The Structure and Function of Human Phosphoinositide 4-Kinases

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A thesis submitted to the University of London for the Degree
Doctor of Philosophy
January 1999

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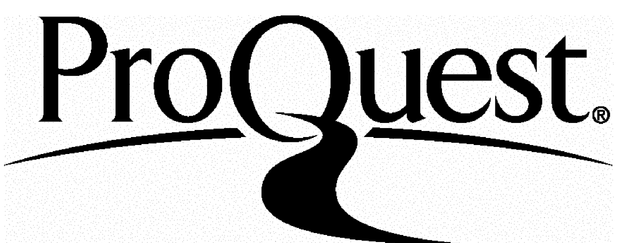

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The Structure and Function of Human Phosphoinositide 4-Kinases

The biosynthesis of phosphosphoinositides underlies a wide range of important biological processes including receptor signalling, cytoskeletal remodelling, and vesicle traffic. This thesis describes the molecular cloning and biochemical characterisation of phosphatidylinositol 4-kinaseβ (PtdIns 4Kβ) and the type IIα phosphatidylinositol(5)phosphate 4-kinase (PtdIns*P*K IIα), two enzymes believed to lie in PtdIns(4,5)P_2 biosynthetic pathways.

I have cloned and characterised two splice variants of a novel wortmannin-sensitive, cytosolic PtdIns kinase, PtdIns 4Kβ, with homology to Pik1p, the archetypal PtdIns 4K from *S. cerevisiae*. The two PtdIns 4Kβ proteins differ by the presence of a short serine-rich insertion which can be phosphorylated *in vitro* by casein kinase II.

Also presented in this thesis is the cloning of PtdIns*P*K IIα and its expression in *E. coli*, *S. frugiperda* and mammalian cells. Recombinant protein has been used to produce a monoclonal antibody which recognises PtdIns*P*K IIα by western blotting, immunoprecipitation and immunofluorescence. Sequence analysis of PtdIns*P*K IIα showed no statistically significant homology with any known phosphotransferase enzyme. However, identification of the nucleotide-binding residue using a combination of affinity labelling and mass spectrometry has permitted identification of the active site. The importance of the nucleotide-binding residue, and of others predicted to be important in catalysis, has been confirmed by analysis of mutant proteins generated by site-directed mutagenesis. The unique substrate specificity of PtdIns*P*K IIα suggests that it may lie in a previously undescribed PtdIns(4,5)P_2 biosynthetic pathway. The possible significance of this pathway is discussed.

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Table 8.3 Pairwise homology scores for selected PtdIns 4K catalytic domains

1.0 Introduction

1.1 Bioactive Phospholipids

The lipid component of cellular plasma membranes plays important structural roles in maintaining a selective physical barrier between the extracellular environment and the interior of the cell. Subcellular membranes, such as those that define the nucleus, the Golgi, mitochondria, and vesicles, serve to compartmentalise biological processes. Each of these membranes is composed of a distinct fraction of lipids which perform structural roles, maintaining the integrity of the membrane. However, certain membrane lipids are precursors of molecules which are key mediators of fundamental biological processes. Several general functions of these lipids can be identified. First, the generation of second messenger molecules in response to extracellular signals, second, interaction with specific proteins to regulate their activity and/or their subcellular localisation, and third, to cause localised structural changes in membranes.

Membrane phospholipids which are precursors of second messenger molecules include the phosphoinositides (PIs*, the subject of this study), sphingomyelin which is the precursor of ceramide and sphingosine-1-phosphate, and phosphatidylcholine (PtdCho) which is a precursor of phosphatidic acid (PtdOH), diacylglycerol (DAG), lyso-PtdCho and arachidonate. The metabolism of these membrane lipids is consequently of great biological significance and, although the potential for crosstalk between these latter signalling pathways and the intensively studied PI pathways is considerable, this Chapter will not address these pathways except in cases where it is directly relevant (for review see (Divecha and Irvine, 1995; Liscovitch *et al.*, 1994; Spiegel *et al.*, 1996).

A vast body of work has accumulated on the properties of PIs and their roles in signalling however, due to length constraints, it is impossible to give a full review of the literature. Therefore, the present discussion will concentrate on aspects of PI signalling which are most relevant to PtdIns$P$ kinases (PtdIns$P$Ks) and PtdIns 4-kinases (PtdIns 4Ks). Where possible review articles will be cited.

1.1.1 Phosphoinositides

The first elements of the PI cycle were described some 45 years ago with the discovery that cellular PtdIns was rapidly phosphorylated in response to extracellular stimuli (Hokin and Hokin, 1953). In the years that followed, a variety of PI metabolic enzymes were

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* The generic term phosphoinositide(s) (PI(s)) is used here to describe all inositol phospholipids, which includes phosphatidylinositol (PtdIns) and its polyphosphorylated forms (phosphoinositides). Where possible, specific isomers will be referred to *e.g.*, PtdIns(3,5)P_2 and where the isomer is not known the empirical formula (*e.g.*, PtdInsP_2) will be used. Inositol phosphates are not included in this definition of PI but will be referred to using similar nomenclature *e.g.*, Ins(1,4,5)P_3 and InsP_3 *etc.*,

described including several PI kinases and PI-phospholipases. At this time evidence for the involvement of PtdIns in cellular signalling was largely circumstantial and the relationship between PtdIns metabolism and intracellular calcium was unclear. In his seminal review on PI signalling, Michell suggested that the agonist-regulated hydrolysis of PtdIns produced a soluble inositol phosphate which could act as an intracellular second messenger (Michell, 1975). This was later confirmed by the demonstration that inositol was necessary for the Ca^{2+} response in 5-hydroxytryptamine-treated fly salivary glands (Fain and Berridge, 1979). Subsequently, in the early 1980's a succession of studies detailed the biochemistry behind the Ca^{2+} response: firstly it was demonstrated that PtdIns(4,5)P_2 was hydrolysed in preference to PtdIns and that Ins(1,4,5)P_3 was the likely second messenger (Michell *et al.*, 1981), secondly, that Ins(1,4,5)P_3 was derived from PtdIns(4,5)P_2 (Berridge, 1983), and finally, that Ins(1,4,5)P_3 was responsible for mobilising cellular calcium (Streb *et al.*, 1983). The subsequent discovery of the DAG-activated protein kinase C (PKC, Section 1.8) led to the current model of receptor-regulated PLC signalling, linking PtdIns(4,5)P_2 hydrolysis to Ca^{2+} and PKC signalling.

In this model, PtdIns in the plasma membrane is converted by PI kinases to PtdIns(4,5)P_2, the substrate for receptor-regulated phospholipase C (PLC) activity. PtdIns(4,5)P_2 hydrolysis by PLC leads to the generation of Ins(1,4,5)P_3 and diacylglycerol (DAG), regulators of intracellular calcium release (Berridge, 1993 for review) and PKC (Nishizuka, 1984), respectively. This model (Figure 1.1) has prevailed with few revisions, although much complexity has been added with the discovery of a class of D-3 phosphorylated lipids, and novel kinases and phosphatases (see Figure 1.2).

1.1.2 Phosphoinositide Metabolism

As shown in Figure 1.2, the inositol headgroup of PtdIns can undergo a number of reactions to produce seven species of polyphosphoinositide which have so far been recognised in the vertebrate cell. PIs of animal cells are distinct from other glycerophospholipids in that the fatty acid groups (R1 and R2 in Figures 1.1 and 1.2) are predominantly stearoyl ($C_{18:0}$) and arachidonyl ($C_{20:4}$), respectively. Cellular levels of certain phosphoinositides, most notably PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, PtdIns(3,4)P_2 and PtdIns(3,5)P_2, change rapidly in response to extracellular stimuli, whereas levels of others such as PtdIns(3)P fluctuate very little. Apart from PtdIns(4,5)P_2, none of the phosphoinositides are known to be physiological substrates of PLC and instead are thought to function either as precursors of messengers that recruit and/or activate effector molecules. The biosynthesis of phosphoinositides is carefully controlled by a large number of PI kinases and PI phosphatases some of which are known targets for cell surface receptor signalling.

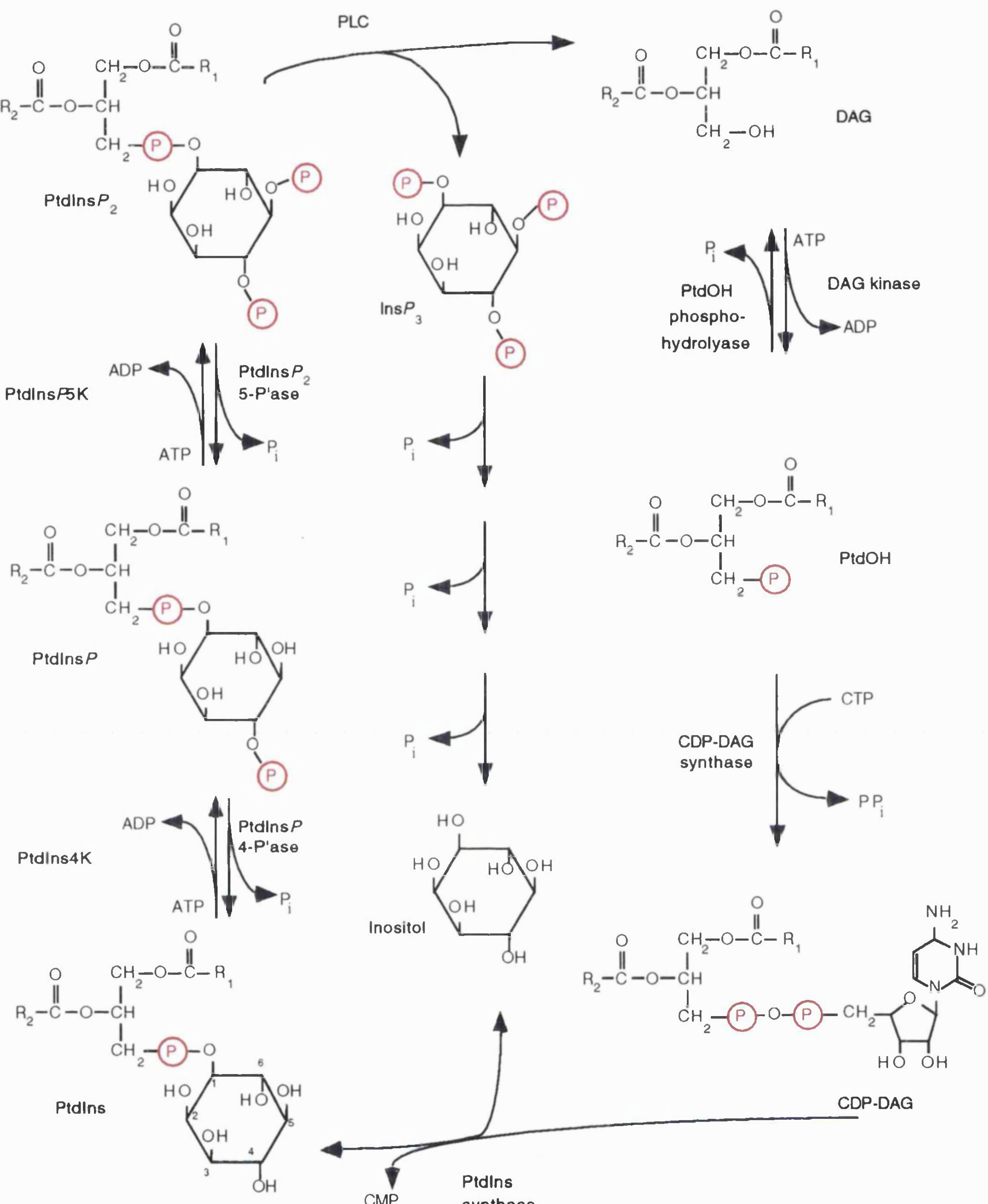


Figure 1.1 The phosphoinositide cycle

Ins(1,4,5)P_3 and DAG generated by the hydrolysis of PtdIns(4,5)P_2 can be used to resynthesise PtdIns via Ins and the activated precursor CDP-DAG, a reaction catalysed by PtdIns synthase. The scheme shown is simplified for the sake of clarity and it should be noted that several of the steps shown above are known to take place in distinct subcellular compartments. Inositol phosphates are also known to undergo a variety of phosphorylation and dephosphorylations which are beyond the scope of this thesis. For more detailed description of the PI headgroup reactions see Figure 1.2. Figure adapted from Hsuan and Tan, 1997.

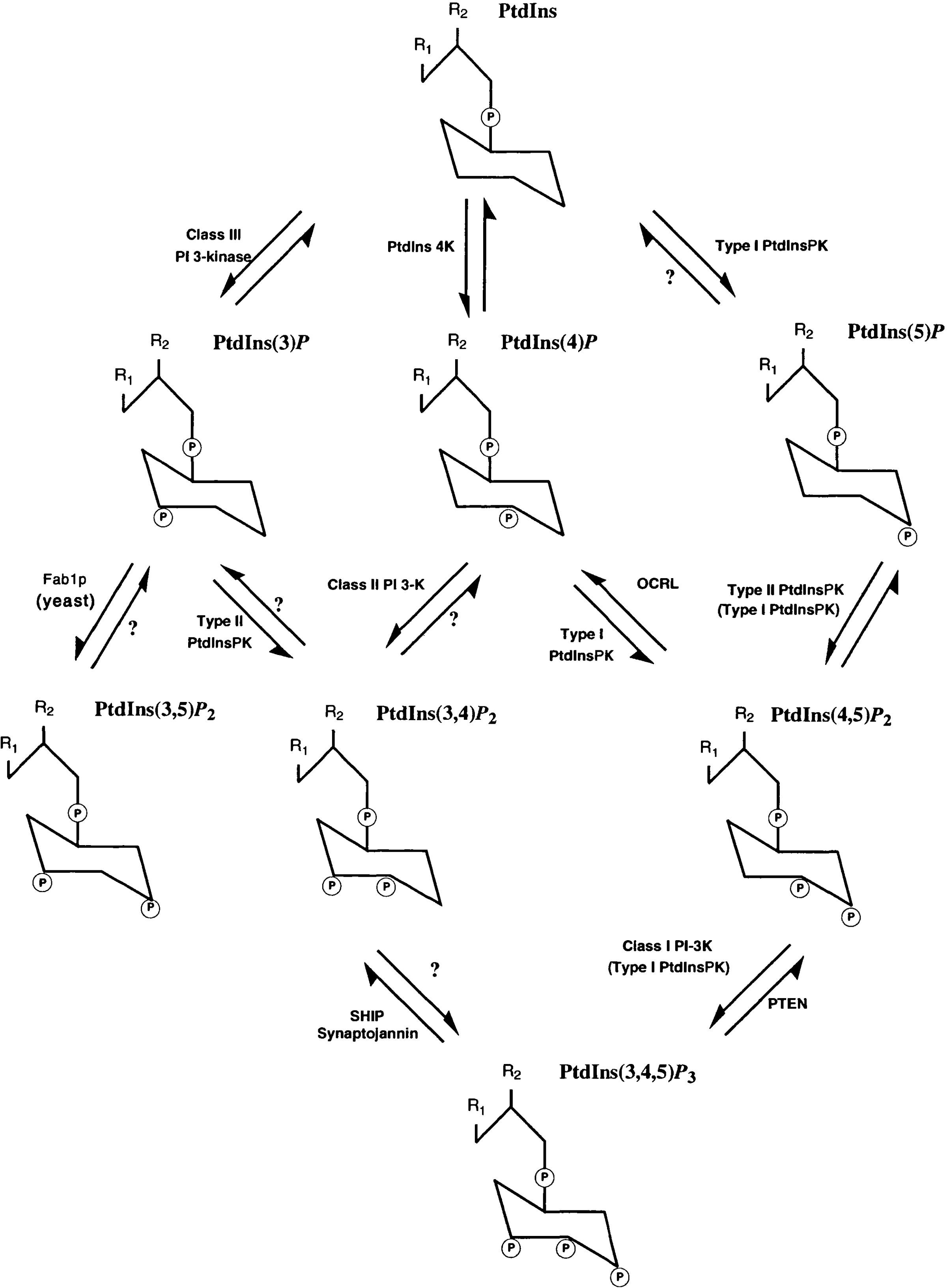


Figure 1.2 Phosphoinositide pathways

The known biosynthetic routes for the interconversion of phosphoinositides by PI kinases and PI phosphatases are shown for enzymes known to catalyse these steps *in vitro* or *in vivo* .

