previous studies from this laboratory have led to the isolation and characterization of a series of TPA resistant U937 cell lines (Kiley et al., 1997). Detailed analysis of three of these resistant clones has demonstrated that although PKC expression and behaviour is grossly normal and that certain acute responses remain intact, there is a subcellular "mis-localization" of PKCβ2 in three independent clones studied in detail (Kiley and Parker, 1997). In U937 cells, this PKC isotype is associated with the microtubule network, and on stimulation with TPA there is a general reorganization of the microtubules. In the TPA resistant cell lines, no reorganization of the microtubules occurs in response to TPA, correlating with the loss of PKCβ2 from this compartment. Consequent to this defect, TPA does not induce an acute upregulation of cell surface integrins derived from perinuclear granules. However a partial reversal of the resistant phenotype can be elicited by nocodazole treatment, suggesting that the microtubule depolymerization and subsequent granule release play essential roles in the differentiation process triggered by TPA (Kiley and Parker, 1997).

Here, initial studies with this model system were directed at the question "Is PLD involved in the acute response to phorbol esters?" This principally involved the ERK1/2 pathway, since activation of this has been shown to be essential for the process of differentiation (Whelan,1999).

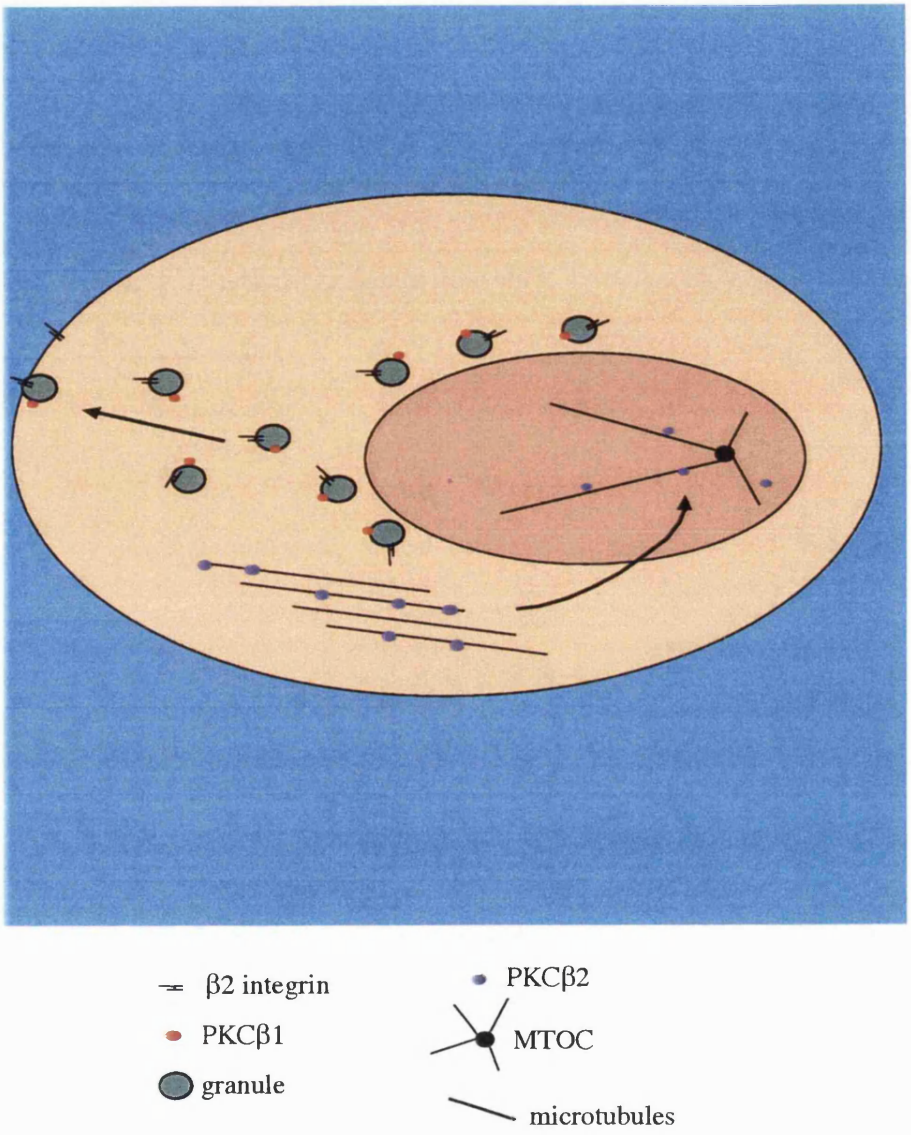


Figure 4.1
Model of differentiation promotion in U937 cells
Acute TPA treatment releases granules with associated PKCβ1 and β2 integrins. MAPK gets activated and cells go on to differentiate.
MTOC (microtubule organising center)

4.2 Results

4.2.1 Differentiation: heterogeneity of different clones

There is a substantial degree of heterogeneity amongst different U937 cell lines. This is reflected in a significant variation in their response to differentiating agents which can trigger between 50% and >95% differentiation. To ensure that the isolates worked with here behaved in a homogeneous manner, lines were checked for their differentiation response to TPA. Wild type cells were exposed to 300nM TPA for 72h and the degree of adherence monitored. Exposure of U937 cells to TPA results in differentiation, characterised by growth arrest and cell adherence (see Figure 4.2). Adherence was obtained for 98% of cells as judged by shake off, but <8% of resistant cells. This was considered satisfactory for the use of these clones (data from resistant clones by R.Whelan in the laboratory)

4.2.2.Activation of ERK in U937 cells.

Prolonged activation and nuclear retention of ERK has been implicated in differentiation (Traverse, 1992); sustained activation has been shown to be important also in U937 cell differentiation (Whelan, 1999). To define the behaviour of phorbol ester induced ERK activation in U937 cells, log-phase cultures were treated with 300 nM TPA for 5, 10, 20, 30, 40, 50 and 60 minutes. The state of phosphorylation of ERK was examined by Western blotting with a phospho-ERK1/2 specific antibody. Figure 4.3 shows that activation was maximum by 5 minutes and was still elevated 60 min after TPA stimulation. To confirm the likely upstream kinase involved in ERK1/2 activation, the MEK inhibitor PD98059 was exploited. Treatment of cells with 30μM PD98059 inhibited ERK activation(Figure 4.4). This inhibitor has proved useful in that it has also permitted assessment of the requirement for this pathway in differentiation. Treatment of cells with 30μM PD98059

control

TPA 72 hours

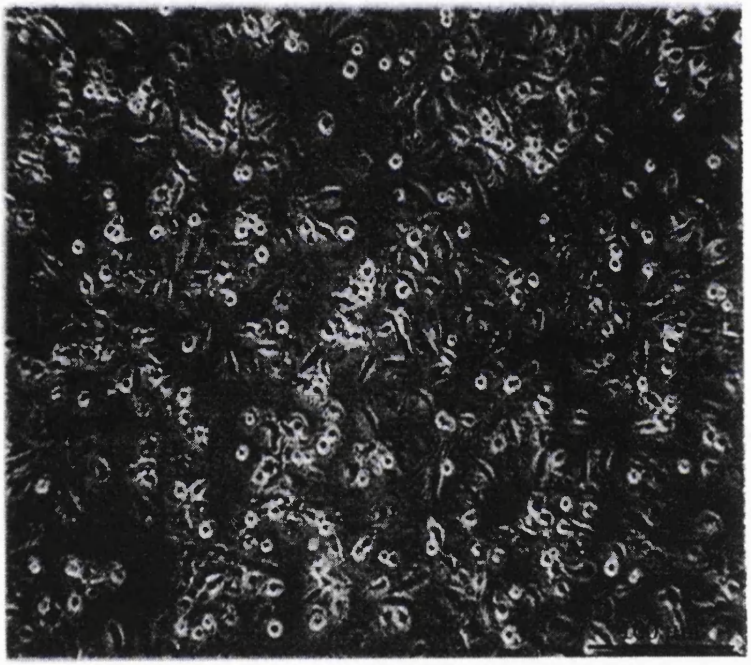


Figure 4.2.
Treatment of U937 cells with phorbol ester.
U937 cells were incubated in the presence or absence of 300nM TPA for 72 hours. Pictures were taken under phase microscope. After 72 hours of TPA treatment, U937 cells have adhered to the substrate and differentiated.The scale bar represents 100 μm.

Figure 4.3.
Activation of p42 MAPK in wt U937cells.
Logarithmically growing wt U937 cells were exposed to TPA (300nM) for the times indicated.The cells then were harvested, washed in PBS, extracted in SDS-PAGE sample buffer and analysed by Western blotting with phospho-specific extracellular signal-regulated kinase1/2 (ERK 1/2/) antibody. The upper band represents ERK1 and the lower ERK2.This is representative of two similar experiments performed.

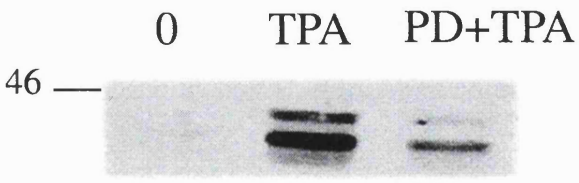


Figure 4.4.
PD98059 inhibits MAPK activation
U937 cells were pretreated with PD98059(PD)for 10 minutes before TPA stimulation for 10 minutes. Cell were harvested and analysed by western with phospho-specific ERK1/2 antiserum. This is representative of two similar experiments performed.

completely prevented growth arrest and adherence (Whelan et al., 1999). This pattern of behaviour was entirely consistent with the previous analysis on these cells.

4.2.3 Primary alcohols inhibit activation of ERK

To assess whether PLD might influence PKC activation of ERK1/2 primary alcohols were used. The use of primary alcohols as a way of investigating PLD function has been used broadly in different systems [Ella, 1997]. Phospholipase D can use primary alcohols as an acceptor molecule for its reaction instead of water; it does so with a 500 fold greater efficiency, so that the physiological product of the PLD reaction, phosphatidic acid, is not formed but rather a non-physiological phosphatidylethanol. This diversion from the generation of the normal product underlies the inhibitory action of primary alcohols on PLD function. To investigate if PLD was involved in the activation of ERK1/2, cells were treated with primary alcohols (ethanol or butan-1-ol) for 10 minutes prior to TPA stimulation. Butan-2-ol, a secondary alcohol was used as a control, since this is not a substrate for the PLD transphosphatydilation reaction.

Alcohols can be toxic, hence the concentrations were titrated down to a level that did not induce loss of viability. Ethanol at 2% blocked TPA-induced ERK1/2 activation as judged by the lack of immunoreactivity with phospho-specific antisera (Figure 4.5). The butan-2-ol control did reduce the level of ERK activation but this was consistently much less than the effect of butan-1-ol. On this basis, it was concluded that a PLD function is likely to be involved in the TPA-induced ERK1/2 activation.

To ensure that the effects of the alcohols was not non-specific, other cell lines were also investigated. It is clear that not all TPA-induced ERK1/2 activation events are sensitive to primary alcohols, consistent with the view that this is a specific effect in U937 cells (Figure 4.6).

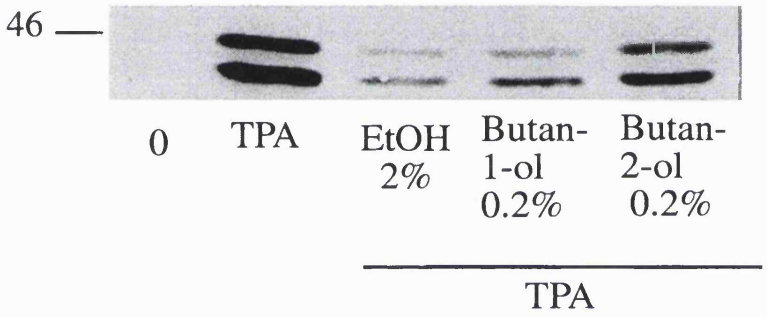


Figure 4.5.
MAPK activation in the presence of alcohols
MAPK phosphorylation was assessed in U937 cell extracts by western blot analysis using phospho-specific polyclonal antibodies against ERK1/2. Cells were treated with primary and secondary alcohols for 10 minutes prior to a 10 min incubation with 300 nMTPA. This is one of 4 replicate experiments.

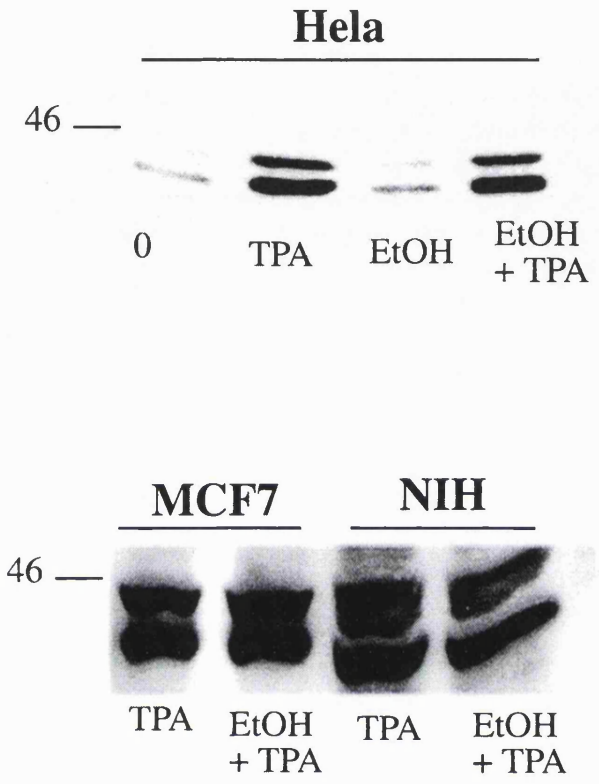


Figure 4.6.
Different cell lines showing ERK activation is not blocked by primary alcohols.
ERK phosphorylation was assessed in cell extracts by western blot analysis using phospho-specific polyclonal antibodies against ERK1/2. Cells lines as indicated were treated with ethanol for 10 minutes prior to a 10 min incubation with 300 nMTPA.

4.2.4 Catalytic activity of PKC is required for ERK activation

Since it was established that phorbol esters have a potential to activate the ERK cascade, it was supposed that PKC, the major cellular receptor for these agents mediates this activation process. This can be distinguished from other phorbol ester receptors through sensitivity to catalytic site inhibitors. Furthermore these agents can be used to distinguish between catalytic and allosteric requirements for PKC itself. To asses the requirement of catalytic activity of PKC, BIM (Bisindolylmaleimide-I) was used. BIM is a relatively potent and specific PKC catalytic inhibitor (Martiny-Baron et al., 1993).
This is one of the best PKC selective inhibitors (Parker,1999) and has recently been tested against a spectrum of protein kinases and shown to retain good but not exclusive specificity (Davis, 2000). Cells were treated with BIM for 15 min prior to activation with TPA. As shown in Figure 4.7, ERK1/2 activation is blocked. It is concluded that catalytic activity of PKC is required at some point in the cascade for the signal to reach MEK/ERK.