? indicates that the activity that catalyses this step is not known. For details see text.

1.2 Receptor-Linked Phosphoinositide Metabolism

Two major classes of cell surface receptor are known to regulate the metabolism of the membrane phospholipid PtdIns; the heterotrimeric GTPase (G-protein) coupled receptors (GPCRs) and the protein tyrosine kinase linked receptors. GPCRs are responsible for transducing signals in response to neurotransmitters and many hormones (such as catecholamines, gonadatrophins, parathyroid hormones), as well as odourants and light. GPCRs are integral membrane proteins with seven transmembrane segments, a structural feature which is characteristic of this large family of receptors. The G-proteins themselves are inactive heterotrimers of α, β and γ subunits associated with the cytoplasmic domains of receptors (Hamm and Gilchrist, 1996). When the receptor is activated by ligand binding, the associated α subunit undergoes a conformational change which lowers its affinity for GDP and promotes exchange for GTP (thus activating the α subunit's intrinsic GTP phosphohydrolase activity). In its active GTP-bound state the heterotrimer dissociates into an α subunit and a tightly associated $\beta\gamma$ dimer both of which have potential effector functions. The subunits remain dissociated until the GTP bound to the Gα subunit is hydrolysed, a period determined by rate of hydrolysis which varies depending on the type of Gα subunit and the action of specific GTPase-activating proteins (GAPs). Once the bound GTP has been hydrolysed to GDP the subunits reassociate with the receptor in the inactive state (Neer, 1995, for review).

Growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), and fibroblast growth factor (FGF) bind to transmembrane receptors with intrinsic protein tyrosine kinase activity. Generally, ligand binding and the subsequent conformational alteration of the extracellular domain promotes dimerisation which leads to receptor autophosphorylation (reviewed in Ullrich and Schlessinger, 1990). The process of receptor activation has been well studied in the PDGF- and EGF receptors and leads to phosphorylation of multiple tyrosine residues generating numerous sites for the interaction of proteins containing SH2 and phosphotyrosine-binding (PTB) domains (Claesson Welsh, 1994; Hsuan and Tan, 1997). As discussed later, tyrosine phosphorylation is a key event which, along with other regulated molecular interactions, enables the assembly of multi-enzyme signalling complexes.

A number of other receptors such as the T cell receptor, B cell receptor, IgM, high affinity IgE receptor, hepatocyte growth factor (HGF), integrins, and cytokine receptors lack protein kinase activity but can induce tyrosine phosphorylation of specific proteins by interaction with non-receptor tyrosine kinases such as members of the Src, Syk, and Jak/Tyk families. In the case of the antigen receptors of lymphocytes these tyrosine kinases link ligand binding to PI 3-kinase and PLCγ phosphorylation (Sjaastad and Nelson, 1997; Weiss and Littman, 1994, for review)

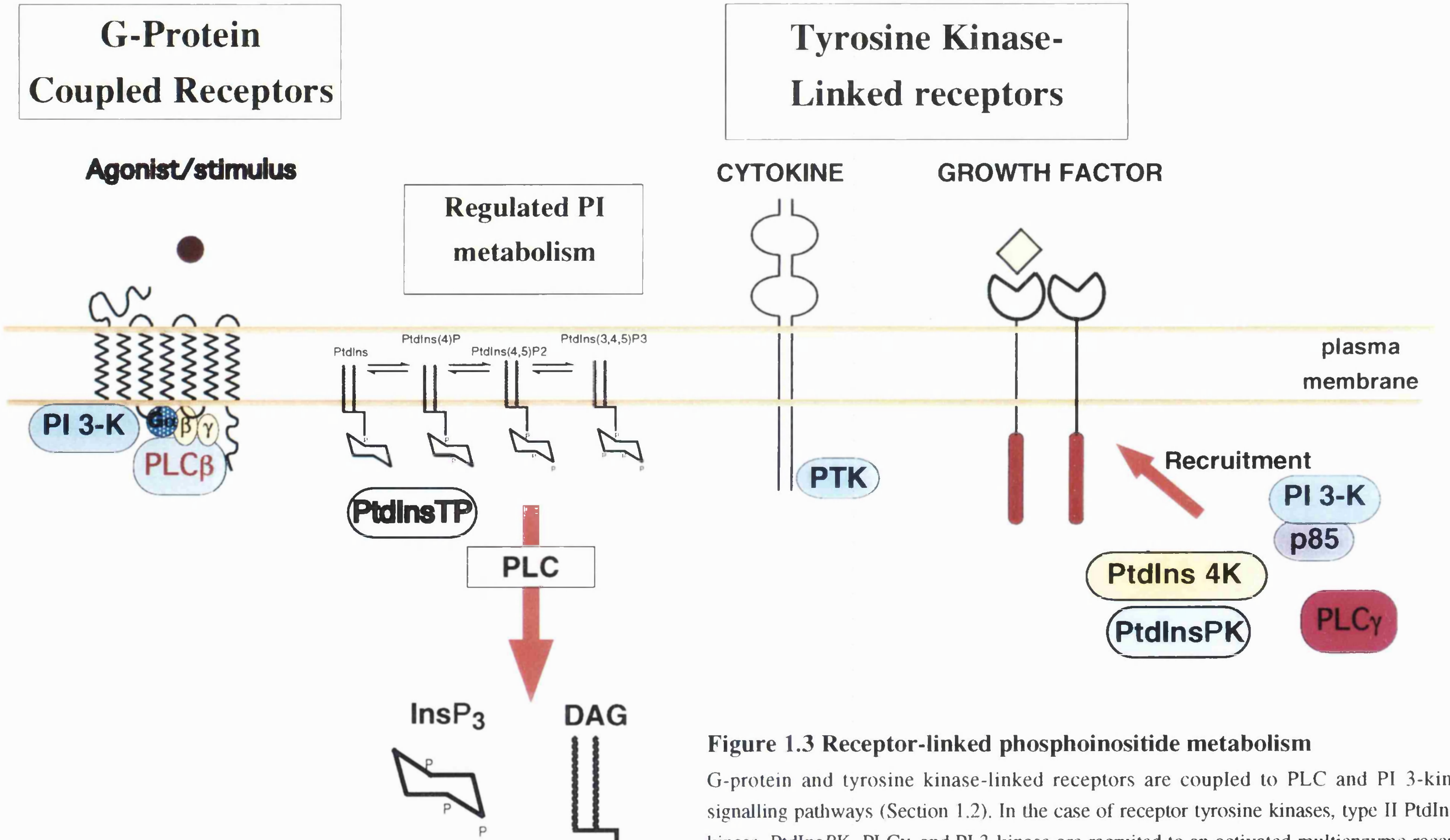


Figure 1.3 Receptor-linked phosphoinositide metabolism

G-protein and tyrosine kinase-linked receptors are coupled to PLC and PI 3-kinase signalling pathways (Section 1.2). In the case of receptor tyrosine kinases, type II PtdIns 4-kinase, PtdIns*P*K, PLCγ, and PI 3-kinase are recruited to an activated multienzyme receptor complex (Section 1.2.3). Recruitment of PI kinases and PLC is thought to bring these enzymes to their membrane-bound substrates. PtdInsTP is required for both RTK and GPCR PLC signalling (Section 1.7.1).

1.2.1 Compartmentation and the Organisation of Signalling Complexes

How activated cells control the supply of phospholipid signalling precursors is still poorly understood and many questions remain to be answered, perhaps the most pertinent of which is: how are PI kinase activities localised and regulated by signalling against high background activities in the unstimulated cell? The answer to this question may lie in the empirical observation that there exists a pool of PtdIns, frequently referred to as the agonist-sensitive pool, which is defined as the fraction of total cellular PI metabolised in response to receptor agonists. The remaining fraction, the agonist-insensitive pool, is resistant to metabolism even during prolonged stimulation. At present, this pool can only be defined in abstract rather than physical terms and in several cases distinct metabolic pools have not been observed (reviewed in Monaco and Gershengorn, 1992). However, its existence has been repeatedly documented in numerous cell types (Monaco and Gershengorn, 1992).

It is often difficult to relate the *in vitro* properties of PI-metabolising enzymes to a cellular model, however, it is clear that the activity of these enzymes is frequently sensitive to substrate presentation. For example, PtdInsTP is required for agonist-dependent PtdIns(4,5)P_2 synthesis and hydrolysis (Cunningham *et al.*, 1995; Hay *et al.*, 1995; Kauffmann Zeh *et al.*, 1995; see also Section 1.5.2) type I PtdIns*P*Ks and PLCγ are activated in PtdOH-containing micelles (Jenkins *et al.*, 1994; Jones and Carpenter, 1993; Moritz *et al.*, 1992), and PtdIns 4-kinases and PLCγ are activated by detergent whilst PI 3-kinase is inhibited (Pike, 1992). If these properties reflect *in vivo* specificities, then factors which effect substrate presentation such as the presence of accessory lipids or PtdInsTP may delimit agonist-sensitive pools (reviewed by Hsuan and Tan, 1997; Liscovitch and Cantley, 1995).

Hypothetically, the agonist-sensitive pool may be defined by being physically separated from the insensitive pool, perhaps at a subcellular locale to which signalling enzymes have access. This proposition is supported by the fact that there are many enzymes which undergo recruitment to specific compartments on stimulation including PLC, PI 3-kinase and PtdIns*P*K (Sections 1.4; 1.5.3; 1.5.2). Furthermore, it is well known that different subcellular membranes have distinct lipid compositions and that, as mentioned above, several PI metabolising enzymes have specific requirements for accessory lipids *in vitro*, suggesting that activity may be limited to specific membrane compartments. It has also been hypothesised that intramembrane compartmentation may segregate substrates and signalling enzymes in so-called membrane rafts in which PI turnover may take place (see Section 1.2.4). However, there is no reason to assume that the agonist-sensitive pool is a material entity, instead the phenomenon could be explained by the hypothesis that access to substrate is limited by properties inherent to the enzyme or even the lipid substrate.

Evidence for the latter is provided by the finding that several PI-metabolic enzymes can discriminate between the fatty acid groups of phospholipids (discussed below).

1.2.2 Compartmentation by substrate recognition

Another potential mechanism for compartmentalising PI metabolism has emerged recently and relies on acyl chain specificity of enzymes. The most common acyl chains of agonist-sensitive PIs are stearoyl and arachidonyl, although many other forms exist in the total cellular pool of PtdInsP_2 (Lee *et al.*, 1991). PtdIns-transfer proteins (PtdInsTPs), diacylglycerol kinase (DAGK), and phospholipase D (PLD) have been shown to discriminate between acyl groups (Kasurinen *et al.*, 1990; Pettitt *et al.*, 1997; Tang *et al.*, 1996; van Paridon *et al.*, 1988). In response to many receptor agonists that stimulate PI turnover, cellular DAG levels increase rapidly on stimulation and return to basal levels within 1-2 min. This initial increase is often followed by a second, sustained phase of DAG generation. The DAG in the initial phase is predominantly made up of polyunsaturated species, whereas the second phase is primarily made up of specific saturated species and is accompanied by a rise in Cho levels, thus suggesting the involvement of PLD (Pettitt *et al.*, 1994). The finding that PtdIns(4,5)P_2- and PtdCho-derived DAG have characteristically different fatty acid compositions, and that in porcine aortic endothelial cells, DAG resulting from PLD activity is not an activator of PKC (Pettitt *et al.*, 1997), provides a model for the metabolic compartmentation of phospholipid signalling which may extend to other lipid metabolising enzymes.

1.2.3 Integrated signalling complexes

Functional multienzyme complexes take advantage of the rapid kinetics offered by having several biosynthetic enzymes present in a single aggregate. Examples of such complexes can be found throughout biology and include the photosystems of plants and algae, The concept of a multienzyme signalling complex is consistent with several compartmentation models of PI signalling because these often involve the recruitment of proteins to membrane-bound receptors in close proximity to lipid substrates. The best characterised examples are the high-affinity phosphotyrosine-SH2 complexes formed on activation of RTKs. PLCγ and PI 3-kinase co-purify with activated PDGF- and EGF receptors (Anderson *et al.*, 1990; Hu *et al.*, 1992; Sections 1.4 and 1.5.3) and associate primarily through SH2 domains, as do many other signalling molecules (reviewed by Hsuan and Tan, 1997; Pawson, 1995). PtdIns 4K, PtdIns*P*K, and PtdInsTP all associate with activated EGF receptors through unknown mechanisms (Cochet *et al.*, 1991; Kauffmann-Zeh *et al.*, 1995; Kauffmann-Zeh *et al.*, 1994; Thompson *et al.*, 1985).

Recruitment of PtdIns 4K, PtdIns*P*K, PLCγ and PtdIns transfer protein to activated EGF receptors has been proposed to constitute such a signalling complex in which PtdIns

at the plasma membrane is sequentially phosphorylated by the kinase activities and hydrolysed by PLCγ (Figure 1.3; Hsuan, 1993; Kauffmann Zeh *et al.*, 1995).

1.2.4 Supramolecular signalling complexes

Recently, evidence has emerged suggesting that PI signalling complexes are organised into higher order membrane structures in the form of discrete, detergent-insoluble microdomains. These microdomains were originally thought to take the form of caveolae, small flask-shaped invaginations of the plasma membrane which are enriched in caveolin, a 22-24 kDa integral membrane protein (Parton, 1996, for review). The hypothesis that membrane signalling complexes can be organised into caveoli (Lisanti *et al.*, 1994) is supported by the finding that a variety of receptors, G-proteins, and non-receptor tyrosine kinases are concentrated in caveolin-rich fractions (Parton, 1996). Furthermore, it has been proposed that caveolin may directly regulate a range of signalling molecules including the EGFR, Ras, and heterotrimeric G-proteins (Parton, 1996).

In many cases however, the experimental evidence for these associations has been based on Triton X-100 insolubility and high bouyancy upon density gradient ultracentrifugation. Recent evidence suggests that caveoli prepared using both detergent and non-detergent methods may be substantially contaminated with non-caveolar membrane fragments (Waugh *et al.*, 1998; Waugh *et al.*, 1999, and references cited therein). Both caveoli and membrane rafts are rich in glycosphingolipids and cholesterol, and are therefore insoluble in detergents such as Triton X-100. Consequently, the presence of signalling molecules in detergent-insoluble fractions may in fact be due to their presence in glycosphingolipid-rich (DIG) domains and other membrane rafts rather than caveolae (Simons and Ikonen, 1997, for review; Waugh *et al.*, 1998; Waugh *et al.*, 1999).

Density-gradient fractionation studies of A431 cells have shown that EGF and bradykinin-simulated PI turnover take place in buoyant, detergent-insoluble, caveolin-rich fractions (Hope and Pike, 1996; Pike *et al.*, 1996) which also contain a type II PtdIns 4K (Waugh *et al.*, 1998). Interestingly, only small changes in PtdInsP_2 levels were observed in other fractions indicating that buoyant, detergent-insoluble fractions are the primary source of agonist-sensitive PtdInsP_2 in A431 cells (Pike *et al.*, 1996). DIGs may also be important for maintaining hormone responsiveness of A431 cells: methyl-β-cyclodextrin (cyclodextrin) is a cholesterol-binding agent that can selectively and reversibly remove cholesterol from intact cells and has recently been used to investigate the role of DIGs in EGF and bradykinin-stimulated PLC signalling. A431 cells treated with cyclodextrin had severly impaired inositol phosphate responses to EGF and bradykinin and furthermore, caveolin, EGF receptor, Gq and PtdInsP_2 redistributed to other membrane fractions (Pike and Miller, 1998). All of these effects were reversible on incubation with cholesterol indicating that it was the removal of cholesterol which was responsible for these

observations. Together, the studies described above suggest that higher order membrane organisation is required for PI signalling which may serve to compartmentalise substrates and enzymes and perhaps segregate potentially cross-reacting components. However, it has yet to be shown that the intramembrane compartmentation of PI signalling is a general phenomenon and the membrane fractions in which signalling takes place needs to be characterised in detail.

1.3 Modular Interaction Domains

Carefully coordinated, non-covalent molecular interactions are an important characteristic of signalling cascades which permit the localisation and appropriate assembly and disassembly of complexes of signalling molecules. These interactions frequently require the presence of non-catalytic domains capable of binding cognate ligands. A well studied example is the generation of phosphotyrosine residues upon RTK activation, leading to the rapid recruitment of cytosolic molecules that interact through phosphotyrosine binding motifs. These agonist-driven changes in subcellular localisation may have important regulatory implications, for example it may bring a key enzyme to the site of its substrate (or substrate to enzyme), cofactor or activator. Some substrates are present at a very low cellular concentration but recruitment to a subcellular compartment where the local concentration of substrate is much higher can result in more favourable kinetics. In other cases an interaction between phosphotyrosine and SH2 domains may also activate an enzyme through an allosteric mechanism as in the case of PI 3-kinase/p85 holoenzyme which is stimulated by binding to PDGF receptor phosphopeptides (Panayotou *et al.*, 1992; Shoelson *et al.*, 1993). SH2, PTB, SH3, PH, C_2, PDZ, and other domains, comprise a group of independently folding and functionally autonomous protein modules which have been identified in recent years. Many examples of these exist in eukaryotes as diverse as *Saccharomyces*, *Dictyostelium*, *Drosophila* and mammals, indicating a very ancient ancestry. These domains are small (80-160 amino acids) and thus often amenable to study by NMR. In many cases individual structures and binding specificities have been elucidated (Pawson, 1995) and such domains continue to be identified in a growing number of signalling molecules. Some exist proteins which lack catalytic activity and consist entirely of modular domains and ligand domains. In several cases these are known to function as adaptor proteins, for example the regulatory subunit of p85 (Section 1.5.3). As well as being present in a subset of phosphoinositide kinases, many PH and C_2 domains are known to bind phosphoinositides and inositol phosphates (Irvine and Cullen, 1996). SH2 domain-phosphotyrosine interactions may also be regulated by phosphoinositides (Rameh *et al.*, 1995). Thus the presence of these domains in signalling molecules, in particular SH2 and PH domains, which in some cases are known to regulate membrane localisation, suggests potential regulatory mechanisms.

1.3.1 SH2 domains

SH2 domains are modules of approximately 100 amino acid residues that were originally identified in the Src tyrosine kinase family and have now been found in a very large number of proteins with signalling functions (Pawson, 1995, for review). Binding studies with phosphopeptides indicate relatively high affinities (Kd 1-10 nM). Recently SH2 domains from the p85 subunit of heterodimeric PI 3-kinase have been shown to bind to PtdIns(3,4,5)P_3 *in vitro* this phenomenon was apparently competitive with respect to phosphotyrosine binding (Rameh *et al.*, 1995). The authors suggest that this phenomenon may underly a mechanism for disrupting SH2-phosphoprotein interactions or a way of recruiting proteins to phospholipid-containing membranes. It will be interesting to see whether such experiments can be repeated under more appropriate conditions, that is, using full-length molecules, with membranes rather than phospholipid micelles and in the presence of physiological concentrations of divalent ions such as Mg^{2+}.

1.3.2 PTB and PDZ domains

Another class of of phosphotyrosine-binding module, the PTB domain was originally identified as a novel protein module distinct from SH2 domains (Bork and Margolis, 1995; Kavanaugh *et al.*, 1995). PTB domains actually represent a class of PH domain capable of binding phosphotyrosine (Eck *et al.*, 1996). The structures of the SHC adaptor protein and insulin receptor substrate-1 (IRS-1) PTB domains have been determined (Eck *et al.*, 1996; Zhou *et al.*, 1995b). Unlike SH2 domains, which share significant sequence homology, these PTB domains have little. PDZ and PTB domains have a similar 3-dimensional architecture and peptide binding mechanism (Cowburn, 1997, for review).

PDZ domains were first identified as protein components of synaptic junctions, but now appear to be more widely distributed and are thought to play a role in localising signalling and adhesion molecules (for review see Craven and Bredt, 1998). For example, the PDZ-containing proteins LIN-2 and LIN-7 are required to localise the EGF receptor to junctions between epithelial cells in *C. elegans* (Kaech *et al.*, 1998; Simske *et al.*, 1996). Furthermore a *Drosophila* PDZ-containing protein, InaD, containing five PDZ modules, has recently been described as a component of the *Drosophila* phototransduction cascade. The individual PDZ domains of InaD have been shown to bind the Trp Ca^{2+} channel, PLCβ, and PKC *in vitro* (Tsunoda *et al.*, 1997). Genetic studies with InaD mutants suggest that InaD is a multivalent adaptor essential for the rapid assembly of a supramolecular, G-protein coupled PI signalling complex (Tsunoda *et al.*, 1997).

1.3.3 SH3 domains

SH2 domains are frequently found alongside SH3 domains. These are motifs of approximately 50-75 residues which mediate interactions with proline-rich sequences. Many SH3 ligands have been identified and have at least one PXXP motif (reviewed in Cohen *et al.*, 1995). In addition, the context of the PXXP motif, such as sequences N- and C-terminal of the critical prolines, are likely determinants of binding specificity (Rickles *et al.*, 1995; Sparks *et al.*, 1996)

SH3 domain interactions can have important regulatory implications. In the Src family of non-receptor tyrosine kinases, an intramolecular SH3-ligand interaction maintains the kinases in an inactive state (Moarefi *et al.*, 1997; Xu *et al.*, 1997) and mutations in the SH3 domain which destabilise this interaction enhance their oncogenic activity (Hirai and Varmus, 1990; Jackson and Baltimore, 1989; Seidel-Dugan *et al.*, 1992). In other cases SH3 binding stimulates activity: for example, the GTPase dynamin can bind and be stimulated by isolated SH3 domains from c-Src and Grb-2 (Gout *et al.*, 1993). In both cases modulation of activity is likely to involve an allosteric mechanism.

There is also evidence that some SH3 domains, such as that of PLCγ, may localise proteins to the cytoskeleton (Bar-Sagi *et al.*, 1993). SH3 domains have been identified in a number of cytoskeletal proteins including myosin I, spectrin and ABP-1 (Stoffler *et al.*, 1995; Lehto *et al.*, 1988; Labeit and Kolmerer, 1995) and numerous proteins associated with cytoskeletal function. Like SH3 domains, the recently identified WW domains also bind proline rich sequences (Chan *et al.*, 1996), however, some WW domains show distinct binding specificty from SH3 domains and the consensus sequence PPXY has been identified (Chen *et al.*, 1995).

1.3.4 PH domains

PH domains were originally identified in the PKC substrate pleckstrin and have since been found in a large number of molecules (Saraste, 1995) including PLC isoforms and PtdIns 4Ks amongst many others. The structural definition of PH domains is not always easy since they can vary considerably in sequence and their functional significance is not always well understood. Now that the 3-dimensional structures for several PH domains have been solved, it is clear that, despite a lack of sequence identity between PH domains, all share a common protein fold and can interact with a diverse range of protein and non-protein ligands. The best characterised function of PH domains appears to be binding to phosphoinositides and inositol phosphates, but it should be stressed that not all PH domains bind phosphoinositides with high affinity and specificity. Nevertheless, several PH domains have emerged as important regulatory domains in a number of proteins which are recruited to membranes by phosphoinositides (Lemmon *et al.*, 1997, for review) and as such, are potential effectors of phosphoinositide kinases.

The importance of PH-dependent membrane localisation is exemplified by the regulation of the PH-containing protein kinase B (PKB, discussed in detail in Section 1.5.3.e) and the non-receptor tyrosine kinase Btk. Btk is essential for B-cell proliferation and lies downstream of the B-cell receptor which regulates both PI 3-kinase and PLCγ pathways (reviewed in Weiss and Littman, 1994). Btk contains a PH domain which is mutated in X-linked agammglobulineamia in humans and in X-linked immunodefficiency in Xid mice, both disorders in which B-cells fail to proliferate. The Xid mutation in Btk causes the substitution of a basic residue for a cysteine in the PH domain causing reduced affinity for PtdIns(3,4,5)P_3 *in vitro*, whereas the wild type PH domain specifically binds PtdIns(3,4,5)P_3 with high affinity (Fukuda *et al.*, 1996; Rameh *et al.*, 1997a; Salim *et al.*, 1996). This mutation has been proposed to prevent localisation to the plasma membrane (Li *et al.*, 1995b) where Btk substrates exist. One of these substrates is PLCγ, which also contains a PH domain but, in contrast to the well-studied PLCδ PH domain, the PLCγ PH domain displays a preference for PtdIns(3,4,5)P_3 and is recruited to PtdIns(3,4,5)P_3-containing plasma membranes (Falasca *et al.*, 1998) where it is phosphorylated and activated by Btk- thus linking PI 3-kinase signalling with Ca^{2+} release. PH domains which show specificity for PtdIns(3,4,5)P_3 have been found to be important effectors of PI 3-kinase signals (discussed further in Section 1.5.4).

The PH domain of PLCδ is specific for PtdIns(4,5)P_2 and Ins(1,4,5)P_3 has the highest known affinity of any PH domain (Lemmon *et al.*, 1995). The isolated PH domain localises to the plasma membrane in rat basophillic leukemia cells, NIH 3T3 cells, and COS-7 cells. Upon stimulation with platelet-activating factor (PAF) or angiotensin II (AT II) the PH domain transiently redistributes to the cytoplasm before relocalising to the plasma membrane. This process has been imaged in real-time using green fluorescent protein-PH domain constructs (Stauffer *et al.*, 1998; Varnai and Balla, 1998) and was shown to be inhibited by the PLC inhibitor U73122, but not wortmannin. This implies that stimulation with PAF and AT II, both G-protein agonists which trigger the hydrolysis of PtdIns(4,5)P_2, causes the PH domain to dissociate from the plasma membrane as the PtdIns(4,5)P_2 is depleted by PLC activity. However, it is not known whether the rise in Ca^{2+} or the released Ins(1,4,5)P_3, which can compete for PLCδ1 PH domain binding, also contribute to this phenomenon.

Some PH domains may mediate interactions with G$\beta\gamma$ subunits (Touhara *et al.*, 1994). For example the β-adrenergic receptor kinase (Touhara *et al.*, 1994), which may require the synergistic actions of G$\beta\gamma$ and PtdIns(4,5)P_2 which have overlapping binding sites (Pitcher *et al.*, 1995). PH domains may also modulate enzyme activity: the N-terminal PH domain of PLCδ1 may have a regulatory function by localising the enzyme to PtdIns(4,5)P_2-containing membrane and promoting processive catalysis (Lomasney *et al.*, 1996, and Section 1.4), and the PH domain-dependent *in vitro* activation of dynamin GTPase by PtdIns(4,5)P_2 has been described (Lin and Gilman, 1996; Salim *et al.*, 1996).

1.3.5 C_2 domains

C_2 or CalB domains are approximately 130 residue domains that bind inositol phosphates (reviewed by Irvine and Cullen, 1996), phospholipids and proteins. These have been found in numerous proteins implicated in signalling and vesicle traffic including PI 3-kinases (Molz *et al.*, 1996; Virbasius *et al.*, 1996), all PI-specific PLC isoforms, the vesicle docking protein synaptotagmin (Damer *et al.*, 1994) and PKCs amongst others (Ponting and Parker, 1996; Rizo and Sudhof, 1998, for review). In addition to phosphoinositide binding, C_2 domains can also mediate protein-protein interactions (Li *et al.*, 1995a) and calcium-dependent dimerisation between C-terminal C_2 domains of synaptotagmin I has been observed *in vitro* (Chapman *et al.*, 1996).

Dispite much study, the function of the C_2 domains of PI 3-kinases and PLC isozymes remains unclear. C_2 domains are not obligatory Ca^{2+}- and phospholipid-binding modules since they can also bind protein ligands and some are also Ca^{2+}-independent (reviewed in (Rizo and Sudhof, 1998). C_2 domains also exhibit quite different phospholipid binding specificities: whilst the C_2A domains of the synaptotagmins and the C_2 domain of PKCβ preferentially bind acidic phospholipids, the C_2 domain of cytosolic phospholipase A2 interacts with neutral phospholipids (Nalefski *et al.*, 1998; Shao *et al.*, 1997; Sudhof, 1995). The C_2 domain of PLCδ1 is required for activity towards membrane substrates (Ellis *et al.*, 1993; Yagisawa *et al.*, 1994) and structural studies of have revealed that the C_2 domain may localise PLC isoforms to the plasma membrane, perhaps in a Ca^{2+}-dependent manner (see below).

1.4 Phospholipase C

PtdIns(4,5)P_2 generated in response to extracellular signals, as well as being the physiological substrate for PI 3-kinases, is the preferred substrate for hydrolysis by multiple PI-PLC isoforms. This reaction generates two highly important second messengers: DAG, an important activator of protein kinase C activity (Section 1.8 and Nishizuka, 1988, for review) and Ins(1,4,5)P_3, which regulates the release of Ca^{2+} from intracellular stores (Berridge, 1993; Clapham, 1995, for review). Many receptor systems can trigger Ins(1,4,5)P_3 and DAG responses through activation of different phospholipase C isoforms. PtdCho-specific PLC activities which generate PtdOH (a precursor of DAG) are known in prokaryotes, however, whilst there is some evidence for stimulation of this pathway in mammalian cells, there is no reported purification of such an enzyme.

Given the importance of PtdIns(4,5)P_2 hydrolysis in response to extracellular stimuli, the molecular mechanisms regulating PLC activity have been relatively well studied. To date, ten mammalian PI-specific PLC isofoms have been cloned and can be divided into three types; PLCβ (composed of subtypes β1, β2, β3 and β4), PLCγ (γ1and γ2) and PLCδ (δ1, δ2, δ3 and δ4) (Lee and Rhee, 1995, for review). Several features are

common to all PLC isozymes; all have an N-terminal PH domain (Parker *et al.*, 1994), and all hydrolyse PtdIns, PtdIns(4)*P* and PtdIns(4,5)P_2 *in vitro* although PtdIns(4,5)P_2 is the preferred substrate at physiological concentrations of Ca^{2+} ions (Katan, 1997). Phosphoinositides containing a phosphate group at the D-3 position are not substrates for any known PLC activity. This is consistent with the idea that PI 3-kinases act through separate pathways to PLC enzymes.