4.2.5. The effects of inhibitors of clathrin mediated receptor endocytosis

As discussed in the Introduction to this chapter, TPA treatment of cells leads to a series of events that includes the release of granules and the upregulation of integrins. To determine whether a cell surface receptor mediated process may be involved in ERK activation and if this is restricted to the cell surface, we tested some inhibitors of clathrin mediated endocytosis, such as monodansylcadaverine (MDC) and hyperosmolar sucrose, 0.45M (which block clathrin association). Precedents that endocytosis may be involved in such responses is exemplified by studies on the β-adrenergic receptor (Claing et al., 2001). Figure 4.8 shows that MDC and hyperosmolar sucrose markedly reduced the TPA dependent activation of ERK. This suggest that an endocytic mechanism may be involved. It also questions whether the catalytic requirement for PKC occurs proximal or distal to this event.

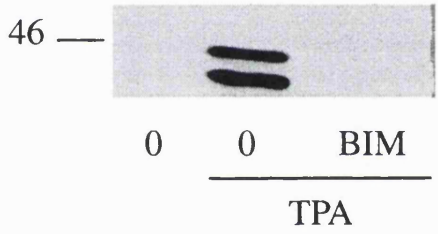


Figure 4.7.
BIM inhibits MAPK activation
wtU937cells preincubated in the presence or absence of 10μM BIM for 10 minutes were cultured in the presence of absence of 300nM TPA for 10 minutes. Extracts were analyzed by western blotting as described previously with phospho specific ERK1/2 antibodies. This is one of 4 replicate experiments.

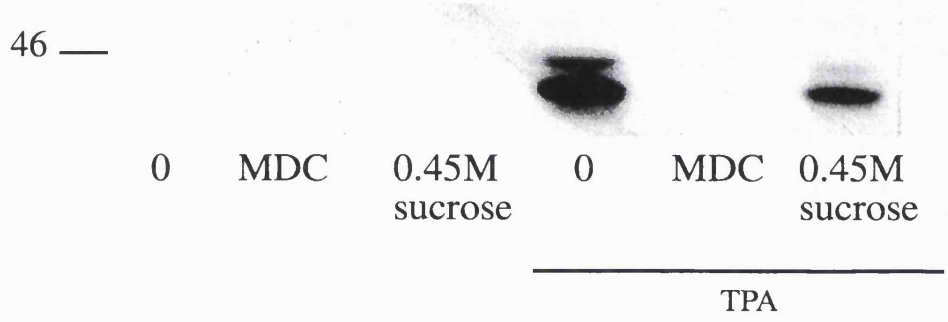


Figure 4.8.
Activation of ERK in presence of endocytosis blocking agents.
U937 cells were pretreated with hyperosmolar sucrose or 200μM monodansylcadaverine (MDC) for 15 minutes before stimulation with TPA (300 nM) for 10 minutes. Cells were harvested and sample buffer was added inmediately. Extracts were analysed by Western blotting with phosphospecific ERK1/2 antibody.

4.2.6 What receptor mediated mechanism might account for activation of MAPK?

To start to trace back the trigger for ERK activation, the potential role for integrin function was investigated. Although TPA has been shown to upregulate β2-integrin at the cell surface, there is nevertheless significant amounts of this integrin already present at the cell surface. This was exploited by determining whether ICAM-1 a β2-integrin substrate could directly activate ERK1/2.

Cells were allowed to settle onto 1 μg/ml of human ICAM-1 Fc (provided by Nancy Hogg) coated coverslips for 5, 10 or 15 minutes in the presence of 5 mM $MgCl_2$ and 1 mM EGTA, these last components were required for having the right conformation of the integrin to bind the ligand. Following incubation, cells were immediately put into sample buffer and run on an SDS-PAGE gel to check ERK activation. In some experiments, cells were pre-treated with cadaverine prior to ICAM-1 binding. As shown in Figure 4.9, ICAM-1 produced a robust activation of ERK1/2. Furthermore, consistent with the view that this may play a role in the TPA-induced response, this ICAM-1 dependent activation was also blocked by MDC. Thus the integrin-dependent activation of ERK1/2 in these cells, like that induced by TPA, appears to require endocytosis. This led us to suppose that the TPA response may require integrin activation.

4.2.7 Is there release of an autocrine factor?

Evidence is beginning to emerge suggesting that proteolytic cleavage of cell surface proteins, or ectodomain shedding, is an important mechanism whereby cells can regulate the repertoire and activation state of proteins expressed on their surface. Several types of membrane proteins undergo ectodomain shedding, including cytokines(TNF-α,IL-6R,TNF R I-II, MCSF R I), growth factors (TGF-α,HB-EGF) and adhesion molecules (L-selectin). Cell surface proteolysis appears to be largely mediated by members of the

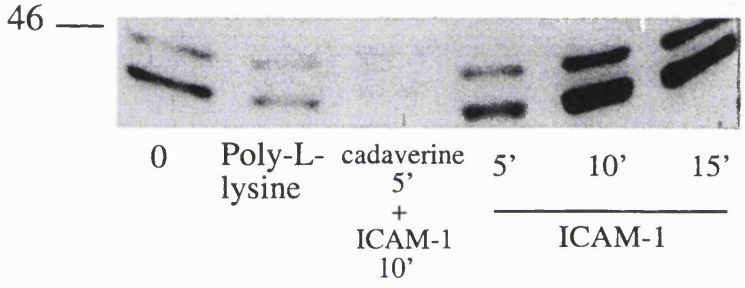


Figure 4.9.
Inhibition of ICAM-1-induced MAPK phosphorylation by cadaverine.
U937 cells were allowed to settle onto coverslips coated in the presence or absence of 1μg/ml human ICAM-1 Fc (kindly provided by Dr. Nancy Hogg Laboratory) for 5, 10 or 15 minutes.Where indicated cells were preincubated with 200nM monodansylcadaverine for 5 minutes. Activation of MAPK was checked by western blotting with phospospecific ERK1/2 antibodies.

metzincin superfamily of zinc dependent proteases which include the matrix metalloproteinases (MMPs).

Human U937 promonocytic leukemia cells, like circulating macrophages and neutrophils, contain intracellular granules that undergo stimulus-induced translocation to the cell surface. These small intracellular vesicles are alternatively referred to as secondary or specific granules and contain β2 integrins, CD11b and CD11c, cytochrome b558 of the NADPH oxidase complex and a variety of proteases including lysozyme and gelatinase. If the TPA-induced ERK1/2 activation was mediated by cell surface integrin, then it was considered possible that the TPA-induced release of granules may be sufficient to cause proteolytic activation of integrin ligand(s).

So to test if an autocrine mechanism of this type was involved in MAPK activation, we investigated the effect of the metalloproteinase inhibitor marimastat on TPA induced ERK activation. Marimastat is a broad spectrum MMP inhibitor that is non- membrane permeant but acts on the surface exposed MMPs. It has a collagen-mimicking hydroxamate structure, which facilitates chelation of the zinc ion in the active site of the MMPs. This compounds inhibit MMPs potently and specifically (Wojtowicz, 1997). Cells were preincubated with 5μM marimastat for 10 minutes prior to stimulation with 300nM TPA for 10 minutes. Figure 4.10 shows a partial inhibition (50%, determined by scanning densitometry of 3 experiments) of ERK activation by marimastat. This is consistent with a contribution through the proposed pathway. It was not possible to obtain a greater effect of marimastat, which may reflect either the operation of two independent pathways or that the ability of marimastat to access the released MMPs is limited, perhaps by the kinetic constraints of the system - note that preincubation does not give access to the targets, marimastat effectiveness is dependent upon instantaneous action at the plasma membrane.

Detection and quantitation of matrix metalloproteases release was checked by zymography (Leber and Balkwill, 1997). Zymography is an electrophoretic method for measuring proteolytic activity. The method is based on a sodium dodecyl sulfate gel

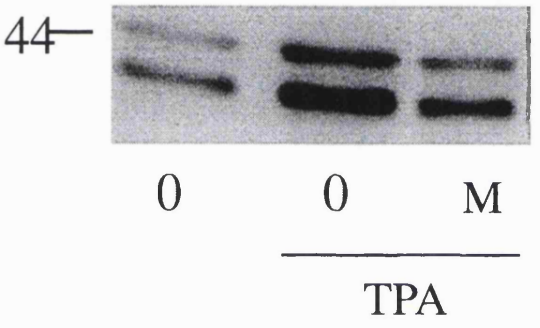


Figure 4.10.
Marimastat partially blocks MAPK activation
U937 cells were preincubated with 5μM marimastat (M) for10 minutes prior to stimulation with 300nM TPA for 10 minutes. Extracts were analyzed by western blotting using phospho-specific ERK1/2 antibodies.

impregnated with a protein substrate which is degraded by the proteases resolved during the incubation period. Coomassie blue staining of the gel reveals sites of proteolysis as white bands on a dark blue background. Within a certain range the band intensity can be related linearly to the amount of protease loaded. Note that MMPs are released from cells in a proteolytically inactive pro-form (zymogen) which is about 10 kDa larger than the activated form. U937 cells were treated with TPA during the times showed and spun down to take the conditioned media after treatment. Sample buffer not containing b-mercaptoethanol was added and samples were run onto gelatin containing gels (see Materials and Methods). Unfortunately the amount of MMP released in response to TPA was too low for reproducible detection (data not shown). Sensitivity is an intrinsic limitation of this method.

4.2.8 Visualisation of granules moving after TPA treatment

All the data shown hint at the possibility of an input of TPA via PKC and PLD at the level of release of the granules to the plasma membrane, upregulating total integrin levels and releasing marimastat-sensitive proteinase(s), that may be responsible for triggering the subsequent signalling event. While the data was consistent with this notion, it was important to establish the granule release component.

Cells were treated with TPA for 10 minutes and granule movement monitored. Because U937 are suspension cells they had to be spun down onto poly-L-lysine coated coverslips before performing immunofluorescence. The cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% saponin in the presence of BSA and FCS. Then first and second antibodies were incubated before mounting the coverslips onto mowiol. Slides were checked under confocal microscopy. Figure 4.11 shows the movement of perinuclear granules with PKCβ1 to the plasma membrane after TPA stimulation for 10 min. This confirms that granules are being released under these conditions.
Various strategies were employed to monitor the movement of integrin to the plasma membrane. Initially I developed a C-terminal antiserum to β2-integrin which although it

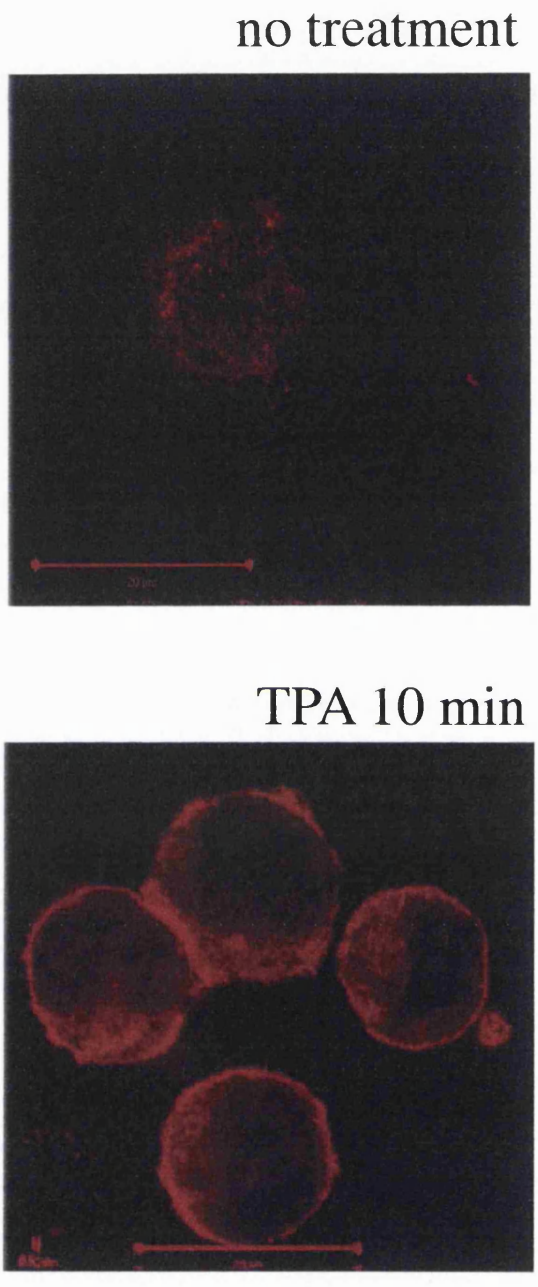


Figure 4.11.
Cellular localisation of PKCβ1 in U937.
U937cells were treated with TPA for 10 minutes and plated into poly-L-lysine coated coverslip before fixaxion and permeabilisation. They were observed under confocal microscopy.(Bar size:20 μm)

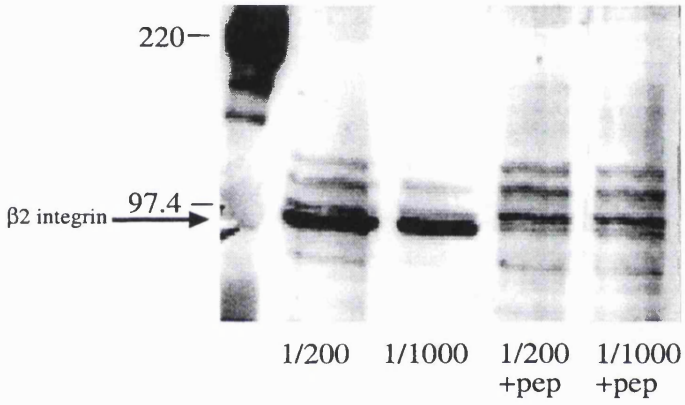


Figure 4.12.
Immunodetection of β2(CD18) integrin.
An antiserum raised to a carboxyterminal peptide of β2(CD18) integrin was raised in rabbits (see Methods). The serum was tested on extracts of U937 cells and showed specific immunoreactivity with an antigen of the appropriate size (arrowed). The dilutions of the antiserum are indicated and the presence of competing antigen (+pep).

worked in western analysis (Figure 4.12) was not found to be satisfactory for immunofluorescence. Similarly, I used the 44 monoclonal antibody (provided by Nancy Hogg) that recognises the N-terminus of CD18, to assess upregulation by FACS. Consistently I found that the change in immunoreactivity in response to TPA was too close to the basal level of cell surface. A variety of protocols (fixatives, permeabilisation agents) were tested. Hence this antiserum also proved unsuitable for immunofluorescence.

To try and exploit a western procedure for which had a suitable reagent (see Figure 4.12), trypsin was used to clip/degrade cell surface proteins prior to or post TPA treatment. The objective was to assess the protected (intracellular) pool of β2-integrin. Despite use of different concentrations of trypsin and combinations of protease inhibitors (trypsin inhibitor and serum), it was not possible to control the proteolysis and hence to define the changing intracellular pool. Cells were treated in the presence or absence of TPA 10 minutes. After that, trypsin was added to the media for 10 minutes at 37°C to cleave the integrins (and other proteins) that had already reached the plasma membrane. Integrin was monitored by western blot. Two different immunoreactive bands should appear, the ones cleaved, i.e. those exposed to trypsin at the plasma membrane, and those protected within the cell. There was no protected pool, even in the TPA non-treated cells (Figure 4.13).