Type	Modular domains	Activation by G-protein subunits
PLCβ	PH C_2	Stimulated by Gqα. Order of sensitivity: β1~β3>β4>β2 Stimulated by βγ: β2 + β3 >> β1 PLCβ4 is not stimulated *in vitro*.
PLCγ	PH and "split" PH two SH2 SH3 C_2	May bind Gqα *in vitro*.
PLCδ	PH EF C_2	not significantly activated by Gβγ *in vitro*

Table 1.1 The mammalian phospholipase C family

Summary of the properties of mammalian PI-PLC isoforms. "split" PH refers to a putative PH domain with an intervening sequence (Ponting and Parker, 1996). EF; EF hand motif.

The regulation of PLCβ isoforms by Gqα is well established (Lee and Rhee, 1995), for review) and the four β isoforms are all stimulated to different degrees by Gqα subunits which (*in vitro* at least) appear to interact with a unique C-terminal region close to the catalytic domain. PLCβ isoforms are also stimulated by Gi/o βγ subunits by an interaction with the N-terminal PH domain (the properties of the different PLC isoforms are summarised in Table 1.1). PLCβ1 also has the unusual property of acting as a GTPase activating protein for Gq/11 (Berstein *et al.*, 1992). This phenomenon suggests a negative feedback loop in which the effector (PLCβ1) rapidly terminates its own activation by accelerating the hydrolysis of GTP bound to its physiological regulator, Gq/11. Rapid deactivation at the level of the G protein signal may provide a fast response of the type required for biological processes such as phototransduction which occurs via Gq activation of PLCβ in *Drosophila* and *Limulus* species.

The physiological role and regulation of PLCδ isoforms is not well understood in a signalling context although a weak and possibly insignificant activation has been observed with Gβγ subunits *in vitro* (Park *et al.*, 1993). Recently, two mechanisms for the regulation of PLCδ enzymes have been proposed, The first is via G_h, a novel heterodimeric GTP-binding protein which has been found associated with agonist-bound α1-adrenergic receptors (α_1-AR). The multifunctional $G_h\alpha$ subunit (which also possesses

a transglutaminase activity) activates PLCδ1 in a GTP-dependent manner *in vitro* and forms a complex with PLCδ1 in cells stimulated via the α_1-AR (Das *et al.*, 1993; Feng *et al.*, 1996) and furthermore, COS cells overexpressing $G_h\alpha$ show enhanced PLC activation induced by ligation of α_1-AR (Nakaoka *et al.*, 1994). Together these data suggest that $G_h\alpha$ directly couples PLCδ1 to α_1-AR. A second potential pathway for the activation of PLCδ is via p122, a protein with homology to Rho-GAP which specifically stimulates the PLCδ 1 isoform *in vitro* (Homma and Emori, 1995).

The PH domain of PLCδ1 has been implicated in the regulation of enzyme activity since it has a relatively high affinity for PtdIns(4,5)P_2 and the domain is required for membrane localisation *in vivo* (Garcia *et al.*, 1995; Lemmon *et al.*, 1995). It has been suggested that interaction of the PH domain with PtdIns(4,5)P_2-containing membranes may be required for processive, but not non-processive catalysis (Cifuentes *et al.*, 1993). This hypothesis has been strengthened by the publication of mutagenesis data confirming that the N-terminal PH domain of PLCδ1 is necessary for the dose-dependent stimulation of enzyme activity by PtdIns(4,5)P_2 (Lomasney *et al.*, 1996). Because the PH domain can competitively bind Ins(1,4,5)P_3 with high affinity, a mechanism of product inhibition has also been proposed (Lemmon *et al.*, 1995; Lomasney *et al.*, 1996). PLCδ1 PH domains transiently delocalise from the plasma membrane on stimulation with PLCβ agonists PAF and AT-II (Stauffer *et al.*, 1998; Varnai and Balla, 1998). It is not known if a rise in cytosolic Ins(1,4,5)P_3 concentration is responsible but reassociation with the plasma membrane can be prevented by inhibition of PtdIns(4,5)P_2 resynthesis (Varnai and Balla, 1998).

All PLCs are activated by Ca^{2+} ions *in vitro* but PLCδ isoforms are the most sensitive to Ca^{2+} concentration, suggesting that cellular Ca^{2+} may be an important regulatory signal *in vivo*. The recently solved crystal structure of PLCδ1 suggests that the putative calcium-dependent lipid binding site of the C_2 domain may bind the plasma membrane in the same plane as the closely associated active site (Essen *et al.*, 1996). Thus it has been proposed that the C_2 domain may fix the catalytic domain in a productive position on the plasma membrane. Furthermore, deletion of the C_2 domain leads to a loss of activity towards membrane substrates, suggesting that the C_2 domain could provide a point of regulation by Ca^{2+} (Ellis *et al.*, 1993; Yagisawa *et al.*, 1994). It should be noted that a Ca^{2+}-binding EF-hand motif has been identified in PLCδ isozymes (Bairoch and Cox, 1990) but this has no established regulatory function (Nakashima *et al.*, 1995). In contrast to the C_2 domain which has a rigid interface with the catalytic domain, the N-terminal PH domain is thought to have a flexible connection with the rest of the enzyme (Essen *et al.*, 1996). It is thought that the PH domain may play a role in tethering PLCδ1 to PtdIns(4,5)P_2 in the plasma membrane prior to full activation; this is consistent with the observations of the isolated PH domain and the processive catalysis model (see above).

A rat liver isoform, PLCδ4 has been characterised at the molecular level (Lee and Rhee, 1996; Liu *et al.*, 1996). Liu *et al.* reported highest levels of expression in regenerating rat liver and intestine and a nuclear localisation, whereas Lee and Rhee reported no detectable expression of protein or message in these tissues. Although the data from the two groups is contradictory, the nuclear localisation and the specific expression of PLCδ4 during the transition from G to S phase in the cell cycle is intriguing as it suggests a role for PI in the nucleus (Liu *et al.*, 1996, and Section 1.9).

PLCγ isoforms are primarily regulated by tyrosine kinase-linked receptors (Lee and Rhee, 1995, for review). Two sets of phosphorylation events appear to regulate PLCγ function *in vivo*: stimulation of enzyme activity by phosphorylation and the recruitment of cytosolic PLCγ to the activated receptor. As mentioned above (Section 1.2), the activation of receptor tyrosine kinases by ligand binding results in tyrosine autophosphorylation and the phosphorylation of many signalling molecules including PLCγ. Expression of mutant forms of PLCγ in mammalian cells has been used to investigate the importance of individual tyrosine residues. *In vivo*, PLCγ1 is phosphorylated by the EGFR, PDGFR and other growth factor receptors and non-receptor tyrosine kinases on tyrosine residues 771, 783, and 1254. Mutation of tyrosine 783 to phenylalanine totally blocks activation (Kim *et al.*, 1991; Meisenhelder *et al.*, 1989) and full activation requires phosphorylation of tyrosine residues 783 and 1254. Purified, tyrosine-phosphorylated PLCγ has increased activity *in vitro* (Wahl *et al.*, 1992) but the mechanism of activation remains enigmatic, since mutation at residues 771, 783 and 1254 has no effect on activity *in vitro*. (Kim *et al.*, 1991). Autophosporylation of the EGFR generates five potential SH2 ligands each of which can bind PLCγ1 (Soler *et al.*, 1994). In contrast the FGFR and NGFR posses only one tyrosine that is responsible for PLCγ binding (Mohammadi *et al.*, 1991; Obermeier *et al.*, 1993).

It is interesting to note that tyrosine phosphorylated PLCγ translocates to the cytoskeleton upon stimulation in hepatocytes (Yang *et al.*, 1994), reminiscent of earlier reports of phosphoinositide kinase activities associated with the cytoskeleton (Payrastre *et al.*, 1991). It is not known which component of the cytoskeleton mediates this association but the SH3 domain of PLCγ is required (Bar-Sagi *et al.*, 1993). It is also unclear whether this translocation brings PLCγ into contact with its substrate or an unknown protein required for activation.

Non-receptor tyrosine kinases can also phosphorylate and activate PLCγ isoforms in response to the ligation of certain cell surface receptors (Section 1.2). The activated tyrosine kinases often phosphorylate the associated receptor such that PLCγ can then bind via its SH2 domains and become phosphorylated by the tyrosine kinase. PLCγ1 has been found to associate directly with Src and Syk *in vivo* and is phosphorylated by Src, Fyn, Lck, Lyn and Hck *in vitro* (Lee and Rhee, 1995; Noh *et al.*, 1995, for review).

Several lines of evidence suggest that PLCγ isoforms may not be regulated exclusively by protein tyrosine kinase-linked receptors. PLCγ1 is one of the few proteins which can co-precipitate with Giα subunits from EGF stimulated hepatocytes (Yang *et al.*, 1993) and the EGF-induced calcium response can be inhibited by microinjection of anti-Gα antibodies (Yang *et al.*, 1993). PLCγ1 can also be regulated through a pertussis toxin-sensitive pathway in rat hepatocytes (Johnson *et al.*, 1986) and renal epithelial cells (Teitelbaum *et al.*, 1990) but in other cell types treatment with the toxin had no effect (Liang and Garrison, 1992). Tyrosine phosphorylation of PLCγ has been observed in response to several GPCR agonists including AT-II, PAF and thrombin. Activation of both receptor and non-receptor tyrosine kinases by GPCRs has been demonstrated (for example see Daub *et al.*, 1996), however the mechanism of this activation is unclear.

There is also accumulating evidence for tyrosine kinase-independent pathways that activate PLCγ. PLCγ isoforms are stimulated by PtdOH (Jones and Carpenter, 1993) and by arachidonic acid in the presence of neuronal microtubule-associated protein tau *in vitro* (Hwang *et al.*, 1996). PtdOH and arachidonic acid are the products of PLD and PLA_2 activities, respectively, and both enzymes are coupled to various receptors which are known to signal through PLC. More recent data suggests that PI 3-kinase signalling may contribute to the activation of PLCγ isoforms. Firstly, the Tec family of non-receptor tyrosine kinases contain an N-terminal PtdIns(3,4,5)P_3-binding PH domain which is important in the PI 3-kinase dependent recruitment of these proteins to the plasma membrane (August *et al.*, 1997; Sections 1.34 and 1.5.4). Recruitment of Tec and Btk by PtdIns(3,4,5)P_3 to the plasma membrane is thought to lead to phosphorylation and activation of PLCγ (Fluckiger *et al.*, 1998; Scharenberg *et al.*, 1998). A second mechanism involves the recruitment of PLCγ isozymes by direct interaction of PtdIns(3,4,5)P_3 with their SH2 domains (Bae *et al.*, 1998; Rameh *et al.*, 1998). However, this has only been demonstrated *in vitro* with synthetic PtdIns(3,4,5)P_3 and PtdIns(3,4,5)P_3-dependent PLCγ signalling via the Tec kinases remains a more convincing pathway *in vivo* which may be responsible for the PI 3-kinase-dependent calcium mobilisation in HepG2 cells (Rameh *et al.*, 1998).

PI 3-kinase has also been implicated in the regulation of PLCγ2, an isoform expressed predominantly in haematopoietic cells, which is phosphorylated by non-receptor tyrosine kinases in response to B-cell receptor ligation (Carter *et al.*, 1991; Hempel *et al.*, 1992). In platelets, stimulation of PLCγ2 via the FcγRIIA receptor is dependent on the synthesis of PtdIns(3,4,5)P_3 (Gratacap *et al.*, 1998). While wortmannin treatment of platelets did not alter tyrosine phosphorylation of PLCγ2, it did however, abolish the Ca^{2+} response and the translocation of PLCγ2 to a Triton X-100-insoluble compartment upon FcγRIIA cross-linking (Gratacap *et al.*, 1998). Once again the involvement of PI 3-kinase suggests that PLCγ2 may be activated by a non-receptor tyrosine kinase (see above), this is likely to

follow recruitment to a signalling compartment at the plasma membrane in a similar way that PLCγ is recruited to RTKs. Alternatively, it may be recruited via its N-terminal PH domain which, in the case of PLCγ1 has been shown to bind PtdIns(3,4,5)P_3 (Falasca *et al.*, 1998). Whatever the mechanism of recruitment, phosphorylation of PLCγ is still necessary for full activation.

1.4.1 Genetic models of PLC function

The only known PLC genes of lower eukaryotes *S. cerevisiae* and *D. discoideum* are most similar to the mammalian PLCδ isoforms. Interestingly, *S. cerevisiae* and *D. discoideum* PLC null mutants are viable (Drayer *et al.*, 1994; Flick and Thorner, 1993) suggesting that the lower eukaryotic PLCs lie in redundant pathways. In contrast, mutation of the mouse PLCγ1 gene is lethal at around day E9 (Ji *et al.*, 1998). However, the fact that fibroblasts from these embryos respond normally to RTK activation (Ji *et al.*, 1998) suggests that some redundancy exists in PLC pathways of higher eukaryotes.

More recently, the PLCβ1 and PLCβ4 genes have been targeted in mice. Homozygous PLCβ4 null mutants are born normal but develop postnatal dwarfism. The mutant mice also show defective motor coordination and cerebellar development (Kim *et al.*, 1997). PLCβ1 mice are viable but are prone to epileptic seizures. Tissue from the hippocampus and temporal cortex shows a reduced Ins(1,4,5)P_3 response upon treatment with the muscarinic acetylcholine receptor (mAchR) agonist carbacol. Mutant mice also display a behavioural phenotype including defective spatial learning which, interestingly, can be induced with the mAchR antagonists scopolamine and psycostigmine (Kim *et al.*, 1997).

1.5 The Phosphoinositide Kinases

As a result of early studies, the phosphoinositide kinases of eukaryotes were originally classified as type I, type II, and type III according to their biochemical characteristics. Type I PtdIns-kinase was distributed between the soluble and particulate fractions, was inhibited by detergent and was found to be a heterodimeric complex of 110 and 85 kDa subunits (Carpenter *et al.*, 1990; Escobedo *et al.*, 1991; Morgan *et al.*, 1990). In contrast the type II activity was exclusively membrane-associated, was substantially activated by detergent, inhibited by calcium and adenosine, and was active as a 55 kDa monomer (Hou *et al.*, 1988; Jenkins *et al.*, 1991; Kanoh *et al.*, 1990; Porter *et al.*, 1988; Scholz *et al.*, 1991; Walker *et al.*, 1988). The type III PtdIns-kinase was found to have many properties in common with the type II, except that it was relatively insensitive to adenosine and had a molecular mass of between 80-230 kDa by gel filtration (Endemann *et al.*, 1987; Li *et al.*, 1989; Whitman *et al.*, 1987; Yamakawa and Takenawa, 1988).

The type I designation is now obsolete because these activities correspond to the extensively characterised PI 3-kinases. The type II and type III activities are PtdIns 4Ks

and this terminology remains, reflecting the fact that the first PtdIns 4Ks have only recently be characterised at the molecular level. The biochemical properties of the type II PtdIns 4K differs significantly from those of its type III counterparts and whether the type II PtdIns 4K isoform is structurally related to other PI kinases remains to be determined (see below). The high molecular mass type III activity is most similar to the 230 kDa PtdIns 4K which is related to the yeast PtdIns 4K STT4p. The lower molecular mass type III activity (Yamakawa and Takenawa, 1988) probably corresponds to PtdIns 4Kβ, the cloning and characterisation of which is described in this thesis (Chapter 6).

The cloned PI 3-kinases and PtdIns 4Ks are structurally related and share significant homology in their kinase domains. In contrast the PtdIns*P*K family (Section 1.5.2) forms a separate group of PI kinases with little homology to the PI 3/4-kinase family. Sequence analysis of the PI 3/4-kinases indicates two regions of homology termed LKH1 and LKH2 (for Lipid Kinase Homology; Section 1.5.1.b). These correspond to the catalytic core of the enzyme and the lipid kinase unique domain, respectively (Zvelebil *et al.*, 1996). In addition, several PtdIns 4Ks contain an additional region of homology (LKH3) which is unique to a subset of the PtdIns 4Ks (Figure 1.4b).

1.5.1 The PtdIns 4-kinases

1.5.1.a Type II PtdIns 4-kinase

The tightly membrane-bound, 55 kDa type II activity is of particular interest because it associates with activated receptors and has been implicated in many important cellular processes. However, this isoform has not been satisfactorily purified and currently remains uncloned.

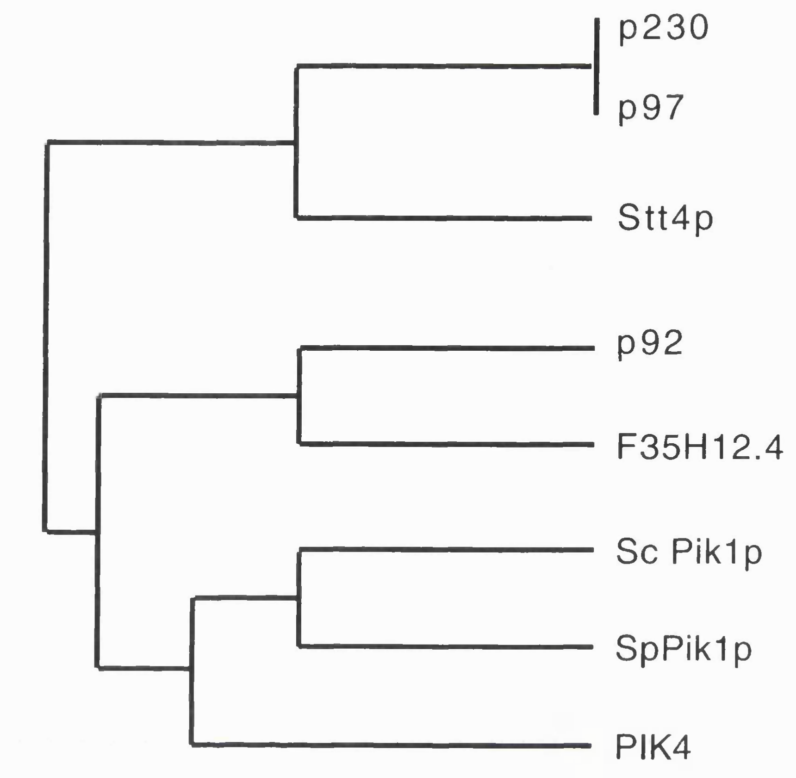
Type II PtdIns 4K activity has been found in stimulated receptor immunoprecipitates from EGF-stimulated A431 cells (Cochet *et al.*, 1991; Kauffmann-Zeh *et al.*, 1994). The site of interaction of the type II PtdIns 4K and an unknown PtdIns*P*K with the EGF receptor has been mapped to the juxtamembrane region by using a series of C-terminally truncated receptor mutants and synthetic peptides (Cochet *et al.*, 1991). The association with receptor mutants was EGF-dependent and did not require the receptor tyrosine kinase activity. The juxtamembrane region is well conserved between the other members of the EGFR family suggesting that these may share a common PtdIns(4,5)P_2 signalling mechanism. Indeed, type II PtdIns 4K can be immunoprecipitated from c-erbB2 overexpressing breast cancer cells (Scott *et al.*, 1991). The juxtamembrane region does not contain a tyrosine phosphorylation site and the mechanism of interaction of these activities

Figure 1.4 The PtdIns 4-kinase gene family

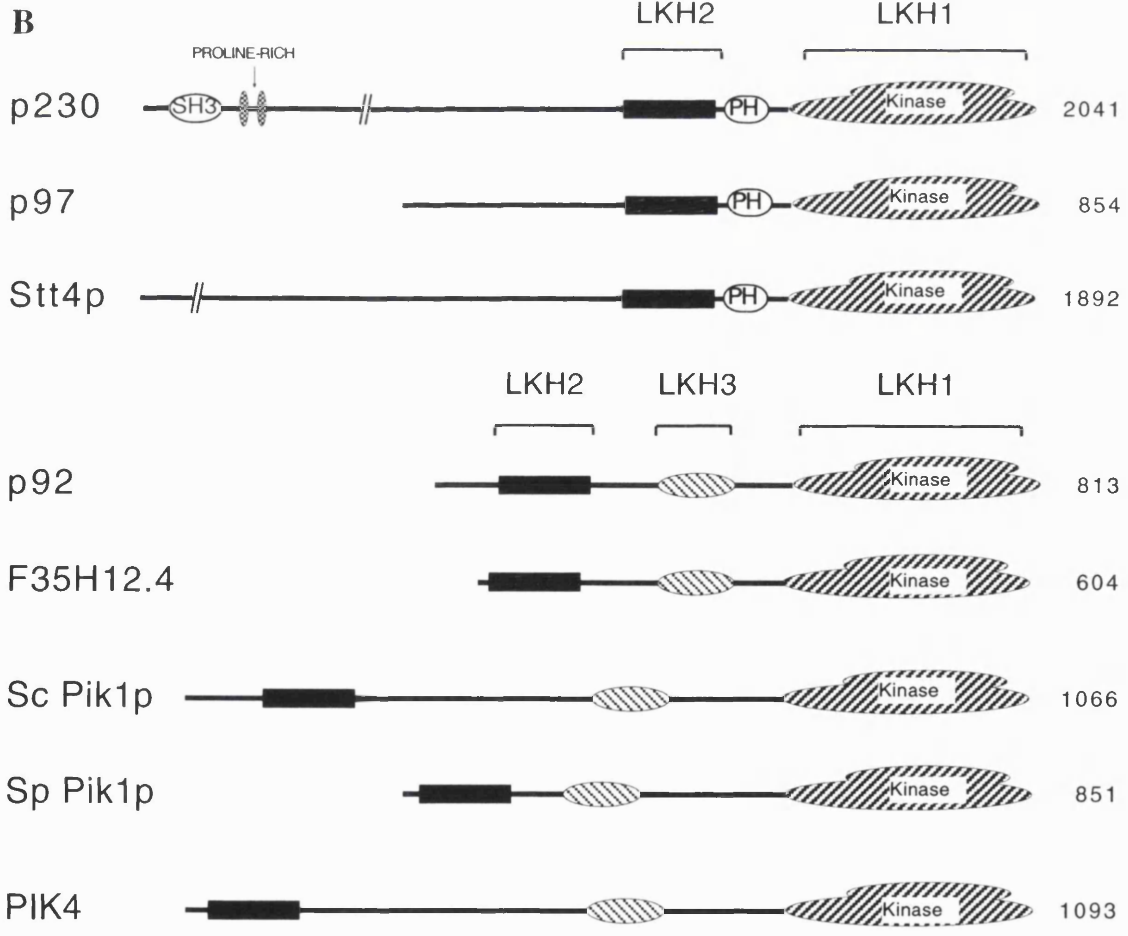
A. The degree of similarity between the kinase domains of the cloned PtdIns 4-kinases is shown as a dendrogram (for pairwise similarity scores see Table 8.3).

B. Schematic of PtdIns 4-kinases showing regions of homology, sites proposed for modular domain interactions, and the number of amino acids. LKH1, lipid kinase homology region 1 (kinase domain); LKH2, lipid kinase homology region 2 (also known as the lipid kinase unique region and found in the PI 3-kinases); LKH3, lipid kinase homology region 3 (a feature unique to th Pik1p-related PtdIns 4-kinases). An alignment of these homology domains is shown Figure 6.3). Acession numbers and references are as follows: p230, rat 230 kDa type III PtdIns 4-kinase (D83538; Nakagawa et al., 1996b); p97, 97 kDa human PtdIns 4-kinase (1172505; Wong and Cantley, 1994), Stt4p, *S. cerevisiae STT4* gene product (D13717; Yoshida et al., 1994); p92, 92 kDa rat PtdIns 4-kinase (1906794; Nakagawa et al., 1996a; Myers and Cantley, 1997; Chapter 6); F35H12.4, *C. elegans* putative PtdIns 4-kinase (U41540); Sc Pik1p, *S. cerevisiae* PIK1 gene product, (S39245; Flanagan *et al.*, 1993; Garcia Bustos *et al.*, 1994); Sp Pik1p, *S. pombe* Pik1 homologue (Z70043); PIK4, *D. discoideum* PtdIns 4-kinase gene product (2120376D; Zhou *et al.,* 1995a).

A



B



is unknown. However, PtdIns 4K and PtdIns*P*K activities can bind to the synthetic peptide in an EGF-independent manner (Cochet *et al.*, 1991), and the high proportion of basic residues in this region suggests the potential for non-specific interaction with phosphorylated proteins such as the type II PtdIns 4K (Kauffmann-Zeh *et al.*, 1994). It is also notable that the type II isoform is activated by positively charged peptides *in vitro* (reviewed by Pike, 1992).

PtdIns 4K activity can also be stimulated by cytokine receptors. Interleukin-1 treatment of fibroblast membranes caused a more than 10-fold increase in a PtdIns 4K activity (Ballou *et al.*, 1991). Furthermore, crosslinking of CD4 receptors on T cells, which signal through the associated non-receptor tyrosine kinase $p56^{lck}$, caused a 5-10-fold increase in CD4-associated PtdIns 4K activity. Interestingly, the C-terminal tail of CD4 contains a basic sequence similar to the juxtamembrane region of the EGF receptor family (Figure 1.5). There is also convincing evidence for the involvement of a type II PtdIns 4K in $\alpha_3\beta_1$ integrin signalling (Berditchevski *et al.*, 1997; Yauch *et al.*, 1998).

EGFR	669-	LFM**RRR**HIV**RKR**TL**RR**LLQ..
c-erbB2	678-	ILI**KRR**QQKI**RK**YTM**RR**LL..
c-erbB3	664-	LYW**R**G**R**RIQN**KR**AM**RR**YLE..
c-erbB4	695-	VYV**RRK**SIK**KKR**AL**RR**FLE..
CONSENSUS		ØØØ**RRR**.I.**KKR**AØ**RR**ØL.
CD4	423-	CRH**RRK**QAE**R**MMSQI**KR**LL..

Figure 1.5 Comparison of juxtamembrane sequences thought to bind PtdIns 4-kinases and PtdIns*P* kinases.

Conserved basic residues are shown in bold (Ø, hydrophobic residue and ".", gap introduced in order to optimise alignment).

The phosphorylation state of the EGF receptor appears to regulate both recruitment and activity of type II PtdIns 4K. Increased PtdIns 4K activity can be found in anti-phosphotyrosine and receptor immunoprecipitates from EGF-treated A431 cells (Payrastre *et al.*, 1990) and PtdIns 4K activity can be competitively eluted with phenylphosphate (Cochet *et al.*, 1991). Interestingly, treatment of imunoprecipitates with a nonspecific phosphatase reduced PtdIns 4K activity to levels similar to those seen with unstimulated cells (Kauffmann-Zeh *et al.*, 1994). Although a serine/threonine-specific phosphatase had a similar effect, a purified tyrosine phosphatase was able to stimulate activity three-fold (Kauffmann-Zeh *et al.*, 1994). However, these experiments were unable to distinguish direct effects of phosphorylation of PtdIns 4K from indirect effects possibly mediated by changes in the phosphorylation state of the EGF receptor on other proteins in the immunocomplex. Although association with the receptor was dependent on receptor autophosphorylation, tyrosine phosphorylation of PtdIns 4K actually inhibited activity.

The mechanism of type II PtdIns 4K regulation by the EGF receptor remains incompletely defined as does the identity of the serine/threonine kinase responsible for its activation.

Little data exist for the direct regulation of PtdIns 4K activity by heterotrimeric G-proteins, although two areas of study deserve mention. Firstly, some evidence suggests that a pool of PtdIns(4)*P* is sensitive to cholera toxin (which catalyses ADP-ribosylation and the subsequent inactivation of Gsα subunits). This surprising observation is of added interest because the toxin was able to specifically inhibit increases in the level of PtdIns(4)*P* in EGF-treated A431 cells (Pike and Eakes, 1987), indicating that a G-protein may be involved in EGFR signalling. The identity of the PtdIns 4K isoform involved in these studies has not been determined.

Secondly, a chromaffin granule-associated PtdIns 4K that may be involved in exocytosis has been found to lie down stream of the small GTPase Rho (also see Section 1.11.2). PtdIns 4K activity was also dependent on mastoparan, a peptide toxin from wasp venom which activates Gα proteins, indicating that the chromaffin granule-associated PtdIns 4K was also under the control of heterotrimeric G-proteins (Gasman *et al.*, 1998). Interestingly, the effect of mastoparan on chromaffin granule-associated and on MDCK cell membrane-bound PtdIns 4K have been noted previously (Eng and Lo, 1990; Husebye and Flatmark, 1988). However, because purified type II PtdIns 4K preparations displayed mastoparan-dependent activity it was suggested that the effect was not the result of Gα activation, but rather the well established stimulation of type II activity by cationic peptides (Pike, 1992). Nevertheless, the chromaffin granule-associated activity, which has properties consistent with a type II activity, may indeed be specifically stimulated by Gα since antibodies to the mastoparan interacting site of Goα were able to block the stimulation of PtdIns 4K activity (Gasman *et al.*, 1998). This observation supports the hypothesis that type II PtdIns 4K can be regulated by heterotrimeric G-proteins.

1.5.1.b *STT4*-related PtdIns 4-kinases

The membrane-associated, wortmannin-sensitive yeast PtdIns 4K STT4p was originally identified in a screen for mutants which conferred sensitivity to the antifungal compound staurosporine. Staurosporine is a selective inhibitor of PKC and another staurosporine-sensitive mutant, *stt1* was found to be allelic with the yeast PKC gene *PKC1* whose overexpression confers resistance to staurosporine (Yoshida *et al.*, 1992). Thus mutations in the yeast PKC pathway can confer resistance to staurosporine. Temperature-sensitive *stt4* mutants undergo growth arrest at the non-permissive temperature similar to the *pkc1* phenotype (Levin *et al.*, 1990). Null *stt4* mutants are conditionally lethal, requiring osmotic support in the form of high concentrations of sorbitol in the growth medium and show substantially reduced levels of cellular PtdIns(4)*P* (Yoshida *et al.*, 1994a). Resistance to staurosporine, and the fact that the *stt4* phenotype can be rescued by

overexpression of PKC1p, implicates STT4p in a PKC pathway (Yoshida *et al.*, 1994b). More recent work has investigated the genetic interaction between *STT4*, a yeast PtdIns*P*K *MSS4* (see Section 1.5.2.d), PKC1, and the yeast PLC, *PLC1* (Cutler *et al.*, 1997). Briefly, overexpression of *STT4* and *MSS4* conferred resistance to wortmannin, an inhibitor of STT4p. Deletion of *PLC1* also conferred wortmannin resistance, suggesting that STT4p and MSS4p lie in a linear pathway and that PtdIns(4,5)P_2 is an essential product of these enzymes. However, it is unlikely that STT4p is linked to PKC via PtdIns(4,5)P_2 hydrolysis, since *PLC1* overexpression does not confer wortmannin resistance and the yeast PKC1p is not activated by DAG *in vitro* (Kamada *et al.*, 1996). It is possible that the STT4p-MSS4p PtdIns(4,5)P_2 biosynthetic pathway activates PKC1p via an alternative pathway. A strong candidate is the ROM2p-RHO1p pathway, whose components form a Rho-type GTPase switch that controls PKC1p (Nonaka *et al.*, 1995; Ozaki *et al.*, 1996). PtdIns(4,5)P_2 generated through the STT4p-MSS4p route may activate the ROM2p-RHO1p switch via the ROM2 PH domain in a manner analogous to the mammalian PI-regulate exchange factors ARNO, cytohesin and Grp1 (Section 1.5.4.b). It should be noted however that the PH domain of ROM2p has not been characterised with respect to phospholipid binding specificity.