4.3 Summary

It appears that PLD is involved in some way in MAPK activation via TPA in U937 cells. The phosphorylation of the ERK1/2 proteins is blocked by primary alcohols and not by secondary alcohols. The primary alcohols can be used by PLD as an acceptor molecule for the transphosphatidylation reaction. So in some part of the path leading to ERK activation PLD appears to be present. There are as yet no other agents that can be employed in the acute inhibition of PLD and hence it was not possible to confirm this conclusion by other means. However the potential for non-specific effects was reduced

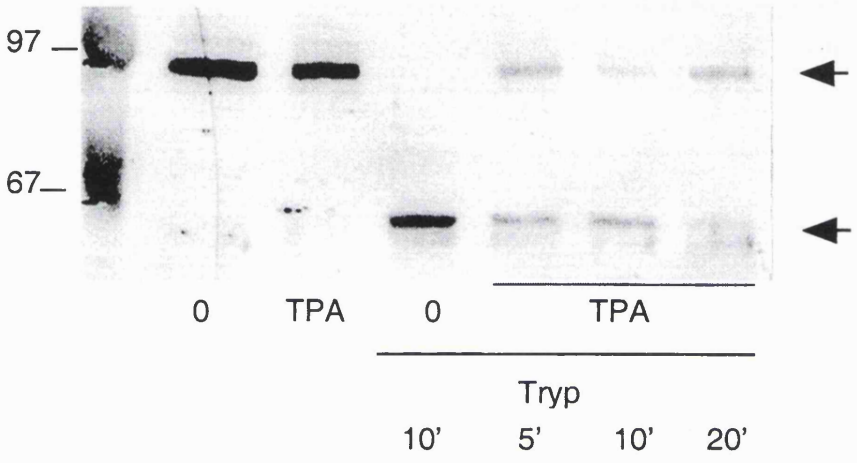


Figure 4.13.
Trypsinization of cd18 in cell surface.
U937 cells were treated in the presence of TPA for 10 minutes.Trypsin was then added to the media for the times shown at 37°C. The size of the integrin was monitored by Western blot. The two bands identified are indicated by arrows. The upper is the full length protein.

when we observed that ethanol did not inhibit TPA-induced ERK activation in other cells tested. While this might indicate that there is something unusual about U937 cells, it also suggests that ethanol does not non-specifically disrupt some conserved PKC>ERK pathway. Using specific catalytic inhibitors of PKC we demonstrated that PKC catalytic activity is needed to activate ERK. In light of the above, this might occur by activation of PLD. This subject will be discussed in next chapter.

The way that TPA triggers ERK activation was further investigated. We considered it possible that this may be indirect in view of the well established degranulation that occurs. To assess this, initial studies were directed at endocytosis as this has been reported to be important in other contexts (Claing et al., 2001). Indeed we observed that when endocytosis was blocked, the activation of ERK1/ERK2 is inhibited. This was indicative of a receptor driven process, that led to subsequent experiments on the potential of an integrin based response. It was shown that activation of ERK could be achieved by direct activation of the integrin pathway using the ligand ICAM-1. Importantly this activation could also be blocked by inhibitors of endocytosis, suggesting that TPA might lead to the cell surface activation of integrins. It is also shown in this chapter that metalloproteinase release plays a role in ERK activation. Using Marimastat, a metalloproteinase inhibitor, we observed a 50% inhibition in ERK activation. As noted above, this partial inhibition may be accounted for by the limited access of the inhibitor to the target. Whether the proteolysed target involved is indeed an integrin ligand or whether the inhibition reflects action of some other released factor would need to be confirmed by direct identification.

U937 release granules to the plasma membrane after TPA stimulation (Kiley and Parker, 1995). Here we monitored this response by immunofluorescence with a PKCβ1 antibody, confirming the appearance of the protein at the plasma membrane. Parallel studies using antisera to β2-integrin were found not to be suitable for demonstration of its movement also.

In Figure 4.14, an hypothesis is outlined for TPA-induced ERK activation in the U937 cell model. After acute TPA treatment, granules containing integrin and PKCβ1 are delivered to the plasma membrane. This leads to release of metalloproteinase(s) and subsequent triggering of a cell surface receptor (possibly an integrin). The receptor is endocytosed and presumably in an endosomal compartment, engages the upstream pathway responsible for ERK activation.

The studies described in this chapter initially provided evidence for an involvement of PLD in the TPA activation of ERK. It is evident from the above that there are multiple steps at which PLD might contribute to this response. Subsequent chapters address directly PLD activation and its likely role.

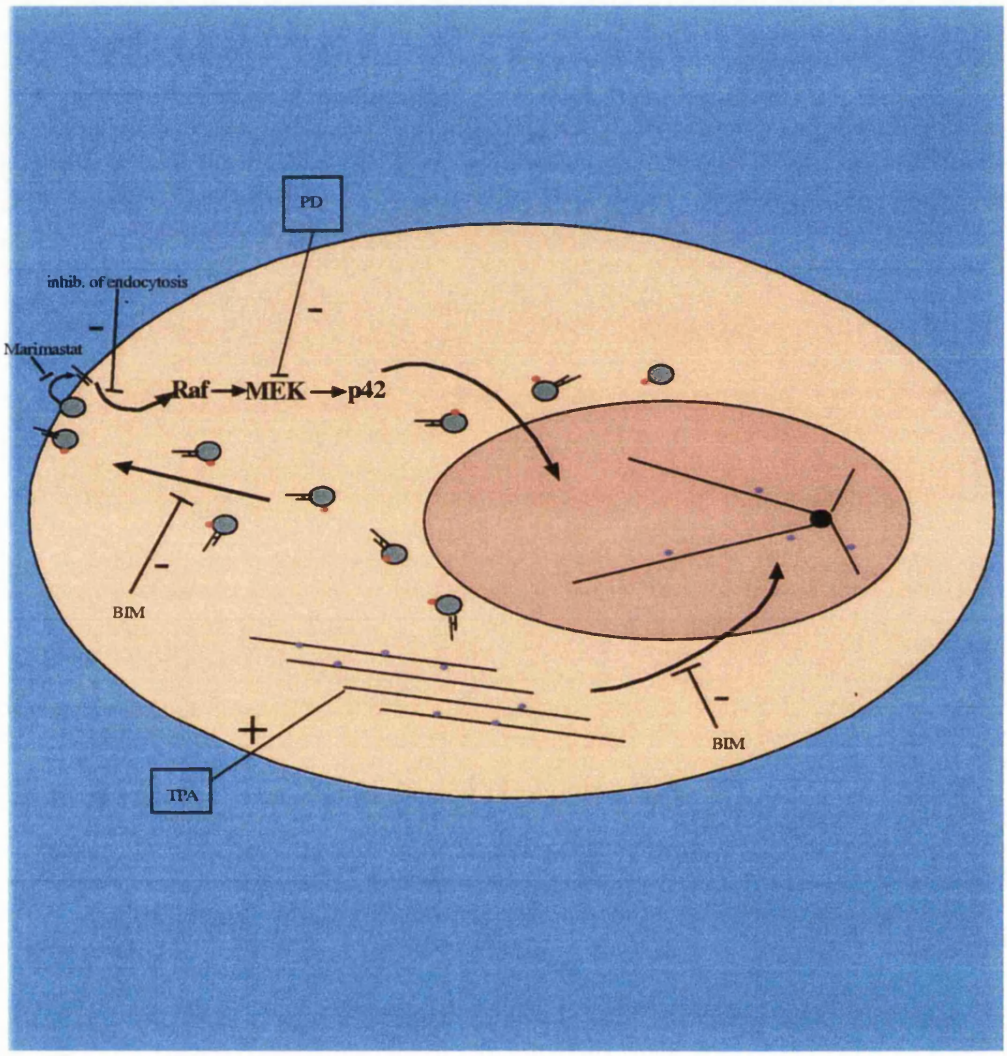


Figure 4.14
Hypothesis in U937 model
After acute TPA stimulation granules containing integrins and PKCβ1 are released to the plasma membrane after microtubule rearrangement. The signal sent to MAPK can be blocked with inhibitors for PKC, metalloproteinase, endocytosis and MEK. Primary alcohols also block this signal implying that PLD plays a role in this system. This question is addressed in the next chapters.

Chapter 5

PKC dependent PLD activation

5.1 Introduction

It was shown in the previous chapter that in U937 TPA stimulated cells triggered MAPK activation and that this was hypothesised to be effected via an autocrine mechanism related to the granule release. This MAPK activation is inhibited by primary alcohols implying a role for PLD in this process.

Phospholipase D activity (PLD) is implicated in vesicular trafficking and signal transduction. Ca^{2+} and protein kinase C have been implicated in the control of the exocytic machinery although the exact mechanism has not been defined (Ozawa et al., 1993). The potential role of PLD in regulated exocytosis is indicated by the observation that ethanol can inhibit antigen mediated exocytosis from mast cells (Lin et al., 1991).

Phorbol esters are highly effective stimuli of PLD activity in intact cells, reviewed in (Exton, 1997). Studies have identified two models of regulation of PLD by PKC. The first has been suggested to involve phosphorylation of PLD itself or some associated regulatory component. The second identified is a phosphorylation independent mechanism that has been shown to be independent of PKC catalytic activity, and the likely mechanism of activation is due to co-operative recruitment of PKC and PLD to their target membranes through which certain PKC isozymes can activate PLD1. Proteolysis studies implicate the N-terminal PKC regulatory domain as the site of this interaction.

In this chapter, an *in vivo* PLD activity assay is used in order to study the TPA dependent PLD activation in U937 cells. We have used this assay to try and address the likely role(s) of PKC in this process.

5.2 Results

5.2.1 *In vivo* labelling of U937 cells

Regulation of *in vivo* PLD activity by TPA was investigated in U937 cells. To assess this, cells were labelled in the presence of 2μCi/ml ^{3}H myristic acid. This fatty acid gets incorporated in phosphatidylcholine, the substrate of the PLD enzyme, so that the tritiated PtdCho can be metabolized by PLD. The product of this reaction depends on which acceptor molecule is available for the transphosphatidylation reaction. For example if the cells are left in normal media the acceptor molecule appears to be water. Therefore phosphatidic acid will be the labelled product that can be detected. If primary alcohols, eg. ethanol or butan-1-ol, are included in the media then the products of PLD activity will be phosphatidylethanol (PtdEtOH) or phosphatidylbutanol (PtdBut) respectively. The lipid products of this reaction can be separated by TLC.

To investigate a suitable labelling time of phosphatidylcholine, a time course of phosphatidic acid production was checked. After labelling the cells for the times shown, they were treated with 10 nM TPA for 10 minutes. Lipids were extracted and analysed by TLC (see Materials and Methods) and production of tritium labelled phosphatidic acid observed by fluorography (see Figure 5.1). At 6 hours more label was incorporated thus yielding a better signal facilitating further analysis. For future experiments the 6 hours labelling period was chosen to have a suitable signal.

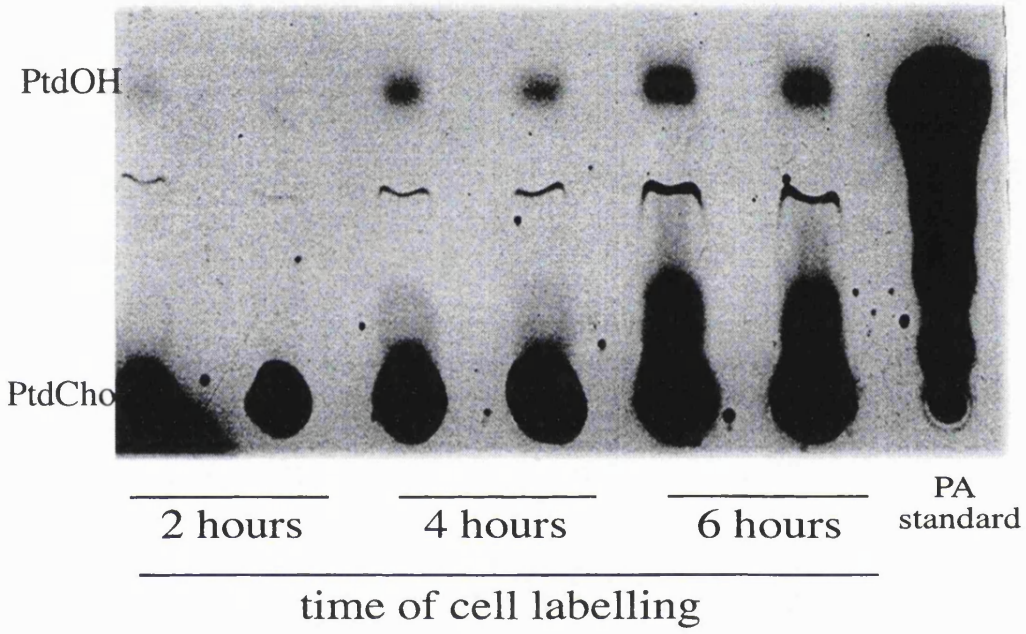


Figure 5.1.
***in vivo* ^{3}H-Phosphatidic acid production in U937 cells**
U937 cells were labelled for the times shown with 2μCi/ml of ^{3}H myristic acid before treatment with 1 μM TPA for 10 minutes. Lipids were extracted and processed as described in Materials and Methodsbefore separation by TLC as shown above.

5.2.2 TPA causes activation of PLD *in vivo*.

Different concentrations of TPA 1, 10, 100 and 1000 nM, were checked for their ability to stimulate PtdEtOH production in ^{3}H-myristate labelled U937 cells. After labelling, cells were stimulated for 10 minutes with these different concentrations of TPA before lipid extraction and TLC analysis. The graph in Figure 5.2 shows that the highest activity observed was in cells stimulated acutely with 1000nM of TPA. This was the concentration used in future experiments.

Figure 5.3. shows the phosphatidylethanol formed during a time course of PLD activation by TPA. In further experiments the lipids were scraped from the silica plates and counted in scintillant to quantify PtdEtOH production. Figure 5.4. shows a graph of PtdEtOH produced in response to TPA. The initial rate of production is essentially linear for the first 10 min only. In all future experiments described in this chapter (except where specified), cells were labelled with ^{3}H myristic acid for 6 hours, pre-treated with 2.5% of EtOH and then stimulated with 1μM TPA for 10 minutes. Lipids were extracted and run on TLC plates as explained in Materials and Methods (Chapter 2).

5.2.3. Drug sensitivity of PLD activation

In initial experiments to map potential intermediates in the activation of PLD, the priming effect of PI3Kinase was initially tested to define whether this is an intermediate in TPA-induced PLD activation. No inhibition of TPA-induced PLD activation with PI3Kinase inhibitors (LY294002 and wortmanin) was observed (Figure 5.5). Similarly, to define whether the proposed involvement of PLD activation in ERK activation was consistent with inhibitor effects, we determined whether the inhibition of ERK activation itself had any effect upon TPA-induced PLD activation. As shown in Figure 5.5., PD98059 (an inhibitor of MEK, therefore of ERK1/2 activation) did not inhibit PLD activation induced by TPA. This is at least consistent with the notion that PLD is upstream of ERK activation.

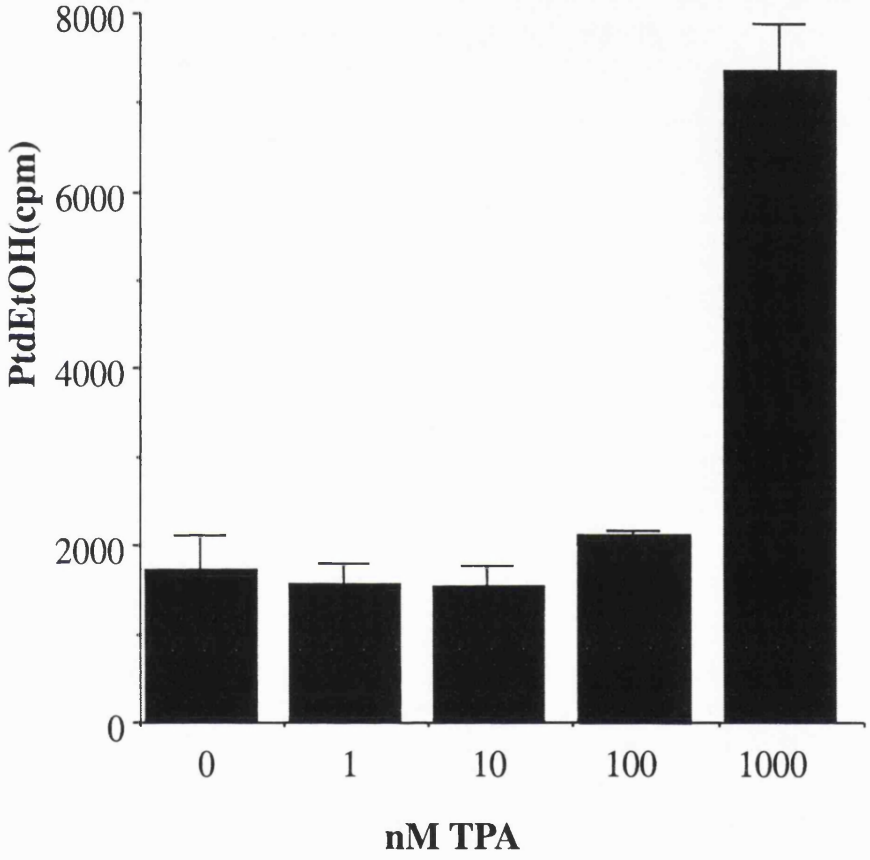


Figure 5.2 .
Dose dependent activation of PLD in U937 cells.
U937 cells were labelled with ^{3}H myristic acid for 6 hours. 2.5% EtOH was added to the media for 10 minutes and then different concentrations of TPA were added for 10 minutes. Lipid extraction was carried out as described in M&M and lipids separated by TLC. Appropiate spots were scraped and counted in scintillant.

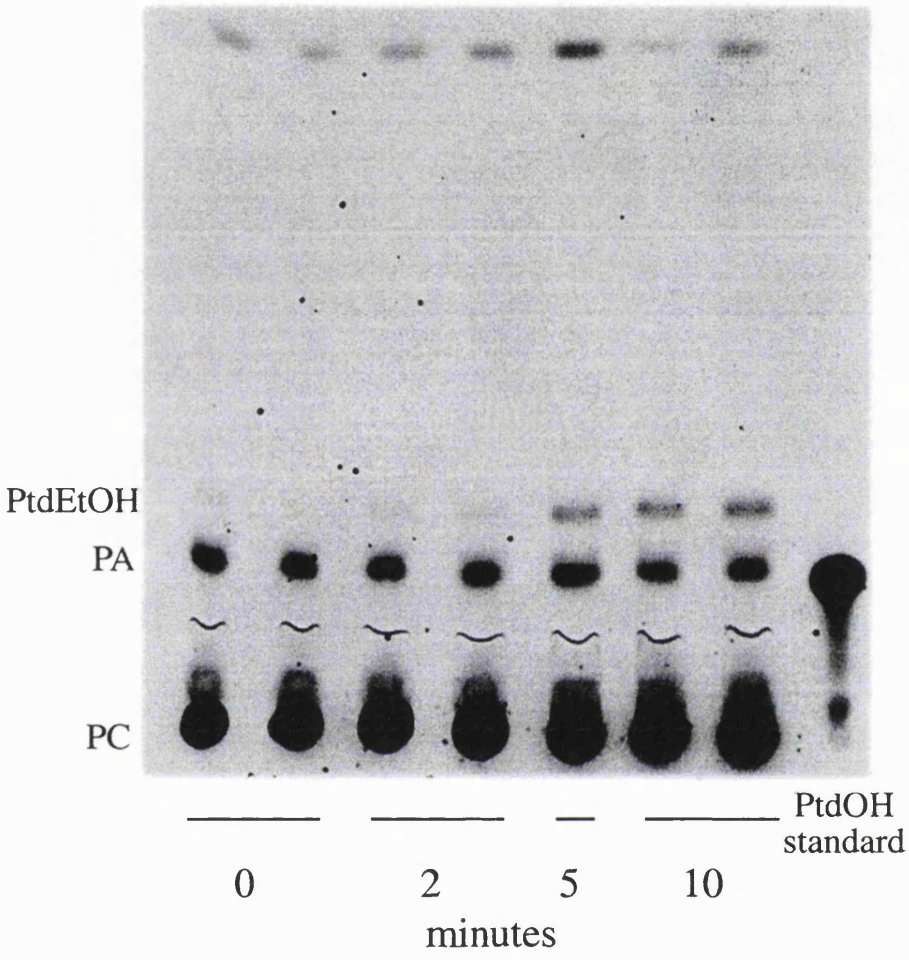


Figure 5.3.
Time course of PLD activation *in vivo* in U937 cells.
Duplicate flasks of U937 cells were labelled with ^{3}H myristate, 2.5% EtOH was added to the media for 10 minutes and then cells were treated with 1μM TPA for the times shown. Lipids were extracted as described in Materials and Methods and separated by TLC to observe PtdEtOH production.

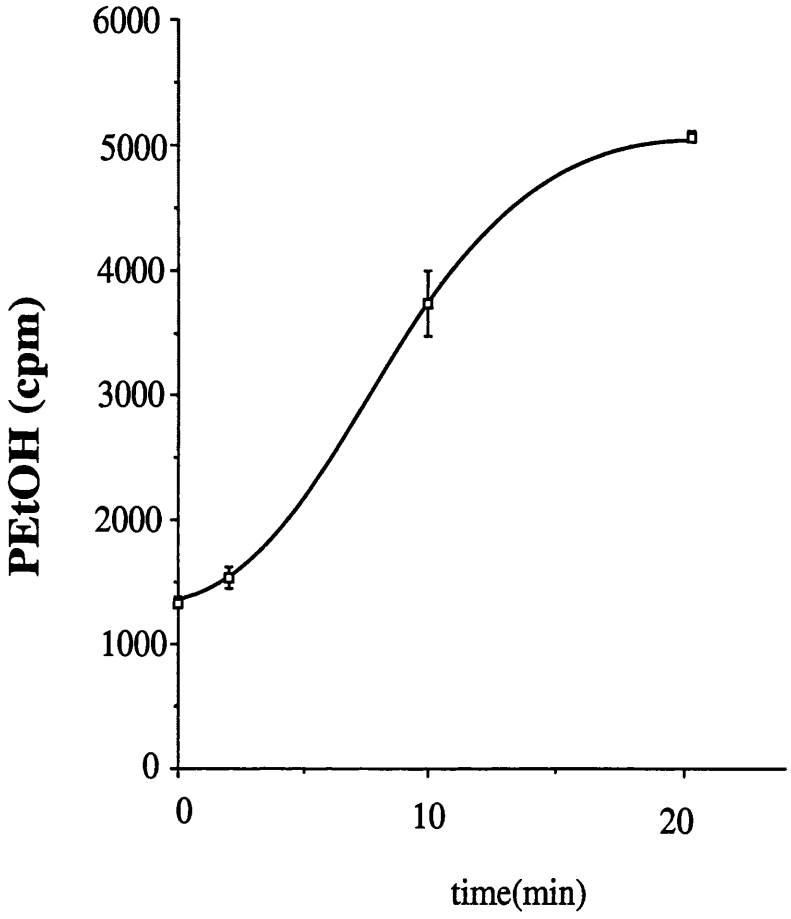


Figure 5.4.
***In vivo* time course of PLD activation in response to TPA.**
U937 cells were labelled for 6 hours as described in Materials and Methods, incubated with 2.5%(v/v) ethanol and challenged with 1μM TPA for the times shown. Lipids were extracted and analysed as described in Materials and Methods. Data is presented as cpm incorporated into PtdEtOH and error bars represent the range of two sets of data.

		%activation
	0	0
	LY	0
	Wort	0
	PD	0
	TPA	100
+TPA	LY	121 +/- 4
	Wort	120+/- 6
	PD	99 +/- 6

Figure 5.5.
Drug sensitivity of PLD activation
^{3}H myristic acid labelled U937 cellswere incubated with 2.5%(v/v) ethanol and treated in the presence or absence of the different drugs for 15 minutesbefore being stimulated with1μM TPA for 10 minutes. Drugs are: wortmannin (Wort); PD98059 (PD); LY294002 (LY). Lipid extraction and quantification of myristate labelled phosphatidylethanol was as described in Materials and Methods. The numbers shown are for one of 2 experiments and indicate the mean and range of duplicate samples.

5.2.4 Is the TPA-induced response via PKC?

As discussed in Chapter 1 there are now known to be five distinct classes of phorbol ester responsive proteins. To determine whether PKC was a requirement for the TPA-induced PLD activation a catalytic site inhibitor was employed since this is the only class of phorbol ester receptors that are protein kinases. Of the variety of PKC inhibitors available, Bisindolylmaleimide (BIM) was used, since this displays a good selectivity profile (although it is not exclusive for PKC). BIM is an ATP competitive catalytic inhibitor of PKCs (Martiny-Baron et al., 1993). Cells were pre-treated with BIM prior to TPA stimulation for 10 minutes. Figure 5.6. shows that BIM inhibits the stimulation of PLD by TPA. Specifically, it was noted that it was the TPA-induced increment of PtdEtOH production that was inhibited. This implies that there is substantial basal (PKC-activity independent) PLD activity under these conditions. The effect of BIM indicates that activation of PLD is dependent on PKC catalytic activity. Although providing some distinction between PKC and other TPA-dependent targets, the result was surprising in some respects as it has previously been demonstrated in vitro that PKC mediated PLD activation does not require PKC catalytic activity (see Chapter 1).

5.2.5. Relationship between PLD and microtubule reorganization

Microfilaments are believed to be involved in a wide variety of cellular activities such as changes in cell shape, motility, endocytosis, exocytosis, intracellular transport, and directed movement of cell surface proteins (Bretscher et al., 1997; Frigeri and Apgar, 1999). Many of these activities are specifically triggered when cells are stimulated through receptors on their cell surface. One consequence of activation is a reorganization of cellular actin that exists in a dynamic equilibrium between monomeric G-actin and polymerised F-actin.

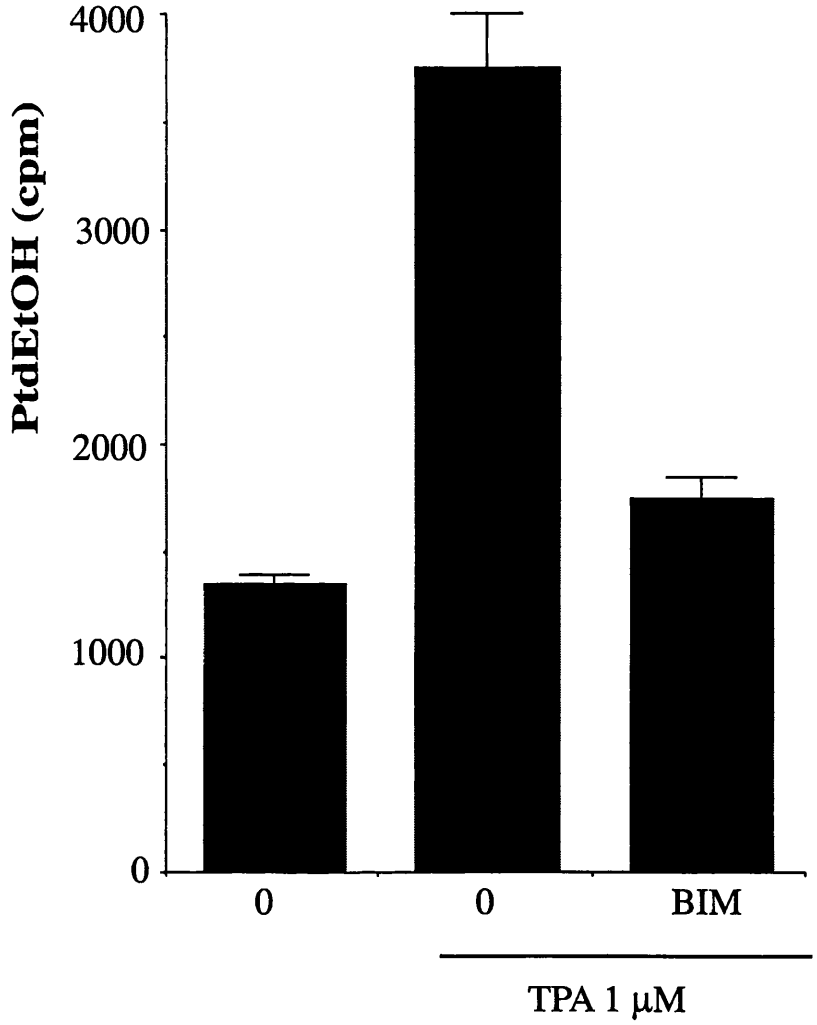


Figure 5.6.
Inhibition of PLD activity by BIM.
^{3}H myristic acid labelled U937 cells were incubated with 2.5%(v/v) ethanol, preincubated in the presence or absence of 10 μM BIM for 15 minutes, and then stimulated with 1μM TPA for 10 minutes. Lipid extraction and quantification of myristate labelled phosphatidylethanol was as described in Materials and Methods. The numbers shown are for one of 3 experiments and indicate the mean and range of duplicate samples.

The mechanisms of transport of β2 integrin containing vesicles to the cell surface has not been described. Drugs that disrupt actin filaments (cytochalasins) are frequently used in combination with fMLP to maximise granule secretion in circulating macrophages and neutrophils. These studies suggest that reorganization of the actin cytoskeleton facilitates integrin vesicle translocation to the cell surface in mature monocytes and granulocytes. Less is known about the role of microtubules in this response. In activated T lymphocytes, the microtubule-organising centre and microtubules undergo a rapid reorientation that effectively directs vesicles containing cytosolic proteases toward the target cell. A similar reorganization of microtubules has been described in promonocytic U937 cells following acute TPA treatment. In resting cells, PKC β2 was found exclusively associated with microtubules (Kiley and Parker, 1995). In other work (Kiley and Jaken, 1990), agonist-stimulation resulted in a tighter association of PKC with cytoskeletal elements. In U937 cells PKCβ2 is loosely associated with microtubules in the resting cell and TPA stimulation stabilises the association of PKCβ2 with cytoskeleton. A potential consequence of this altered interaction would be reorganization of the microtubule cytoskeleton. Phosphorylation is known to increase the interaction between microtubules and microtubule-associated protein in vivo and at least one microtubule-associated protein MAP2, is a known PKC substrate (Brugg and Matus, 1991).

Previous studies from this laboratory have led to the isolation and characterization of a series of TPA resistant U937 cell lines. Detailed analysis of these clones has demonstrated that although PKC expression and behaviour is grossly normal, there is a subcellular mis-localization of PKC β. In wild type cells this PKC isotype is associated with the microtubule network, and on stimulation with TPA there is a general reorganization of the microtubules. In the TPA resistant cell lines, no reorganization of the microtubules occurs in response to TPA, correlating with the loss of PKC β2 from this compartment. Consequent to this defect, TPA does not induce an acute up-regulation of cell surface integrins derived from perinuclear granules.
In order to know if the microtubule network was involved in the activation of PLD, experiments were done trying to disrupt this network.