The first metazoan PtdIns 4K to be cloned was the 97 kDa PtdIns 4Kα, which has an identical domain organisation and displays a high degree of sequence identity (50% in the kinase domain) to STT4p (Figure 1.4b). The 230 kDa rat and bovine brain PtdIns 4Ks have identical C-termini (Gehrmann *et al.*, 1996; Nakagawa *et al.*, 1996), indicating that PtdIns 4-Kα is probably a splice variant of the larger type III enzyme. It should also be noted that no purification studies have confirmed the existence of the 97 kDa PtdIns 4-Kα isoform. The type III PtdIns 4Ks all contain a PH domain positioned between the LKH2 (lipid kinase unique) region and the C-terminal catalytic domain (Figure 1.4b). The PH domain of a plant homologue has recently been shown to bind PtdIns(4)*P* *in vitro* (Stevenson *et al.*, 1998), and although the function of this is unclear, its position close to the kinase domain suggests a regulatory function. In addition to the PH domain, the mammalian p230 isoforms have a predicted N-terminal SH3 domain and a proline rich region (Nakagawa *et al.*, 1996). Like the PtdIns 4Kβ isozyme (see Section 1.5.1.c, below), the STT4p-related PtdIns 4Ks are all inhibited, to various degrees, by wortmannin *in vitro* (Balla et al., 1997; Cutler *et al.*, 1997; Woscholski *et al.*, 1994).

1.5.1.c *Pik1*-related PtdIns 4-kinases

S. cerevisiae Pik1 was purified as a soluble, 125 kDa PtdIns 4K (Flanagan and Thorner, 1992) and was the first PtdIns 4K to be cloned (Flanagan *et al.*, 1993). The function of Pik1p has not yet been elucidated but null mutation is lethal. Pik1p has also been isolated as a component of the nuclear pore complex (Garcia-Bustos *et al.*, 1994), a finding at

variance with the work described above. More recently, direct immunofluorescence has localised Pik1p exclusively to the cytosol (Thorner, 1996) and it is possible that differences in the preparation of yeast cell fractions accounts for the discrepancy.

As shown in Figure 1.4, the PtdIns 4K family can be divided into two groups on the basis of primary structure homology within the C-terminal kinase domain. In addition, the domain organisation of these two groups (referred to here as STT4-like and Pik1-like) are significantly different. The Pik1-related PtdIns 4Ks lack a PH domain but contain a region of homology (LKH3, for lipid kinase homology region 3) that is unique to this group. Also, in contrast to the STT4p-related proteins, the LKH2 region of the Pik1p-related PtdIns 4Ks is positioned close to their N-termini. The functional significance of these differences is not known but they may represent a dichotomy similar to that seen with the type I and type II PtdIns*P*Ks, or the subdivisions of PI 3-kinases, whose members have characteristic substrate specificities and modes of regulation (Sections 1.5.2 and 1.5.3). This is supported by the finding that *STT4* overexpression cannot compensate *PIK1* function and vice versa (Cutler *et al.*, 1997).

The function of the cloned PtdIns 4K isoforms is currently unclear, but they appear to localise to distinct subcellular membranes. p230 PtdIns 4K localises to the endoplasmic reticulum (Nakagawa *et al.*, 1996; Wong *et al.*, 1997) whereas PtdIns 4Kβ localises to the Golgi (Wong *et al.*, 1997). A wortmannin-sensitive pool of PtdIns(4)*P* and PtdIns(4,5)P_2 has been described which is metabolised in response to both G-protein and RTK agonists (Nakanishi *et al.*, 1995), but the finding that all cloned mammalian PtdIns 4Ks can be inhibited by PI 3-kinase inhibitors precludes the determination of which PtdIns 4K isoform is responsible for this pool. The type II PtdIns 4K is not inhibited by wortmannin (Nakanishi *et al.*, 1995; M. Waugh, unpublished data) and can therefore be excluded.

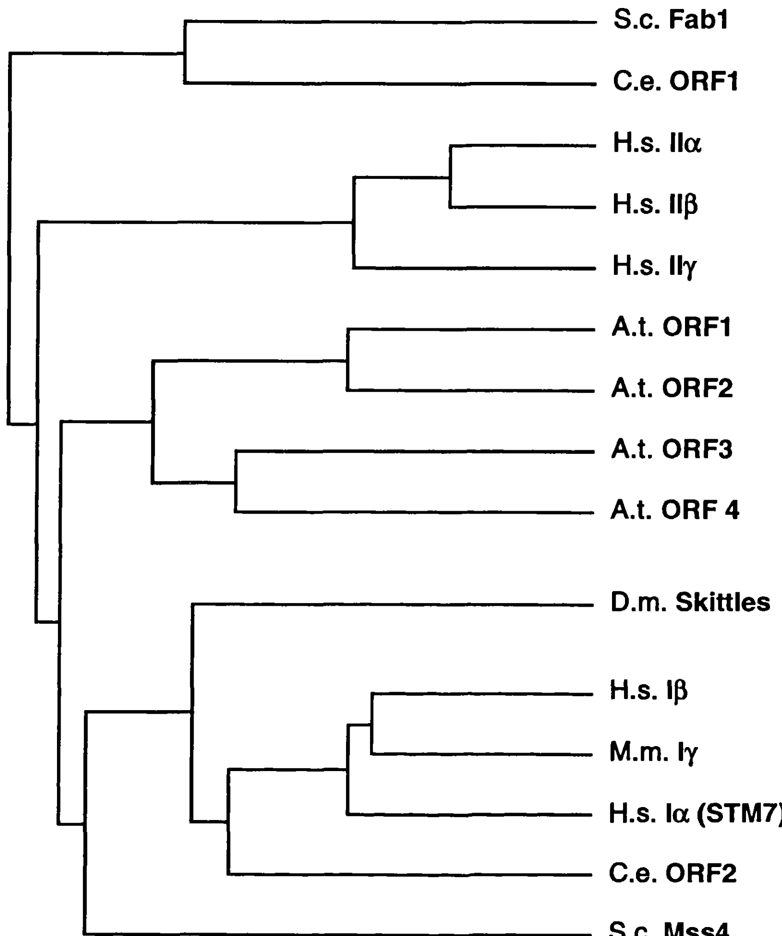
1.5.2 PtdIns*P* kinases

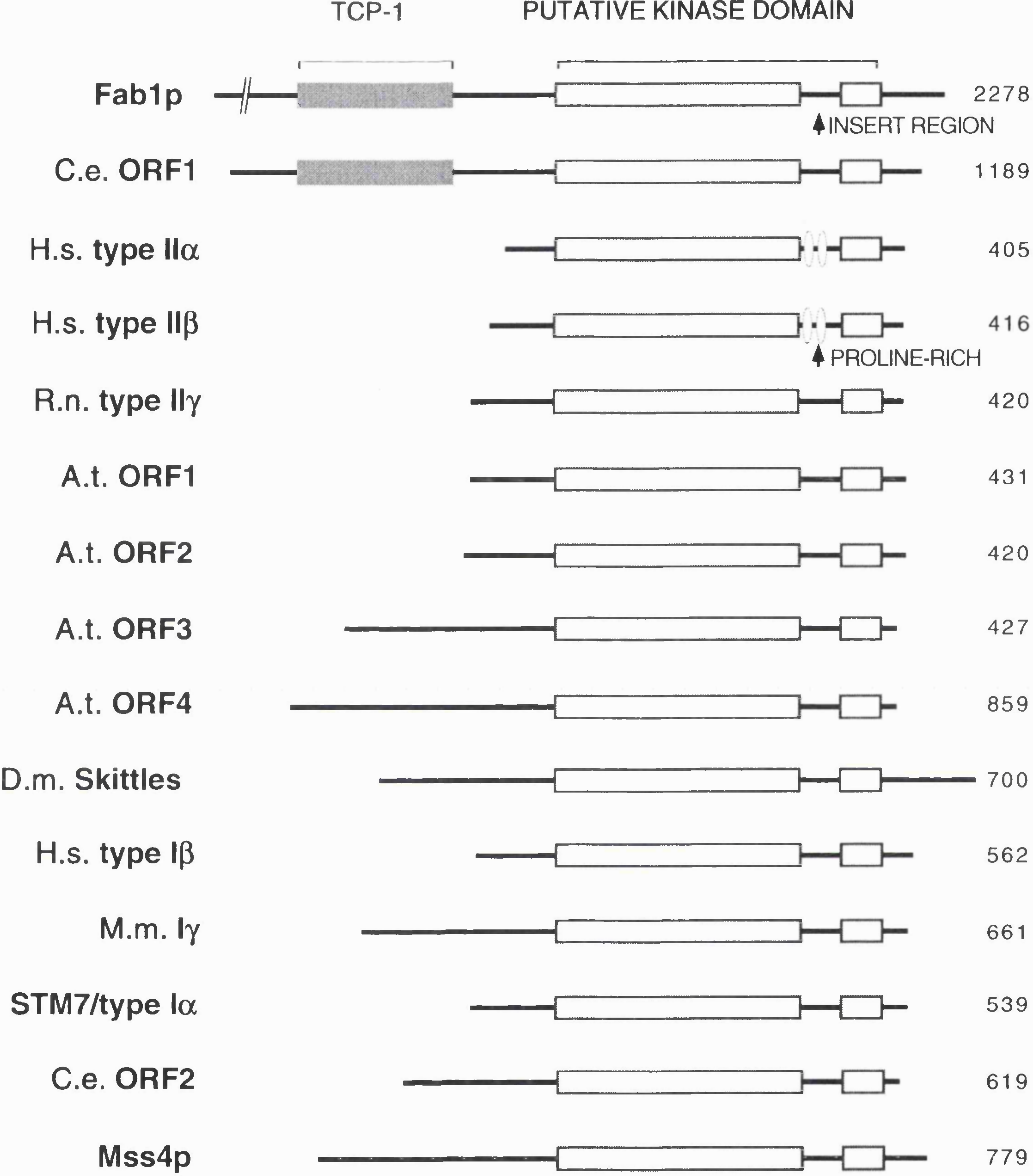
The mammalian PtdIns*P*Ks (sometimes referred to as PIPkins) were previously determined to be a family of at least three immunologically and chromatographically distinct isoforms with approximate molecular masses of 53 kDa, 68 kDa, and 98 kDa, which catalysed the formation of PtdIns(4,5)P_2 from PtdIns(4)*P* (Bazenet *et al.*, 1990; Jenkins *et al.*, 1994). The PtdIns*P*Ks were classified as type I or type II based on their biochemical properties (described in more detail in Chapter 3). Evidence in the literature rapidly accumlated indicating that the type I and type II isoforms had profoundly different properties. Most notable was the finding that type I enzymes were active towards membrane PtdIns(4)*P* substrate and were activated by phosphatidic acid, whereas the type II isoform was not active toward membranes and was not stimulated by phosphatidic acid (Bazenet *et al.*, 1990; Jenkins *et al.*, 1994; Moritz *et al.*, 1992).

Figure 1.6 The PtdIns*P*K family

A. Similar to 1.5a, a dendrogram is shown of cloned PtdIns*P*K family members' catalytic domains.

B. Schematic similar to Figure 1.5b, showing PtdIns*P*K homology regions and proposed molecular interaction domains. The size of each PtdIns*P*K is given in amino acids. Acession numbers/references: *S.c.* Fab1, *S. cerevisiae FAB1* gene product (U01017; Yammamoto *et al.*, 1995); *C.e.* ORF1, *C. elegans* Fab1p homologue (Z67879); *H.s.* IIα, (U14957; Borononkov and Anderson, 1995; Divecha *et al.*, 1995; Chapter 3), *H.s.* IIβ, human PtdIns*P*K IIβ (U85245; Castellino *et al.,* 1997), *H.s.* IIγ, human PtdIns*P*K IIγ (Itoh *et al.*, 1998); *A.t.* ORF 1, *A. thalina* putative PtdIns*P*K (AF007269); *A.t.* ORF 2, *A. thalina* putative PtdIns*P*K (AF019380); *A.t.* ORF 3, *A. thalina* putative PtdIns*P*K (U95973); *A.t.* ORF 4, *A. thalina* putative PtdIns*P*K (Y12776); *D.m.* skittles, *D. melanogaster skittles* gene product (U73490); *H.s.* Iβ, human PtdIns*P*K Iβ, (Ishihara *et al.*, 1996); *M.m.* Iγ, murine PtdIns*P*K Iγ (Shibasaki *et al.*, 1998); *H.s.* Iα (STM7), human PtdIns*P*K Iα (Carvajal *et al.*, 1995), *C.e.* ORF2, *C. elegans* putative PtdIns*P*K (AF003130); *S.c.* Mss4, *S. cerevisiae MSS4* gene product (D13716; Yoshida *et al.*, 1994). TCP-1, chaperonin TCP-1 homology domain.


S.c. Fab1
C.e. ORF1
H.s. IIα
H.s. IIβ
H.s. IIγ
A.t. ORF1
A.t. ORF2
A.t. ORF3
A.t. ORF 4
D.m. Skittles
H.s. Iβ
M.m. Iγ
H.s. Iα (STM7)
C.e. ORF2
S.c. Mss4


TCP-1
PUTATIVE KINASE DOMAIN
Fab1p
2278
INSERT REGION
C.e. ORF1
1189
H.s. type IIα
405
H.s. type IIβ
416
PROLINE-RICH
R.n. type IIγ
420
A.t. ORF1
431
A.t. ORF2
420
A.t. ORF3
427
A.t. ORF4
859
D.m. Skittles
700
H.s. type Iβ
562
M.m. Iγ
661
STM7/type Iα
539
C.e. ORF2
619
Mss4p
779

Important work has recently provided an explanation for the differences in type I and type II activities: investigations of *in vitro* substrate specificity using recombinant proteins and synthetic substrates has revealed that type II enzymes can phosphorylate PtdIns(5)*P* at the D-4 position to generate PtdIns(4,5)P_2. The long-held belief that all PtdIns*P*Ks transferred phosphate exclusively to the D-5 position of the inositol headgroup was based on the assumption that the PtdIns(4,5)P_2 product could only be generated by this reaction. It now appears that commercial preparations of PtdIns(4)*P* contain small amounts of the PtdIns(5)*P* isomer, a species which is presumably not found in erythrocyte membranes (Bazenet *et al.*, 1990). Interestingly, type IIα PtdIns*P*K can also catalyse the formation of PtdIns(3,4)P_2 from PtdIns(3)*P*, although with lower activity (Rameh *et al.*, 1997b). The type I PtdIns*P*Ks are *bona fide* PtdIns(4)*P* 5-kinases but can also convert PtdIns(3,4)P_2 to PtdIns(3,4,5)P_3, catalyse the phosphorylation of PtdIns at the D-5 position, and PtdIns(3)*P* at both the D-4 and D-5 position to generate PtdIns(3,4,5)P_3 (Rameh *et al.*, 1997b; Zhang *et al.*, 1997). For both isoforms the formation of PtdIns(4,5)P_2 is the most kinetically favoured reaction *in vitro* and, although the promiscuous substrate specificities of the type I isoforms are intriguing, the physiological relavence of these reactions is unclear at present.

1.5.2.a Regulation of PtdIns*P* kinases

Little is known about the regulation of PtdIns*P*K activity in intact cells. PtdIns(4,5)P_2 is produced is response to both G-protein and RTK agonists (Stephens *et al.*, 1993; Urumow and Wieland, 1988) and PtdIns(4,5)P_2 levels may be regulated by the small GTPase Rho in an integrin-mediated pathway (Chong *et al.*, 1994). Also, as a consequence of recent studies of the *in vitro* substrate specificities of the PtdIns*P*Ks, pathways which produce other PI species such as PtdIns(3,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (produced from PtdIns(3)*P*) must now be considered potential products of the PtdIns*P*Ks (Rameh *et al.*, 1997b; Zhang *et al.*, 1997).