5.2.6. Nocodazole synergies with TPA: depolymerization of microtubules enhances PLD activity

In view of the BIM sensitivity of the PLD response it was important to determine whether the PKC catalytic dependence of the PLD response was in fact secondary to the reorganisation of the microtubules that is known to be catalytic activity dependent. In addition to this, cytoskeletal reorganisation coincides with vesicle translocation and may facilitate transport to the cell surface. Since PLD has been implicated in vesicle transport we wanted to investigate the role of the microtubule cytoskeleton in TPA induced PLD activity in U937 cells. To determine a possible requirement for microtubules, cells were treated with the microtubule-disrupting drug, nocodazole 5 μM, prior to TPA stimulation. The results showed that nocodazole synergised with TPA in the activation of PLD(Figure 5.7).

This experiment was repeated with HeLa cells to asses wether the potentiation of TPA stimulated PLD activity is specific to the U937 cell line. As shown in Fig 5.8.,the synergistic effect of the nocodazole and TPA is also present in HeLa cells. Although there is an increase in PLD activity seen with nocodazole alone, the combination of TPA and nocodazole yields an increase above basal that is more than additive.

The properties of the synergy of nocodazole and TPA were further investigated in U937 cells. Figure 5.9 shows a time course experiment for *in vivo* PLD activity in U937 cells after pre-treatment with nocodazole, 5 μM for 10 minutes and stimulating with TPA for the times indicated. Clearly nocodazole causes both an increase in the rate of production of PtdEtOH and a more prolonged PLD activation.

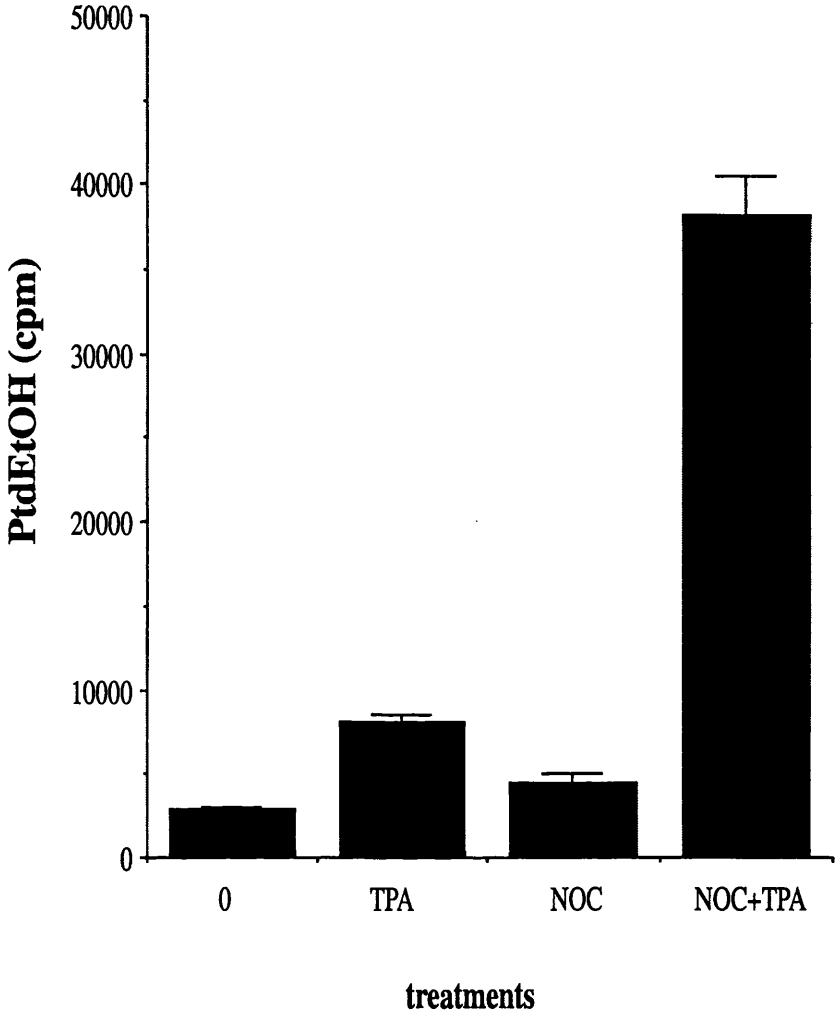


Figure 5.7.
PLD activity in the presence of nocodazole.
U937 cells were labelled with ^{3}H myristate and incubated with 2.5%(v/v) ethanol for 10 minutes,pretreated with 5 μM nocodazole for 15 minutes prior to1 μM TPA stimulation for 10 minutes. Lipids were extracted and PtdEtOH production counted as described in Materials and Methods.
The results are from one of 4 experiments and the error bars indicate the range of duplicate observations.

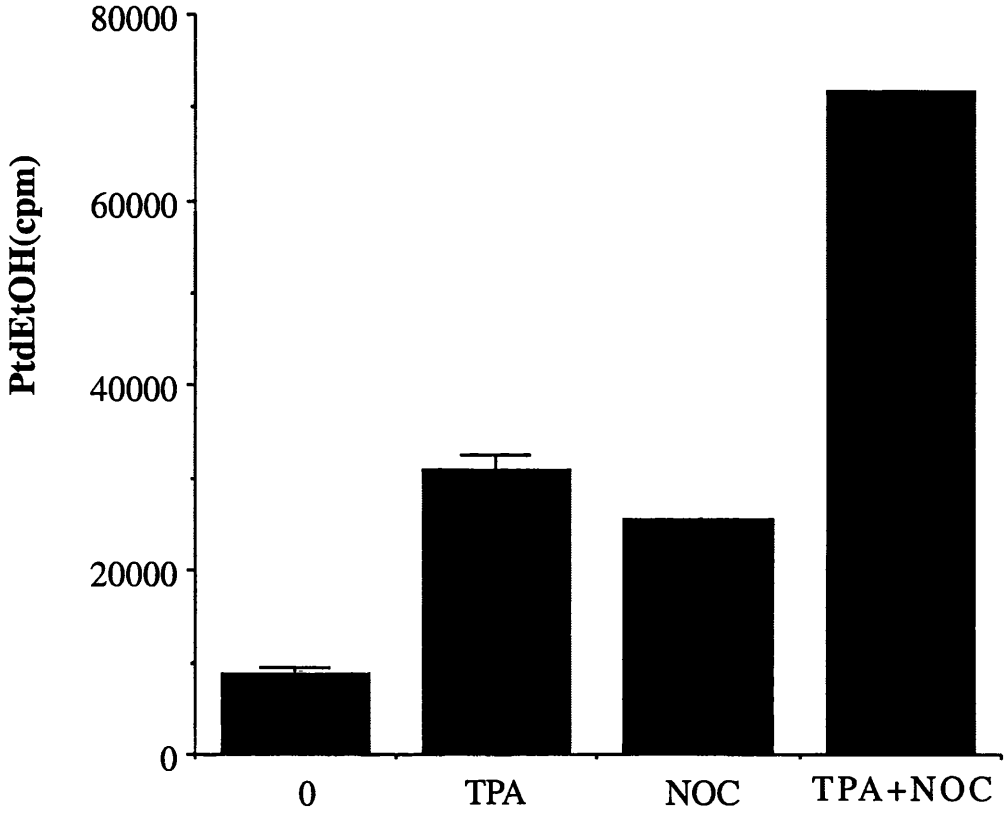


Figure 5.8.
PLD activity in the presence of nocodazole in HeLa cells.
HeLa cells were labelled with ^{3}H myristate for 6 hours as described in Materials and Methods and incubated with 2.5%(v/v) ethanol. Cells were then left untreated(0), treated with 1 μM TPA(TPA) for 10 minutes, or pretreated with 5 μM nocodazole(NOC) for 15 minutes or both (TPA+NOC). Lipids were extracted and PtdEtOH production counted as described in Materials and Methods. This is one of 3 experiments and the error bars indicate the range of duplicates. observations.

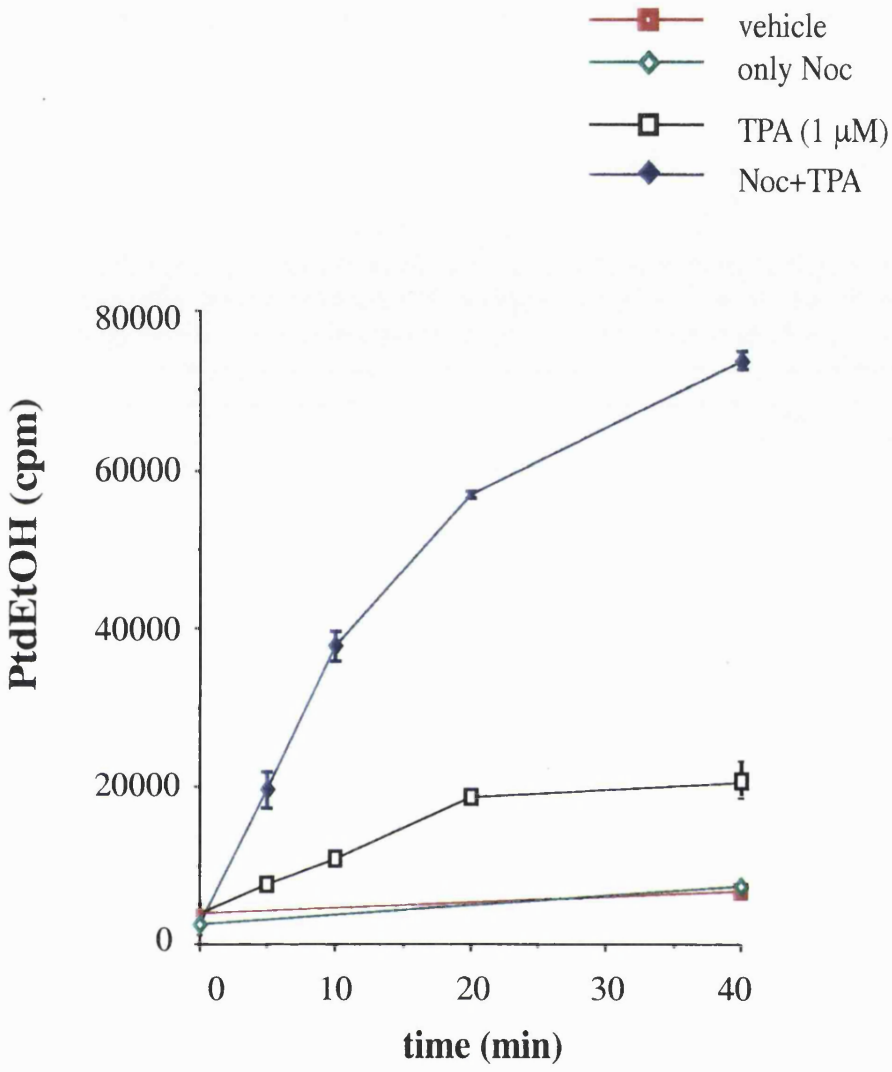


Figure 5.9.
Time course of *in vivo* PLD activity in U937 cells
U937 cells were labelled with ^{3}H myristate and incubated with 2.5%(v/v) ethanol, then pretreated with 5 μM nocodazole for 15 minutes prior to 1 μM TPA stimulation. Reactions were stopped at the times shown (0,5,10,20 and 40 minutes). Lipids were extracted and PtdEtOH production counted as described in Materials and Methods. The results are from one of 2 experiments and the error bars indicate the range of duplicate observations.

5.2.7. Effects of other microtubule depolymerization drugs.

To assess whether the potentiation of TPA stimulated PLD activation is due to microtubule depolymerization and not a non-specific effect of nocodazole, two other structurally unrelated drugs were tested colchicine and vinblastine (Downing, 2000). These agents disrupt the microtubule network in different ways but all of them prevent microtubule formation and destabilise or depolymerize microtubules. Both of these drugs were found to potentiate TPA-induced PLD activation (See Figure 5.10). Interestingly, treatment with Paclitaxel (Taxol), the microtubule stabilising drug, had no effect on TPA-induced PLD activation. None of these drugs has a substantial effect on PLD activity in the absence of TPA (See Figure 5.11).

5.2.8. Does actin depolymerization have the same effect?

β2-integrins can be linked to the actin cytoskeleton in neutrophils. The fact that cytochalasins enhance fMLP-induced granule translocation in this cell type suggests that actin filament reorganization is necessary for vesicle movement from intracellular storage sites to the plasma membrane(Singer et al., 1989). Hence the effect of depolymerisation of actin was also tested in U937 cells.

It has also been reported that the stimulation of actin stress fiber formation was a consequence of PA generation and, therefore, PLD activation(Cross et al., 1996).

U937 cells were pre-treated with the actin depolymerisation drug cytochalasin D(1 μM), before stimulating with TPA. A synergistic effect was also seen implicating actin depolymerisation as also being important for TPA induced PLD stimulation (Figure 5.12).

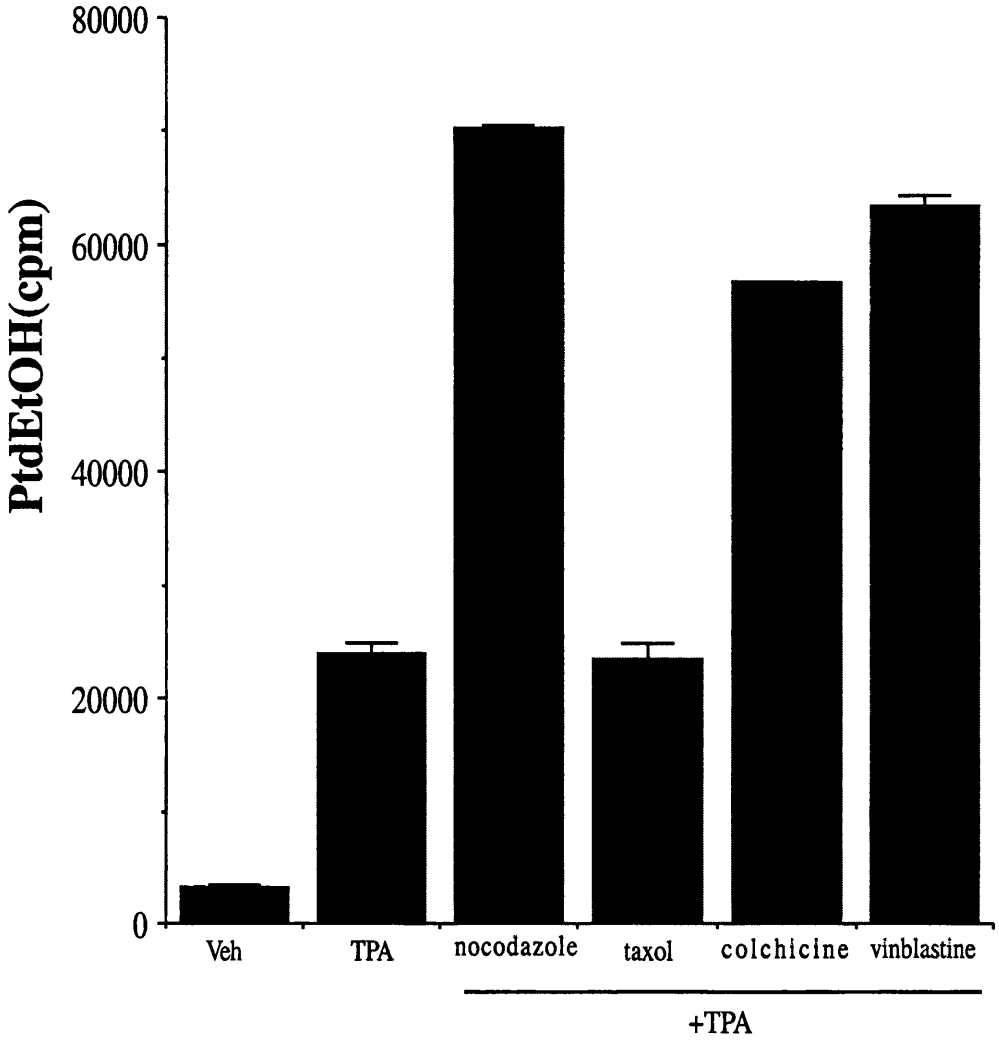


Figure 5.10.
TPA-induced PLD activity in U937 cells in the presence of microtubule depolymerising drugs.
Cells were labelled with ^{3}H myristate and incubated with 2.5%(v/v) ethanol, then pretreated with the drugs indicated for 15 minutes prior to stimulation with TPA for 10 minutes. Lipids were extracted, separated by TLC and counted as described in Materials and Methods.

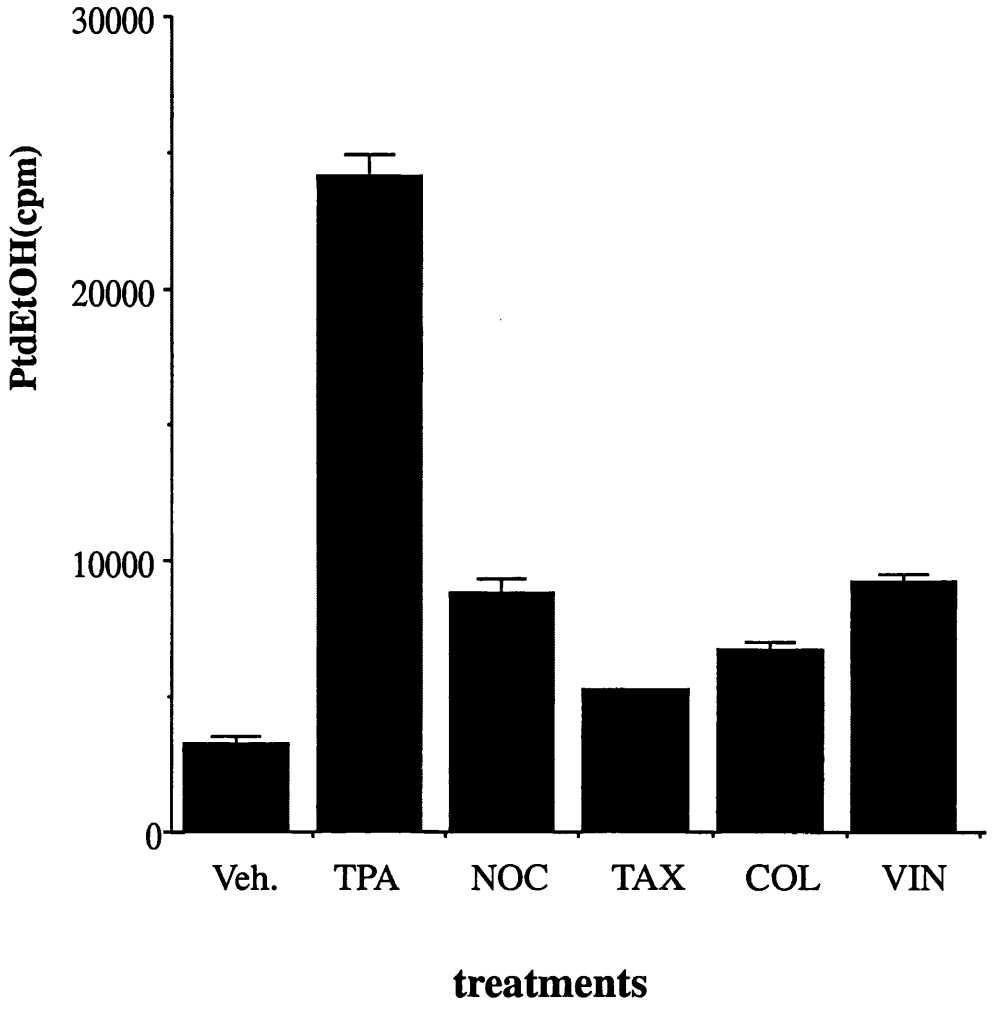


Figure 5.11.
***in vivo* PLD activity in U937 using microtubule depoymerisation drugs in the absence of TPA.**
Cells were labelled with ^{3}H myristate and incubated with 2.5%(v/v) ethanol,then pretreated with the drugs {Nocodazole 5 μM,(NOC), Taxol 10 μM, (TAX), Colchicine 10μM, (COL), Vinblastine 1 μM (VIN)} for 15 minutes. Lipid extraction and counting was as in Materials and Methods.The numbers shown are for one of 2 experiments and indicate the mean and range of duplicate samples.

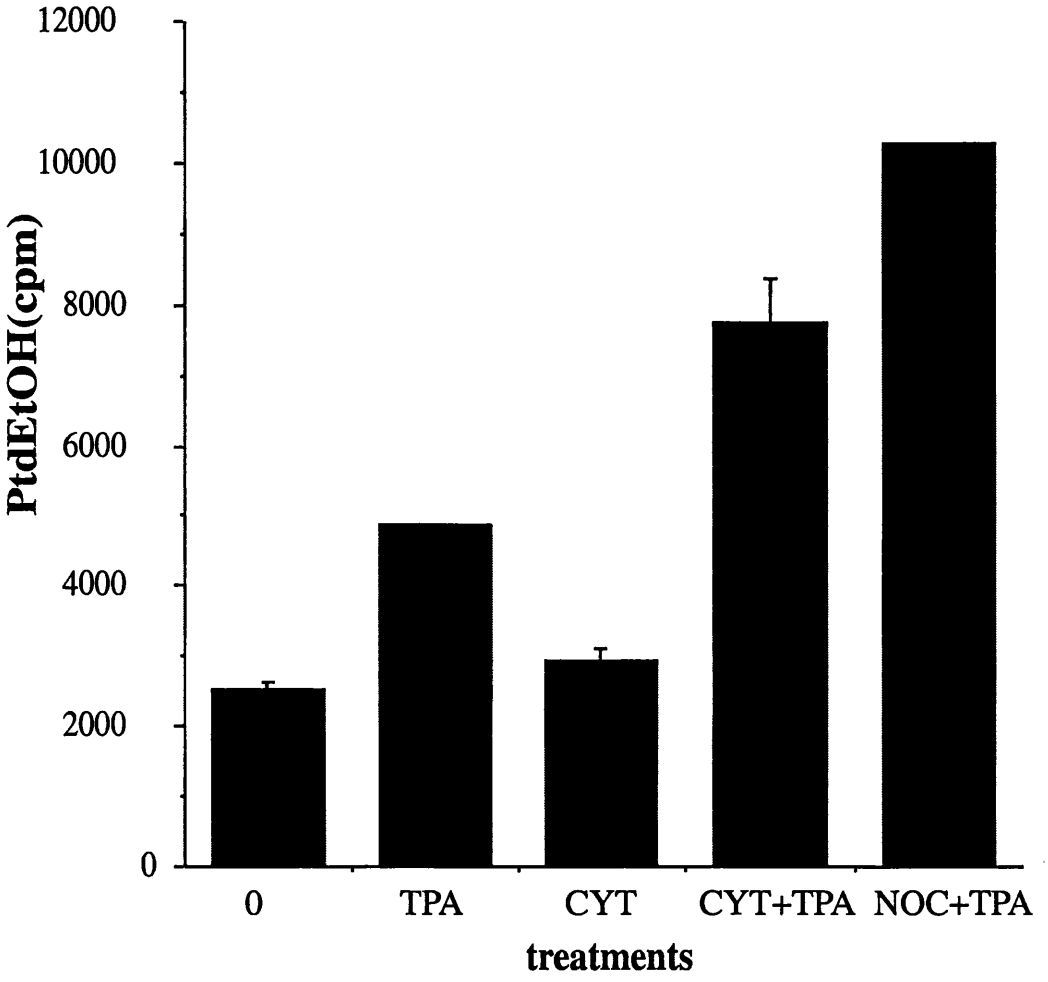


Figure 5.12.
***in vivo* PLD activity in the presence of Cytochalasin D**
Cells were labelled with ^{3}H myristate and incubated with 2.5%(v/v) ethanol, then pretreated with Cytochalasin D for 15 minutes prior to stimulation with TPA for 10 minutes. Lipids were extracted, separated by TLC and counted as described in Methods and Materials.

5.2.9. Nocodazole can overcome the BIM dependent inhibition of TPA stimulated PLD activity

Since PKC activity is essential for the TPA-induced reorganisation of microtubules in U937 cells and as shown above there is a synergy between microtubule disruption and TPA-induced PLD activity, it was possible that the requirement for catalytic function in PLD activation may be due to microtubule regulation. To assess this we determined whether microtubule disruption affected the inhibition by BIM. Figure 5.13 shows that pre-treatment with nocodazole can largely restore the ability of TPA to stimulate PLD activity. This is consistent with the view that PKC is involved in two separate events that leads to PLD activation, one that is dependent on PKC activity and one that is independent of PKC catalytic activity as has been previously described for PLD.

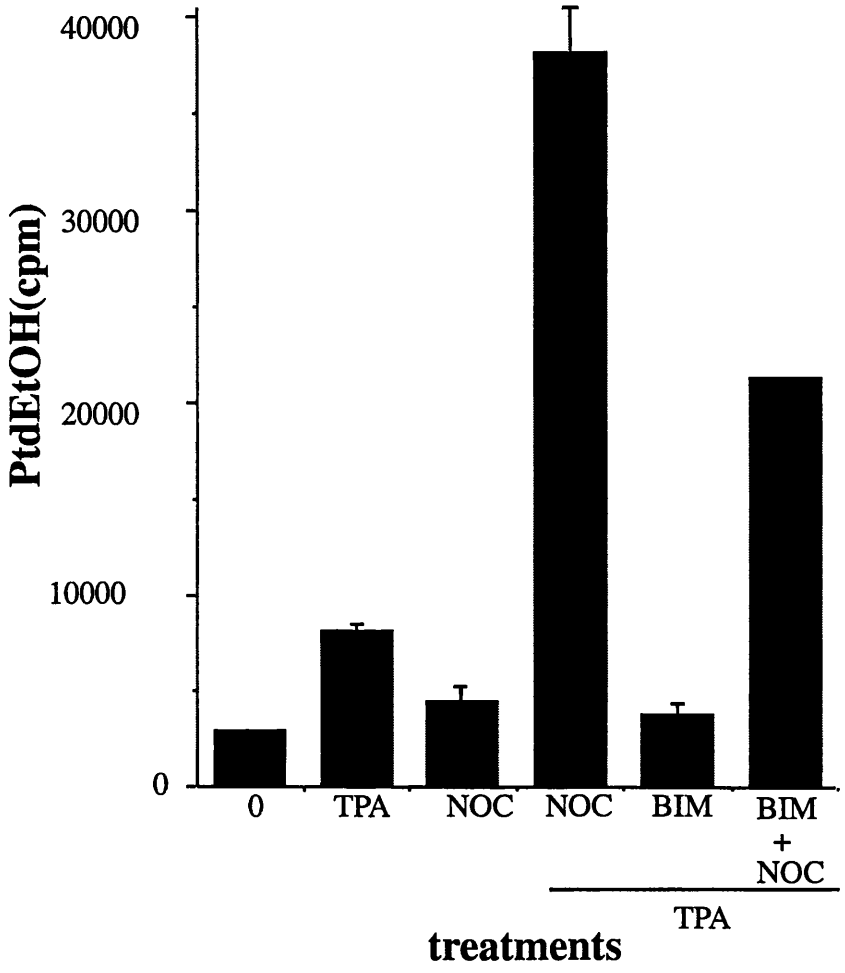


Figure 5.13.
The effect of BIM on TPA-induced PLD activity is attenuated by nocodazole

^{3}H myristic acid labelled U937 cells were incubated with 2.5%(v/v) ethanol for 10 minutes and then preincubated in the presence or absence of 10μM BIM and/or 5 μM nocodazole for 15 minutes before stimulation with 1μM TPA for 10 minutes. Lipid extraction and quantification of myristate labelled phosphotidylethanol was as described in Materials and Methods. The numbers shown are for one of 2 experiments and indicate the mean and range of duplicate samples.

5.3 Summary

In this chapter, the *in vivo* PLD activity assay has been used to check endogenous PLD activation and regulation. It is a powerful tool since overexpression of different PLDs in cells has been demonstrated to have different effects, not all of them controllable. Some papers have shown different compartmentalisation of PLD depending on the level of expression (Freyberg et al., 2001) . Measuring PLD activity *in vivo,* overcomes this problems. But on the other hand has limitations in not knowing which PLD isozymes in particular we are measuring and where.

Inhibitors were used to try to define which pathways were involved upstream of PLD activation. Drugs involved in other signalling pathways were tested in order to know their implication in PLD activation. PI3K and MAPK were tested using some specific inhibitors and the results showed that they were not affecting the *in vivo* PLD activity after TPA stimulation therefore they are not upstream.

The key question to be addressed was whether PKC was playing any kind of role in PLD activation. Since the phorbol ester TPA, an activator of PKC, was shown to activate PLD *in vivo*, we could conclude that possibly PKC was involved in some step in the path leading to production of phosphatidylethanol.

This affirmation is not completely true if the specific inhibitor of the catalytic activity of PKC, BIM, would have given different results. There are other receptors for phorbol esters that could be targeted with TPA; these might produce different signals that could lead to activation of PLD. But since the blocking of catalytic PKC activity with BIM blocks TPA-induced PLD activity about 100 %, we could surmise that PKC activity is involved upstream PLD.

Previous studies from this laboratory have led to the isolation and characterization of a series of TPA resistant to differentiation U937 cell lines and, a partial reversal of the resistant phenotype can be elicited by nocodazole treatment, suggesting that the microtubule depolymerization and subsequent granule release play essential roles in the differentiation process triggered by TPA. Since we were trying to relate PLD activity with the initiation of TPA dependent differentiation in U937 via MAPKinase, it was important to test if nocodazole was having any effect in PLD activity. PLD *in vivo* activity was measure in the presence of nocodazole and it showed a big synergistic effect implying that microtubule rearrangements are involved in making PLD being active. Not only the microtubule network, but the actin cytoskeleton was shown to be influencing PLD activity in vivo in U937, since cytochalasin D, an actin disrupting drug, also synergies with the TPA effect., albeit not as much as

nocodazole. It is clear that cytoskeleton reorganisation is somehow permissive for the TPA-induced activation of PLD.