1.5.2.b Type I PtdIns*P* kinase function

The regulation of PtdIns*P*K activity by small G-proteins has been of particular interest to those working on signalling to the actin cytoskeleton because of the long-held hypothesis that PtdIns(4,5)P_2 has profound and direct effects on cytoskeletal structures (Fukami *et al.*, 1992; Janmey *et al.*, 1987; Janmey and Stossel, 1987; Lassing and Lindberg, 1985). GTP-RhoA has been shown to stimulate PtdIns*P*K activity in fibroblast lysates (Chong *et al.*, 1994) and Rac1 has been shown to stimulate PtdIns(4,5)P_2 biosynthesis in permeabilised platelets (Hartwig *et al.*, 1995) leading to actin filament growth. Both Rac and Rho have been shown to form complexes with PtdIns*P*K activity (Ren *et al.*, 1996; Tolias *et al.*, 1995), but in contrast to the results shown in Figure 1.7, neither of these

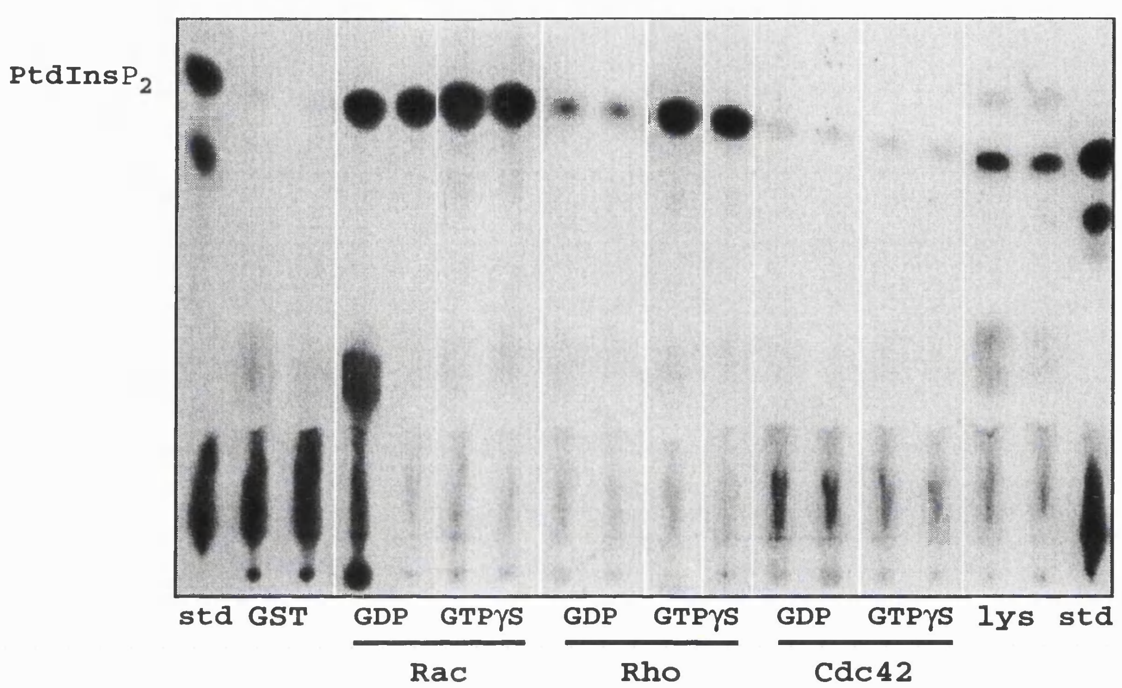


Figure 1.7 GTP-dependent physical interaction of PtdIns*P*kinase activity with Rac1 and RhoA

Triton X-100 lysates of Swiss 3T3 cells were prepared and incubated with GDP- or GTP[γ]S-bound GST fusion proteins and assayed in duplicate for PtdIns*P*K activity as indicated. Lanes: std; [^{32}P]-PtdIns(4,5)P_2 standard. GST; glutahione-S-transferase control. lys; 4 μl of Swiss 3T3 lysate. Results shown are typical of three such experiments. Full experimental details can be found in Section 2.5.2.f.

studies has shown GTP-dependence. There is currently no evidence for a direct interaction between PtdIns*P*K and Rac and Rho and no PtdIns*P*K has been isolated as the result of two-hybrid screening (a technique which has yielded numerous small G-protein targets). The binding of PtdIns*P*K activity to Rac1 may require the Rac1 C-terminal basic region (Tolias *et al.*, 1998). Furthemore, phosphatidic acid activation of the activities bound to Rac1 has been used to demonstrate that the PtdIns*P*Ks involved are type I in character, but exactly which isoforms are responsible remains to be determined.

A fragment of PtdIns*P*K Iβ has been isolated using a genetic screen where it was found to restore mitogenic signalling to a cell line expressing a mutant CSF-1 receptor with impaired tyrosine kinase activity. The catalytically inactive fragment was found to complement signalling by preventing receptor internalisation, thus PtdIns*P*K Iβ may have a role in receptor down regulation (Davis *et al.*, 1997). Although this study did not address how PtdIns*P*K Iβ is involved in this process, it seems likely that the inactive PtdIns*P*K Iβ prevents endocytosis by inhibiting vesicle formation; a process in which the PtdOH-activated type I enzymes have been repeatedly implicated (see Section 1.11).

At least one direct link between lipid kinases and protein kinases has been demonstrated with the recent finding that PKCμ (also known as PKD) interacts with a type II PtdIns 4K and a type I PtdIns*P*K (Nishikawa *et al.*, 1998). PKCμ is a phorbol ester and DAG-stimulated protein serine/threonine kinase which differs from the PKCs in that it contains a putative N-terminal transmembrane domain and a PH domain. Although PKCμ has been included in the PKC family, the catalytic domain and several biochemical properties are more consistent with it being related to the CaMKII-like protein kinases (reviewed in (Mellor and Parker, 1998). Association of PtdIns 4K and PtdIns(4)*P* 5-kinase requires a region between residues 79-340, C-terminal to the PH domain and it is not known whether the interaction is direct or which PtdIns*P*K isoform is involved (Nishikawa *et al.*, 1998). The significance of this complex is unclear: PKCμ is activated by GPCRs and RTKs via conventional and novel PKC isoforms (Sidorenko *et al.*, 1996; Zugaza *et al.*, 1997), but no direct evidence for regulation by phosphoinositides has been reported.

1.5.2.c Type II PtdIns*P* kinase function

PtdIns*P*K IIα translocates to the cytoskeletal fraction of thrombin stimulated platelets and is accompanied by an increase in PtdIns(4,5)P_2 associated with the Triton X-100 insoluble fraction (Hinchliffe *et al.*, 1996). Thrombin stimulation causes the secretion of fibrinogen (a ligand for the integrin $\alpha_{2b}\beta_3$) and stirring thrombin-stimulated platelets promotes collision which causes their rapid aggregation. Because PtdIns*P*K IIα translocation was dependent on stirring and could be inhibited by an antagonist of $\alpha_{2b}\beta_3$, regulation of PtdIns*P*K IIα by an integrin signalling pathway is clearly implicated. This work represents the first suggested role for a type II PtdIns*P*K but the exact function of the

cytoskeletal PtdIns(4,5)P_2 is still unclear. Futhermore, it is not known whether other PtdIns*P*Ks such as the type I isoforms contribute to the PtdIns(4,5)P_2 associated with the cytoskeleton. Agonist-stimulated increases in cytoskeletal PtdIns*P*K activity have been previously documented in platelets (Grondin *et al.*, 1991) and in A431 cells (Payrastre *et al.*, 1991). An integrin-mediated pathway that converts PtdIns(3)*P* to PtdIns(3,4)P_2 has been described (Banfic *et al.*, 1998b; Sultan *et al.*, 1991). PtdIns*P*K IIα is currently the only cloned activity known to catalyse this conversion (note that Zhang *et al.* (1997) have shown that type I PtdIns*P*K can catalyse this reaction but this is not bourne out by the work of Tolias *et al.* (1998). However, recent evidence suggests that PtdIns*P*K IIα is not responsible for PtdIns(3,4)P_2 generation in platelets (Banfic *et al.*, 1998a) and that the this may be due a distinct activity such as the PtdIns(3)*P* 4-kinase described previously in erythrocytes (Graziani *et al.*, 1992) and in platelets (Yamamoto *et al.*, 1990).

The type II PtdIns*P*K undergoes a transient dephosphorylation of multiple serine and threonine residues in thrombin-stimulated platelets (Hinchliffe *et al.*, 1998). Dephosphorylation was accompanied by increased PtdIns*P*K activity, while treatment with the phosphatase inhibitor okadaic acid markedly inhibited activity. The activity of anti-PtdInsPK IIα immunoprecipitates from okadaic acid-treated platelets could only be partially restored by treatment with alkaline phosphatase. The correlation between phosphorylation and activity is interesting but the protein serine/threonine kinases involved have not been identified (Hinchliffe *et al.*, 1998). It is also not clear which type II PtdIns*P*K was immunoprecipitated by the monoclonal antibody used in this study (see (Brooksbank *et al.*, 1993). The specificity of this reagent towards platelet lysates has recently been questioned (Banfic *et al.*, 1998a) and furthermore, the closely related PtdIns*P*K IIγ isoform is phosphorylated in response to a variety of agonists (Itoh *et al.*, 1998, see below).

The PtdIns*P*K IIβ isoform was isolated by virtue of its ability to interact with the juxtamembrane domain of the p55 TNF receptor in a two hybrid screen. The endogenous protein co-immunoprecipitated with the p55 TNF receptor and PtdIns*P*K activity associated with the immune complex increased between two- to three-fold upon stimulation with TNFα (Castellino *et al.*, 1997). It is not known whether the associated PtdIns*P*K IIβ is directly involved in mitogenic signalling through PI 3-kinase or PLC or whether it plays a role in receptor internalisation as suggested for PtdIns*P*K Iβ.

A third type II isoform, the recently cloned rat PtdIns*P*K IIγ protein, localises to the endoplasmic reticulum and is phosphorylated on serine residues in response to EGF, PDGF, bradykinnin and lysophosphatidic acid. The function of this phosphorylation event is not clear since it does not lead to changes in PtdIns*P*K activity. Phosphorylation is reminiscent of results previously ascribed to PtdInsPK IIα (Hinchliffe *et al.*, 1998; see above), which may have been caused by immunological cross reaction.

1.5.2.d Yeast PtdIns*P* kinases

Two PtdIns*P*K homologues can be identified in the *S. cerevisiae* genome, Mss4p and Fab1p. The *fab1* phenotype is characterised by acutely enlarged vacuoles and the accumulation of aploid and binucleate cells in populations of mutants resulting from abnormal chromosomal segregation and spindle morphology (Yamamoto *et al.*, 1995). The yeast vacuole is a highly dynamic structure especially with regard to biochemical processes involving membrane traffic and it has been proposed that Fab1p lies in a vacuolar PI pathway (Yamamoto *et al.*, 1995). Interestingly, mutants of the *S. cerevisiae PLC1* gene, encoding a phospholipase C, also display chromosomal segregation defects (Flick and Thorner, 1993; Payne and Fitzgerald Hayes, 1993; Yoko o *et al.*, 1993) and it is tempting to speculate that Fab1p lies upstream of *PLC1*. However, *PLC1* mutants do not display the primary *fab1* phenotype of enlarged vacuoles which probably causes the nuclear abnormalities indirectly by physical interference with spindle structures. It is likely therefore, that the chromosomal defects attributed to *plc1* mutants have a different and currently unknown basis and that Fab1p lies in a different pathway. This is supported by the recent finding that Fab1p catalyses the synthesis of PtdIns(3,5)P_2 from PtdIns(3)*P* *in vitro* but has no activity against PtdIns(4)*P* (Cooke et al, 1998). *S. cerevisiae* contains low levels of PtdIns(3,5)P_2, but hyperosmotic shock promotes a rapid increase in this lipid (Dove *et al.*, 1997). *fab1* null mutants are unable to synthesis PtdIns(3,5)P_2, but PtdIns(4,5)P_2 levels are not effected (Cooke et al, 1998). PtdIns(3,5)P_2 synthesis in yeast requires a functional *VPS34* gene, indicating that this PtdIns 3-kinase catalyses the first step of this novel pathway. However, *vps34* and *fab1* display different phenotypes: in contrast to the abnormal vacuolar morphology of *fab1* mutants, *VPS34* disruption interferes with protein transport from the Golgi to the vacuole. It is therefore possible that PtdIns(3)*P* and PtdIns(3,5)P_2 have independent functions.

PtdIns(3,5)P_2 and PtdIns(3)*P*, the product of the mammalian Vps34 homologue, are known to exist in mammalian cells (Whiteford *et al.*, 1997; Dove *et al.*, 1997). However, while a Fab1p homologue is present in *C. elegans* (Figure 1.6), no equivalent is known in mammals. Consequently, the pathway responsible for the generation of PtdIns(3,5)P_2 in mammalian systems consequently remains unclear.

Mss4 mutants were identified by virtue of their ability to partially suppress the staurosporine-sensitive *stt4* mutant phenotype when overexpressed. *STT4* is an essential yeast PtdIns 4K which has been implicated in the PKC pathway (Section 1.5.1.b) and the property of partial suppression by the Mss4p PtdIns*P*K may be due to restoration of 5'-phosphorylated phosphoinositide levels. Although the catalytic domain of Mss4p is more similar to the type II enzymes than the type I subfamily (Figure 1.6), Mss4p has been found to have many properties in common with the mammalian type I enzymes; (i) they both localise to plasma membranes, (ii) they are stimulated by PtdOH *in vitro*, (iii)

PtdIns(4)*P* is the preferred substrate *in vitro*, (iv) both affect actin structures *in vivo*, and (v) overexpression of murine PtdIns*P*K Iβ can rescue temperature-sensitive *MSS4* mutants (Desrivieres *et al.*, 1998; Homma *et al.*, 1998). These data indicate that *MSS4* is a functional homologue of the type I subfamily of PtdIns(4)*P* 5-kinases (Desrivieres *et al.*, 1998; Homma *et al.*, 1998). Type II PtdIns(5)*P* 4-kinase activities are not known in lower eukaryotes and the absence of a type II isozyme in yeast suggests it is doubtful that the PtdIns(5)*P* —> PtdIns(4,5)P_2 pathway exists in yeast.

Although yeast genetics provides a powerful system for the study of PI pathways relating to vesicle traffic and cytoskeletal regulation, this organism lacks certain important signalling pathways of higher eukaryotes, most notably the receptor-dependent PI 3-kinase and PLCβ/γ pathways. This is likely to be due to the complex signalling machinery required in the multicellular organism. The *D. melanogaster* and *C. elegans* genomes have recently been found to contain putative PtdIns*P*Ks which may prove to be useful genetic models for the dissection of PtdIns*P*K function in metazoan organisms.