The relationship between microtubule depolymerisation and the requirement for PKC activity was tested in relation to the requirement for PKC activity in triggering PLD activity. This provides the first clear mechanism by which activity can contribute to activation indirectly while there may remain a contribution from the physical interaction of PKC and PLD. These observations may underlie the contradictory observations reported util now. The bypass of PKC catalytic activity inhibition by BIM,by nocodazole, could be interpreted in different ways. Either PKC via its catalytic activity is required for microtubule depolymerisation alone and that TPA triggers PLD through an alternate target, or TPA acts at two levels on PKC (different isotypes) to achieve both effects. Note that microtubule disruption alone is insufficient to trigger PLD activation, i.e. there are two steps involved. Based on the allosteric effect of PKC on PLD in vitro, the pragmatic view would be that it is the latter mechanism. A key to establishing this would be the demonstration that PKC becomes localised to a compartment where PLD is activated. This is the subject of the next chapter.

Chapter 6

A granular PKC-PLD link?

6.1. Introduction

In previous chapters evidence has been presented that there is a PLD dependent step in MAPK activation leading to differentiation. As described before, this process may at some point be influenced by the release of granules with their components to trigger this signal. The granules in U937 that move to plasma membrane after TPA stimulation contain the PKCβ1 isozyme and integrins. As shown in previous chapters and in studies from other groups, there is evidence showing that PKC can play an important role in PLD regulation(Conricode et al., 1992; Hammond et al., 1997; Lopez et al., 1995; Min and Exton, 1998; Mukherjee et al., 1996; Singer et al., 1996). The object of the studies described here was to determine whether PKCβ1 and the U937 cell PLD are colocalised as evidenced by co-fractionation. This was considered the most direct route to define the potential for PKC-PLD interaction in the absence of antisera suitable for detection of endogenous PLD.

6.2 Results

6.2.1 PKCβ1 fractionation.

Endogenous PKC isozymes, β1, β2, ε and ζ are found in different compartments in U937. PKCβ1 is found in perinuclear vesicles that redistribute to the plasma membrane after acute phorbol ester treatment by immunofluorescence. To confirm this distribution a subcellular fractionation method was used.

Log-phase untreated cells were lysed and loaded on to a velocity gradient and then selected fractions were subjected to an equilibrium gradient, that separates fractions depending on density and size. Western blots using the PKCβ1 specific antibody show that the antigen is in the soluble fraction (at the top of the velocity gradient) and that the majority is then recovered in fractions 2, 3 and 4 of the equilibrium gradient (see Figure 6.1.).

6.2.2. PKCβ translocation

U937 cells were treated with TPA during 10 minutes and they were processed as above through the subcellular fractionation protocol and fractions blotted with the PKCβ1 antibody. Consistent with its recruitment to a membrane compartment, PKCβ1 is found to enter the equilibrium gradient after TPA-treatment, being recovered mainly in fractions 5 and 6 (Figure 6.1).

After subcellular fraction the samples were run into gels and stained with coomassie blue. Both gels were similar (Figure 6.2) indicating the reproducibility of the fractionation technique and providing an internal control to show that TPA treatment did not grossly modify behaviour. This translocation of PKCβ1 was consistently seen in 4 experiments.

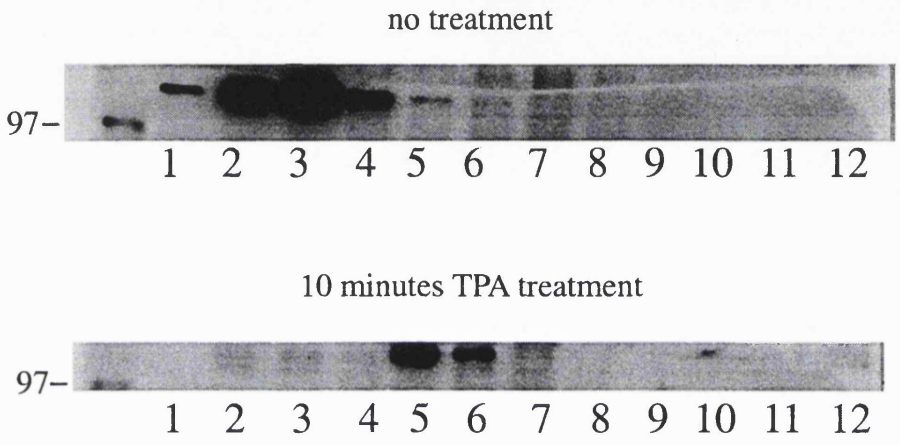


Figure 6.1.
PKCβ1 in subcellular fractions of U937.
U937 cells were incubated in the presence or absence of 1μM TPA for 10 minutes before passing them through a velocity sucrose gradient and an equilibrium sucrosegradient as described at subcellular fractionation protocol in Materials& Methods. Extracts of fractions collected after the equilibrium gradient in sample buffer were analyzed by western blot using PKCβ1antibody. The results are from one of 4 fractionation experiments.

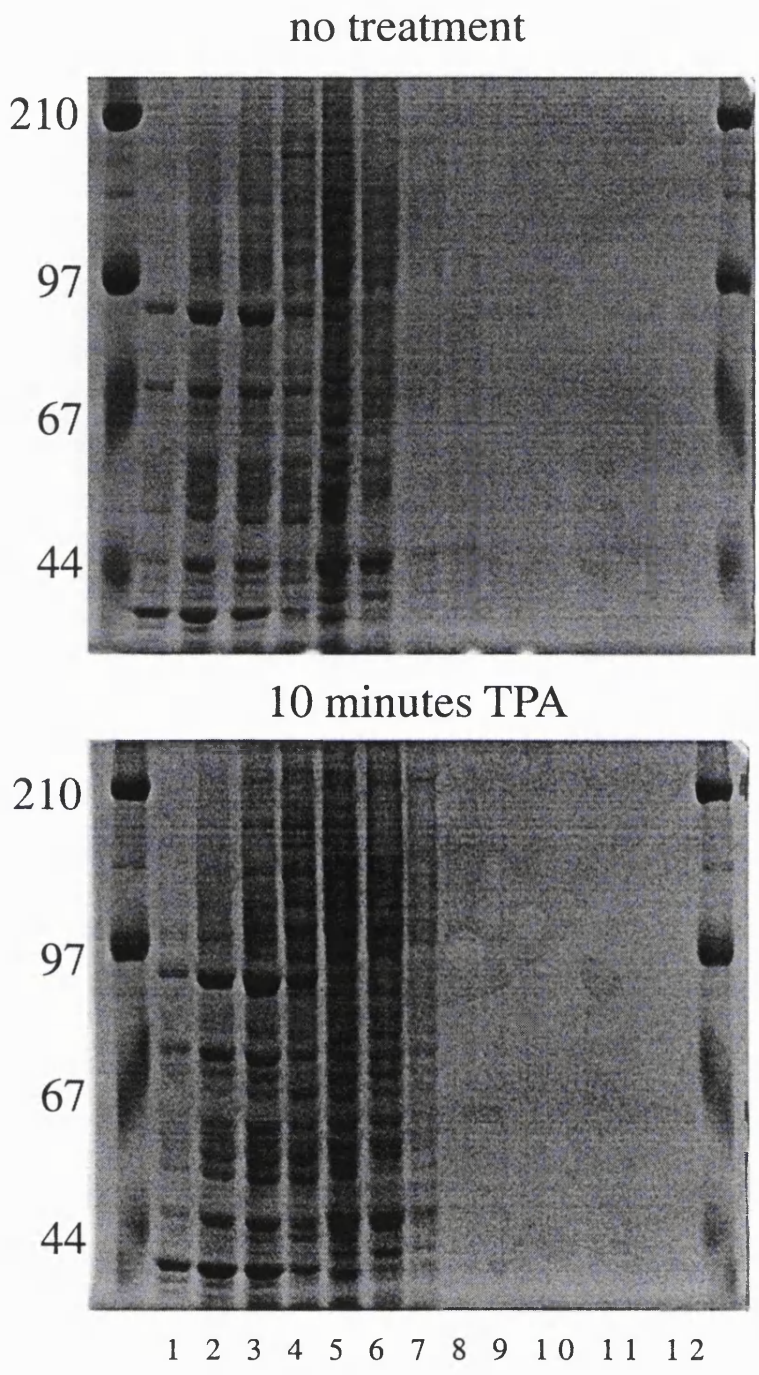


Figure 6.2
Comassie blue stained gels showing fractions after subcellular fractionation protocol of U937cells.
Cell were untreated or treated with TPA for 10 minuters as indicated.The extracts were fractionated (see text) and samples separated by PAGE. The coomassie stained fractions are numbers 1-12 as indicated. The lanes are flanked by molecular weight markers as indicated (kDa).

6.2.3. Does PLD activity colocalize with PKCβ1?

Some studies suggest that depending on cell type and experimental protocol, PLD activity is localised in plasma membranes, intracellular membranes or both. One explanation for this apparent multimodal localization could be that PLD activity is recruited onto more than one cellular compartment upon the mobilisation and membrane association of known PLD activators such as ARF and RhoA.

To check if PLD activity correlated with the PKC translocatation therefore, i.e. that the protein was in the same compartment, an *in vivo* method was developed.

Cells were labelled with ^{3}H myristic acid, treated with TPA in the presence of ethanol and then follow by the subcellular fractionation protocol. After collecting the fractions, a lipid extraction was performed and then the lipids run on a TLC plate (see Figure 6.3) to quantify the formation of phosphatidylethanol in each fraction This was a way of checking if the product of the PLD reaction was retained in the compartment where PKCβ1 was found.

After TPA treatment during 10 minutes the PLD activity was found mainly in fractions 4,5 and 6 substantially overlapping with the PKCβ1 in those fractions (Figure 6.4.).

6.2.4.Subcellular fractionation with CD11b

The available monoclonal antisera to CD11b do not western blot effectively. So to monitor the CD11b component of the β2-integrins, an antibody to the C-terminal cytoplasmic domain was raised. This antiserum can detect CD11b in western blots. This was employed to follow the fractionation of β2-integrins in the U937 cell extracts. As shown in Figure 6.5 this protein was recovered in fractions corresponding to PKCβ1 in TPA treated cells. The two bands observed represent the non-reduced and fully reduced forms; in the absence of reducing agents only the slower migrating form is observed with this antiserum (J.Ivaska personal communication). Prior to TPA treatment the protein was also in these fractions.

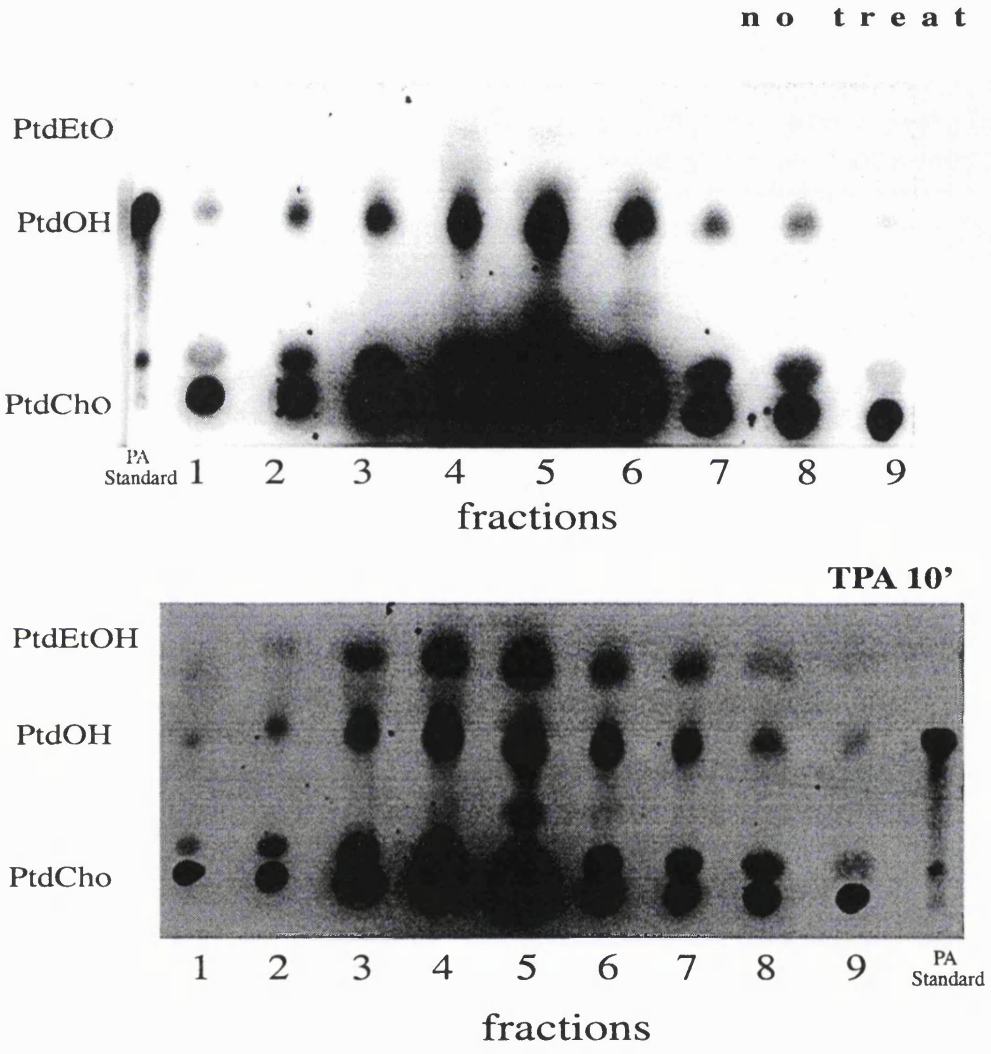


Figure 6.3.
PLD activity in wt U937 vesicle preparation
U937 cells were labelled for 6 hours with ^{3}H myristic acid, prior to treatment with TPA. Then cells were passed through the subcellular fractionation protocol. Samples were lipid extracted and run into TLC plates. Tritiated signals were developed by auroradiography.

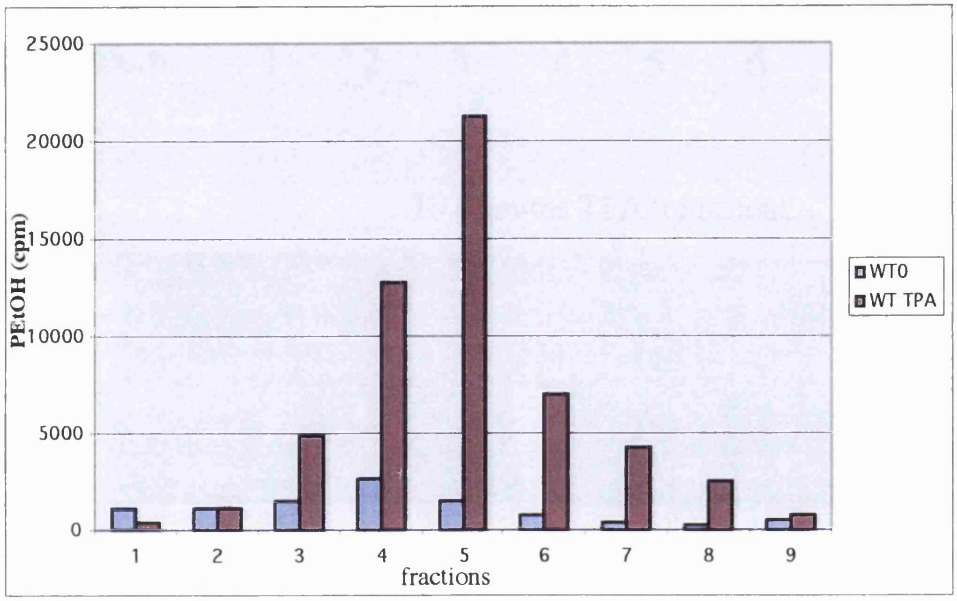


Figure 6.4.
in vivo PLD activity in sucrose fractions
^{3}H Myristic acid labelled U937 cells were incubated for 10 minutes in the presence or absence of 1μM TPA before being passed through the subcellular fractionation protocol described in Materials and Methods. After that lipids were extracted from the sucrose fractions and phosphatidylethanol quantified by scintillation counting.

Based upon the granule to plasma membrane redistribution (Kiley and Parker, 1995) it is concluded that these compartments may well be co-fractionating on these gradients. It should be noted that we adapted the gradient to a very shallow one to optimise separation around the density of the granules (see Chapter 2).

To monitor the plasma membrane in these procedures, the cell surface was biotinylated. Fractionation and detection by streptavidin-HRP however revealed an abundance of labeled proteins without any specific pattern of recovery. This approach did not appear to provide the required marker to aid interpretation of the integrin/PKC/PLD behaviour. Na-K ATPase antibodies were used by western blot but they were not very specific Similarly, zymography was employed for the detection of metalloproteinases present in the granules or released from them. Despite numerous attempts insufficient activity was detected, even though positive controls yielded hydrolytic activity.

6.3. Summary

In this chapter we used fractionation to investigate whether there was evidence for PLD activation in the compartments in which PKCβ1 and/or β2-integrins could be detected. It was clear from the behaviour of PKCβ1 on fractionation that TPA caused its recruitment to a membrane fraction(s). This compartment co-localised with the β2-integrin CD11b and overlapped with the phosphatidylethanol produced by PLD in vivo. The incomplete overlap with phosphatidylethanol indicates that a part of the PLD activation may indeed be associated with the PKC/integrin compartment.

The limitation on this conclusion is that we have not been able to characterise the gradient in a manner that permits us to define fractions as being plasma membrane, granule, etc. In principle it could be concluded that the procedure only served to separate cytosol from membranes. However it is apparent that phosphatidylethanol defines a broader membrane compartment than the two protein antigens and that in fact close scrutiny of the CD11b antigen consistently showed that the proportions of reduced and non-reduced forms were shifted on the gradient. It is not clear why there should be differences in reducibility, perhaps this reflects different conformation states. However this behaviour also indicates that some separation was achieved but that this was insufficient to convincingly demonstrate

'movement' on the gradient. How these issues might be resolved further is discussed in the final chapter.

Chapter 7

Discussion

7.1. Overview.

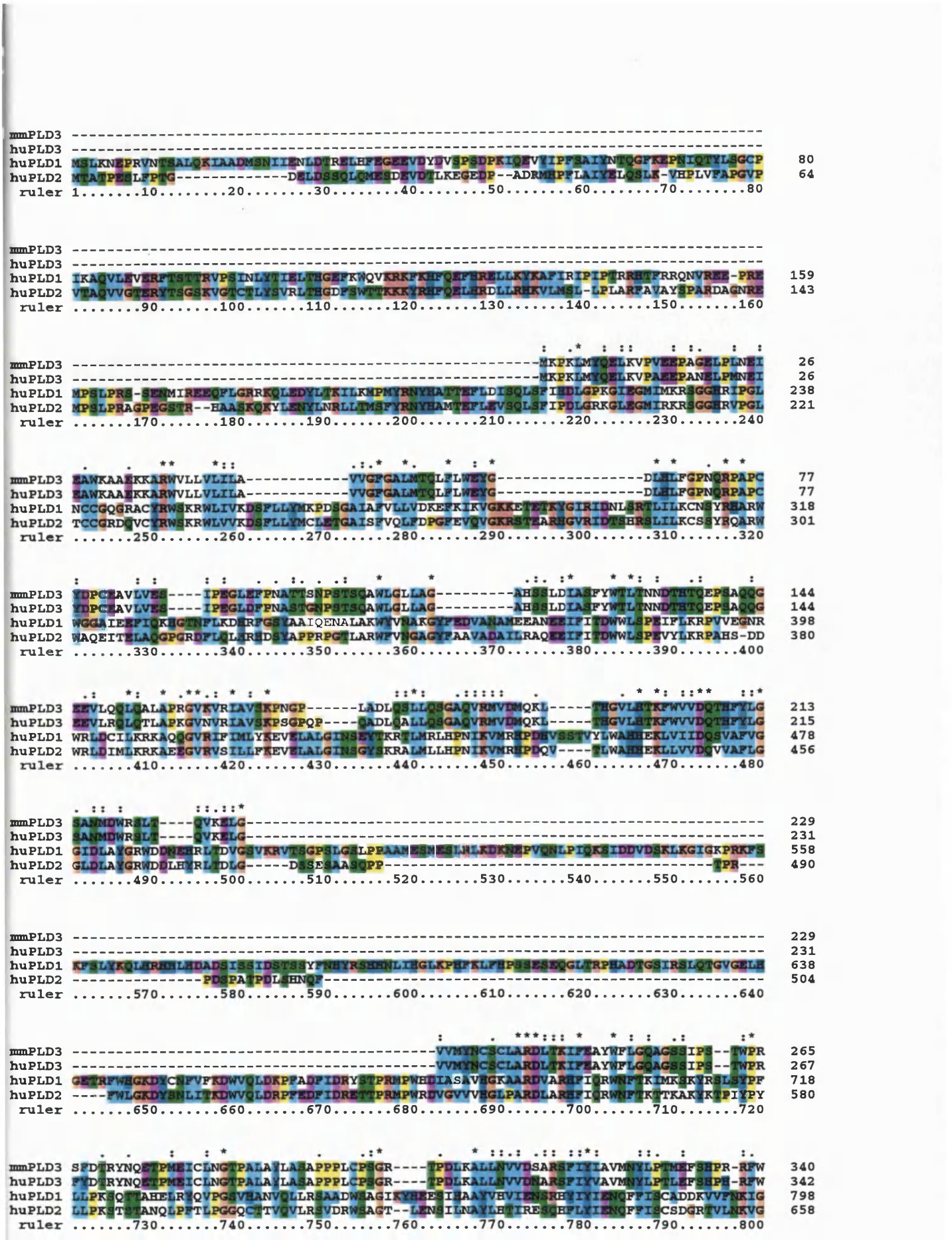
The work in this thesis has been aimed at understanding the TPA-dependent control of PLD and its role in TPA-induced cellular responses. In particular we have sought to define the potential PLD targets and the role of PKC catalytic activity in these events.

At the beginning of this thesis nothing was known about the different isotypes or splice variants of mammalian PLDs excepting an EST in the public database [http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi]. Hence in choosing RBL cells as a secretory model it was considered important to know how many and which PLDs were in these cells in order to approach the PKC-PLD problem. RBL cells are a good example of secretory cells and it has been shown by others during the course of these studies that there is movement of granules containing ectopic GFP-PLD antigen towards the plasma membrane on stimulation (Brown et al., 1998). The identification here of PLD1a and PLD1b in RBL cells suggests that these studies may indeed reflect the underlying behaviour of the endogenous protein (but see below). To try and define the full PLD complement (ie.potential TPA/PKC targets) in RBL cells, the PLD1b fragment derived from this analysis was used for screening a cDNA library to determine whether other related cDNAs were present. Despite an extensive screen, no related sequences were identified. The fact that neither PLD1 splice variant was identified leads to the conclusion that these were simply not present in the library although clearly present in RBL cells.

With the finalisation of the human genome project we now know that there is a third novel PLD as well as PLD1a and 1b splice variants (Hughes and Parker, 2001), and PLD2 (Colley et al., 1997). The third PLD was identified in the draft genome databases; my own in silico screen has revealed this PLD and no others in the Celera databases (see Figure 7.1). This PLD is smaller than the others and apparently it lacks the PH domain (deletion of this domain has been shown to inhibit activity and disrupts PLD1 localisation(Hodgkin et al., 2000)) and also lacks the PX domain that all the other known PLDs retain. In addition PLD3 shows the conserved H (X) K $(X)_4$D, denoted HKD motif, (see amino acids indicated

464-472 in Figure 7.1) characteristic of this family and related to the catalytic activity of the enzyme(Sung et al., 1997); the association of the two halve. of the proteins is essential for activity. In the second HKD motif of PLD3 the D is substituted by an E. Amongst the other conserved sequences in PLD1 and 2 is the sequence GSANIN which in PLD3 is only partly conserved as GTSNWS. Assuming the same catalytic capacity, it is concluded that the poorly conserved substitutions are not important for activity.

The role of PKC in a phosphorylation dependent PtdCho PLD activation process has been implied through the use of phorbol esters which are known to stimulate PKC and PLD. However, failure to achieve routine inhibition of the PtdCho PLD/phorbol ester response with PKC inhibitors (Conricode et al., 1992) has led to the suggestion that phorbol esters may act in part through a PKC independent mechanism. This study has provided at least one explanation for the discrepant results with PKC catalytic inhibitors, by showing that PKC could be involved at different stages in the process in the U937 model. The effect of disruption of the cytoskeleton network is of particular note in that previous studies had shown this element of PKC-dependent action to be critical for MAPkinase activation and differentiation of U937 cells (Whelan et al., 1999). The demonstration here that disruption of microtubules in other cells types also gives an enhanced PLD response to TPA implies that there is some critical relationship between



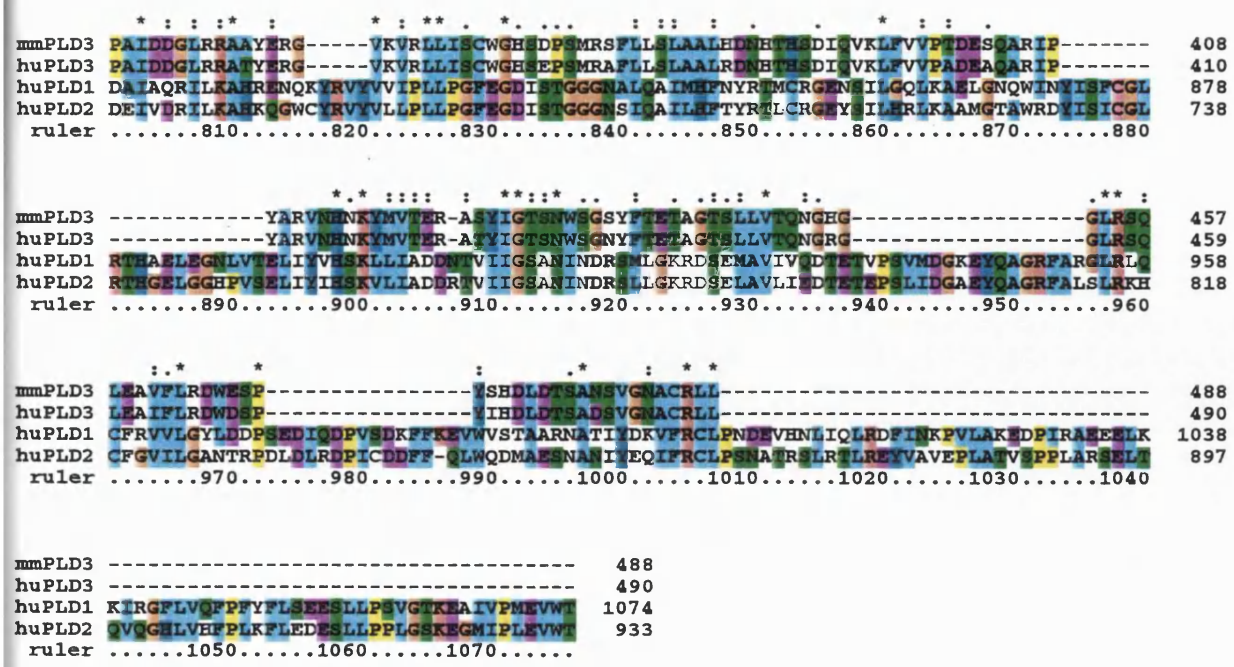


Figure 7.1.
Alignments of PLD1, PLD2 and the novel PLD3 present in the Celera Databases (Dr J. Sgouros and Y. Cabrerizo, unpublished).

the cytoskeleton and the regulation of PLD. Whether this relates in some permissive way to the action of other effectors (such as PKC) by helping PLD to reach the appropriate location for its activation or whether there is a direct binding, synergistic effect of for example G-actin remains to be determined. Other groups have shown a relation between PLD activity and formation of actin stress fibres implying a role for PLD in rearrangement of the cytoskeleton network (Cross et al., 1996; Kam and Exton, 2001; Lee et al., 2001).The studies here indicate that this may be mutual.

To finally resolve some of the outstanding issues in the U937 cell model there are a number of possible approaches. With respect to PLD1 itself there are various generic problems (eg.*in vivo* detection) that are noted below. However it would be possible to use affinity purification with suitable antisera of the fractionated granules to unequivocally define the relative localisation of the PtdEtOH that can be formed *in vivo*, the PLD1 itself, PKCβ and the β2-integrin. It would also be of value to enrich this fraction sufficiently to demonstrate the presence of the metalloproteinases described for this compartment. Positive identification of a particular MMP would help in permitting elucidation of its role in the proposed autocrine triggering of the β2-integrin and subsequent internalisation and MAPKinase activation. Likewise the requirement for internalisation itself is of increasing significance, but may only be clarified with more selective inhibitors and perhaps better means of identifying the compartment in which Raf is activated (Whelan et al., 1999).

7.2. Perspectives

It would be interesting to use immunological and molecular biological tools to answer the key questions relating to subcellular localization and function of different PLD isoforms. In the final analysis our understanding of what controls operate on these proteins will need to be answered by determining which controls really colocalise with or in fact complex to the individual PLDs. Techniques for achieving this are certainly available now, however at present limited either to tagged proteins (ie not the endogenous ones and hence subject to the intrinsic problem of overexpression) or for combinations of proteins where high titre antisera are available. For PLD it has been argued that the levels of expression determine the pattern of localisation (Freyberg et al., 2001) and hence concluded that the endogenous protein is the only one to follow. This is a concern with many approaches to function and certainly argues that a key to moving forwards is the generation of high titre antisera to the different PLD isoforms.

It is of interest to speculate that part of the reason for the distinct concentration-dependent distributions of PLD is that the pattern of behaviour reflects the steady state distribution of a membrane associated trafficking protein that can influence its own traffic. Thus high level expression might bypass some stoichiometric block to exit from the Golgi for example, and lead to accumulation at a different regulated step, eg the endosome. The point is that the compartment defined by overexpression may be entirely relevant to PLD but that it is not the normal rate limiting step in its progress through cellular membranes – of course it might be under certain conditions. No doubt these issues will be resolved in the future.

The descriptive aspects of defining location will need to be complemented by studies on the function of these proteins. This will probably come from both genetic approaches (mouse

knock-outs may be informative) and from reconstitution of trafficking and other processes in which PLD is implicated. One critical issue is whether the generation of PA in membranes has an intrinsic ability to bind proteins (through a PA binding domain?) or whether the main effect of PA is in the distortion of the lipid bilayer in a manner that permits fusion events, the recruitment of proteins by other lipids etc. Probably both sorts of events will prove to be important.

The solution to these general PLD related issues will ultimately inform on the details of how PKC activation by TPA triggers a MAPkinase response that appears to be dependent upon PLD. This may in turn direct a reconsideration of the mechanisms acting between TPA and MAPKinase in other situations.

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