1.5.3 Phosphoinositide 3-kinases

Evidence for the existence of biosynthetic pathways involving PtdIns phosphorylation at the D-3 position was initially provided by the discovery that PtdIns 3-kinase activity co-precipitated with various PTKs including activated PDGFβ (Whitman *et al.*, 1985), v-Src and the middle-T antigen from polyoma virus-infected cells (Courtneidge and Heber, 1987). Soon after it was found that a novel lipid, PtdIns(3,4,5)P_3, was rapidly synthesised on activation of neutrophils (Traynor Kaplan *et al.*, 1989). This work led to the purification and subsequent cloning of the 110 kDa catalytic subunit of bovine PI 3-kinase (Hiles *et al.*, 1992; Morgan *et al.*, 1990), the first mammalian member of an enzyme family that has expanded rapidly in recent years and has been the subject of intensive study. The characterisation of the PI 3-kinases has established diverse cellular roles including regulation of cell survival (Franke *et al.*, 1997), transformation (Chang *et al.*, 1997), and membrane traffic (reviewed by Shepherd *et al.*, 1996). This Section will not attempt to give a comprehensive review of the PI 3-kinase literature but instead a summary of PI 3-kinase structure and function (for review see Toker and Cantley, 1997; Fruhman et al, 1998).

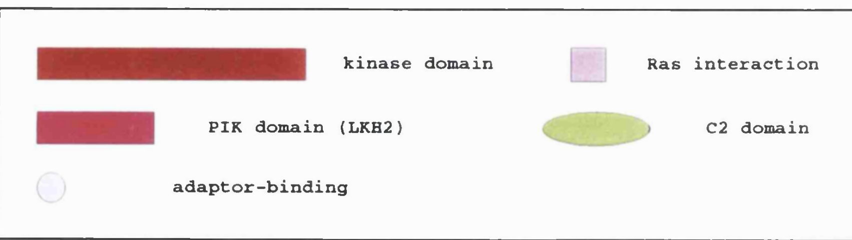
1.5.3.a The PI 3-kinase gene family

The PI 3-kinases have been classified according to their structure, lipid substrate specificities, and their mode of regulation (Domin and Waterfield, 1997). These are summarised in Figure 1.8.

Figure 1.8 The PI 3-kinase family

Summarised properties of the PI 3-kinases (see Section 1.5.3 for details). PI's known to act as substrates *in vitro* are indicated. Definitions are as follows: p110α, β, and δ, all mammalian 110 kDa class Ia PI 3Ks (see 1.5.3.a for relevant references); AGE-1 *C. elegans* class Ia PI 3K (Morris *et al.*, 1996); PIK1, PIK2, PIK3, *D. discoideum* class Ia and class Ib PI 3Ks (Zhou et al., 1995a); p110γ, mammalian GPCR-regulated PI 3K; Cpk-m/p170, murine (Molz *et al.*, 1996; Virbasius *et al.*, 1996); PI3K-C2α, β, and γ human class II PI 3K isoforms (Domin *et al.*, 1997; Brown *et al.*, 1997; Ono *et al.*, 1998); 68D, *D. melanogaster* class II PI 3K (MacDougal *et al.*, 1995); Vps34, *S. cerevisiae VPS34* gene product (Shu *et al.*, 1993); hVps34, human Vps34p homologue (Volinia *et al.*, 1995).

Class	Structure	Isoform	Adaptor	Regulation
I_A	PtdIns, PtdIns(4)*P*, PtdIns(4,5)P_2	p110α,β,δ Dp110 AGE-1 PIK1,2	p85α,β p55α,γ p50α p60	Tyrosine kinases Ras
I_B		p110γ PIK3	p101 p120	G-protein coupled receptors Ras
II	PtdIns, PtdIns(4)*P* proline-rich	Cpk-m/p170 PI3K-C2 α,β,γ 68D	unknown	unknown
III	PtdIns	Vps34 hVps34	Vps15 p150	unknown



The class I enzymes can phosphorylate PtdIns, PtdIns(4)*P*, PtdIns(5)*P* and PtdIns(4,5)P_2 *in vitro* (Serunian *et al.*, 1989). Agonist stimulation of these enzymes *in vivo* increases cellular levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, although PtdIns(3,4)P_2 can be generated by PtdIns(3)*P* 4-kinase activity (Banfic *et al.*, 1998b), or by PtdIns(3,4,5)P_3 (5)-phosphatase activity (Stephens *et al.*, 1991), both of which may contribute to this response. Four class I enzymes have been characterised in mammalian systems, p110α, p110β, p110γ and p110δ (Hiles *et al.*, 1992; Hu *et al.*, 1993; Stoyanov *et al.*, 1995; Vanhaesebroeck *et al.*, 1997). p110-related genes have also been cloned from a range of eukaryotes including *C. elegans*, *D. discoideum* and *D. melanogaster* (Leevers *et al.*, 1996; Morris *et al.*, 1996; Zhou *et al.*, 1995a). All members of this class form heterodimeric complexes with regulatory subunits that couple these enzymes to upstream signalling systems (reviewed in Fruhman, 1998). PI 3-kinases 110α, -β, and -δ (class Ia) are able to interact with the p85 family of adaptor subunits, p85α, p50α, p55α, p85β, and p85γ which arise from three distinct genes (Antonetti *et al.*, 1996; Otsu *et al.*, 1991; Pons *et al.*, 1995). p110γ (class I_B) interacts with an unrelated regulatory sunbunit, p101 (Stephens *et al.*, 1997). The p85 family lack intrinsic enzyme activity but contain SH2, SH3 and proline-rich domains which have the capacity to interact with multiple proteins. It has not been shown that different Class I PI 3-kinases have any preference for individual adaptor subunits (Vanhaesebroeck *et al.*, 1997), however, different p85 isoforms may interact with distinct sets of intracellular proteins (Baltensperger *et al.*, 1994; Reif *et al.*, 1993; Shepherd *et al.*, 1996).

In the case of the class I_A enzymes, adaptor subunits regulate subcellular localisation and activity: many agonists stimulate the phosphorylation of tyrosine residues generating potential binding motifs for proteins containing SH2 domains. The SH2 domains of p85 have been studied in detail and the use of synthetic peptides has been used to determine the binding specificities of p85 SH2 domains. Both SH2 domains bind preferentially to peptides containing pYXXM motif and an additional methionine or valine C-terminal to the phosphotyrosine residue increases binding affinity (Sonyang *et al.*, 1993). This YXXM sequence can be found in many proteins that are known to activate heterodimeric PI 3-kinase for example, polyoma middle T antigen (Druker *et al.*, 1990; Ling *et al.*, 1992) and peptides corresponding to PDGF receptor autophosphorylation sites bind to p85 proteins with high affinity *in vitro*, and stimulate the activity of the associated catalytic subunits (Backer *et al.*, 1992; Carpenter *et al.*, 1993). Recruitment to activated RTKs brings PI 3-kinase to the plasma membrane and also activates the enzyme towards PtdIns(4,5)P_2. The importance of translocation is demonstrated by the observation that constitutively targeting the p110 subunit to the plasma membrane is sufficient to elevate levels of cellular PtdIns(3,4,5)P_3 (Klippel *et al.*, 1996). Ras interaction may also recruit heterodimeric PI 3-kinase to the membrane in a similar way to Raf (Leevers *et al.*, 1994) or alternatively membrane recruitment may bring PI 3-kinase to membrane-bound Ras.

p85 proteins contain a region of homology with Rho-GTPase activating protein (Rho-GAP). However p85 lacks Rho-, Rac-, and Cdc42-GAP activity *in vitro* (Ahmed *et al.*, 1994) and lacks several important residues known to be important for catalysis (Barrett *et al.*, 1997; Musacchio *et al.*, 1996; Rittinger *et al.*, 1997). On the other hand, the p85 Rho-GAP domain has been shown to bind Rac and Cdc42 in a GTP-dependent manner *in vitro* (Tolias *et al.*, 1995; Zheng *et al.*, 1994) and it is possible that Rac and/or Cdc42 regulates PI 3-kinase by localising the complex to an appropriate subcellular compartment. The importance of the regulatory subunit is illustrated by the recent finding that a 65 kDa mutant of p85α containing a portion of the Eph RTK constitutively activates p110 and contributes to transformation (Jimenez *et al.*, 1998).

All class Ia PI 3-kinases are regulated by the small GTPase Ras. p110α activity is stimulated by Ras both *in vitro* and *in vivo* and dominant negative Ras decreases cellular levels of D-3 phosphoinositides (Rodriguez Viciana *et al.*, 1994; Rodriguez Viciana *et al.*, 1997; Rodriguez Viciana *et al.*, 1996). In contrast, the p110γ isoform (class Ib) is unique in that it can be directly stimulated by Gβγ subunits *in vitro* (Stoyanov *et al.*, 1995). Furthermore, increases in PtdIns(3,4,5)P_3 levels upon stimulation with G-protein agonists are mediated by p110γ (Lopez Ilasaca *et al.*, 1997). Although a requirement for the p101 subunit has been shown for full activation (Stephens *et al.*, 1997), the function of this subunit is not fully understood. The predicted Ras-binding domain of p110γ has not been shown to interact with Ras and activation has not been reported.

The class I PI 3-kinase catalytic subunits all posess an intrinsic protein-serine/threonine kinase activity. This activity is responsible for autophosphorylation and trans-phosphorylation of the regulatory subunit, an event which inhibits PI kinase activity (Dhand *et al.*, 1994). Other than p85, only one exogenous protein substrate, the insulin receptor substrate (IRS-1), is known to exist (Freund *et al.*, 1995; Lam *et al.*, 1994). P110δ also has the capacity for autophosphorylation which, like p110α, negatively regulates activity (Vanhaesebroeck *et al.*, 1997). p110γ is also capable of autophosphorylation but no effect on activity has been reported (Stoyanova *et al.*, 1997).

Recently the question of the *in vivo* function of p110γ protein serine/threonine kinase activity has been addressed by using PI 3-kinase catalytic subunits that lack lipid kinase activity but retain protein kinase activity (Bondeva *et al.*, 1998). Chimeric p110γ constructs, containing a short portion of the human PIK-related kinase FRAP catalytic domain C-terminal to the Mg^{2+}-binding loop (the region predicted to determine peptide substrate binding in the protein kinases, see Chapter 4), were capable of autophosphorylation *in vitro* but were unable to cause an increase in cellular levels of D-3 phosphoinositides when constitutively targeted to the plasma membrane. When transfected into COS cells, the chimeric constructs were unable to activate protein kinase B (PKB), a downstream effector of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Section 1.5.4.a). However, transfectants were capable of a lysoPtdOH-stimulated MAPK response which was

inhibited in transfectants containing a catalytically inactive mutant. Furthermore, similar results were obtained in COS cells cotransfected with MEK1 and Erk2 together with the p110γ constructs, suggesting that the protein kinase activity of p110γ was responsible for the activation of this pathway. The finding that p110γ may transduce two distinct signals mediated by the products of it's lipid and protein kinase activities has important implications for PI 3-kinase signalling.

The class II enzymes are larger than the class I enzymes (>170 kDa) and contain a C-terminal C_2 domain (Section 1.3.5). The function of these C_2 domains has yet to be determined but a class II PI 3-kinase has been found to bind lipids in a Ca^{2+}-dependent manner (MacDougall *et al.*, 1995). The physiological role of the class II enzymes is unclear at present however, they are unable to phosphorylate PtdIns(4,5)P_2 and are therefore unlikely to lie in PtdIns(3,4,5)P_3 biosynthetic pathways. Instead, class II PI 3-kinases use PtdIns or PtdIns(4)*P* as substrate *in vitro*, thus class II activities may lie in a distinct pathway for the synthesis of PtdIns(3,4)P_2. PtdIns(3,4)P_2 is normally undetectable in unstimulated cells and it is likely that class II activities are acutely regulated. A role has been suggested for a class II enzyme in the formation of PtdIns(3,4)P_2 upon integrin activation in platelets (Zhang *et al.*, 1998). Interestingly, this reaction appears to procede by the phosphorylation of PtdIns by a class II PI 3-kinase followed by phosphorylation by a PtdIns(3)*P* 4-kinase (Section 1.5.4.a), suggesting that class II PI 3-kinases may act as PtdIns 3-kinases *in vivo*, indeed this is supported by the observation that PtdIns is the prefered substrate for this class of enzymes *in vitro* (J. Domin, personal communication).

Class III enzymes have a substrate specificity restricted to PtdIns and are homologues of the only known yeast PtdIns 3-kinase, Vps34p. The *VPS34* gene was isolated as a vacuolar protein sorting mutant in *S. cerevisiae* where it is essential for the transport of newly synthesised proteins from the Golgi to the vacuole (Schu *et al.*, 1993; Stack and Emr, 1994). Homologues of Vps34p have been identified in *Homo sapiens*, *D. melanogaster*, *Glycine Max*, *A. thalina*, and *D. discoideum* (Hong and Verma, 1994; Linassier *et al.*, 1997; Volinia *et al.*, 1995; Welters *et al.*, 1994; Zhou *et al.*, 1995a). Class III enzymes form heterodimeric complexes with Vps15 proteins which have intrinsic protein serine/threonine kinase activity (Stack and Emr, 1994; Volinia *et al.*, 1995). Levels of PtdIns(3)*P* change very little in stimulated cells and it is thought that mammalian PtdIns 3-kinase performs some role in constitutive membrane trafficking and vesicle biogenesis. In *S. cerevisiae*, Vps34p lies on a PtdIns(3,5)P_2 biosynthetic pathway that is activated by osmotic stress (Cooke *et al.*, 1998), although it is possible that PtdIns(3)*P* also has other functions (Section 1.5.4.d). PtdIns(3,5)P_2 is also present in mammalian cells (Whiteford *et al.*,1997; Dove *et al.*, 1997) but it is not known if this is generated by mammalian

Vps34. Interestingly, the yeast PtdIns(3)P 5-kinase Fab1p contains a PtdIns(3)*P*-binding motif which is also found in other proteins implicated in membrane traffic (Section 1.5.4.d).

1.5.3.b PIK-related kinases

A number of other proteins exists with substantial homology to the PI 3-kinase catalytic domain. Several proteins of between 270 and 600 kDa which in addition to a PI 3-kinase homology domain, are characterised by the presence of a C-terminal extension (Keith and Schreiber, 1995). None of the so-called PIK-related kinases have demonstrable PI kinase activity, however at least one member, DNA-PK, has protein kinase activity towards a variety of substrates (Hartley *et al.*, 1995) and other PIK-related kinases have the capacity for autophosphorylation (Brown *et al.*, 1995).

Members of this group have been implicated in important biological processes: ATM is mutated in ataxia telangietesia (Savitsky *et al.*, 1995), the TOR genes of yeast are involved in control of the cytoskeleton and the cell cycle (Thomas and Hall, 1997, for review) the mammalian TOR homologue RAFT is a target of the immunosuppressent drug rapamycin, and DNA-PK may have a role in DNA repair (for review see Jackson, 1997; Jeggo, 1998).

1.5.3.c Regulation of PI 3-kinases

The class I PI 3-kinases are primarily regulated by extracellular signals via tyrosine kinase and G-protein linked receptors. PI 3-kinase p110γ is activated by Gβγ subunits *in vitro* and is responsible for PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 responses to GPCR agonists (Lopez Ilasaca *et al.*, 1997). Activated RTKs autophosphorylate on key tyrosine residues (see above) promoting recruitment of the p110/p85 complex via SH2 domains of the adaptor subunit. PDGF receptors lacking these tyrosine residues fail to recruit PI 3-kinase and cells expressing mutant receptors are unable to respond to PDGF (Valius and Kazlauskas, 1993). This adaptor-mediated translocation of PI 3-kinase to the receptor complex activates PI 3-kinase activity and is thought to bring PI 3-kinase into close proximity with membrane bound substrate. Many RTKs appear to follow this model as do non-receptor tyrosine kinases, for example the HGF receptor (Ponzetto *et al.*, 1994), the CD4-p56^{lck} complex of T-cells (Prasad *et al.*, 1994), and CD28 (Pages *et al.*, 1994).

In addition, the catalytic subunits of the class I enzymes can all have the potential to interact directly with Ras proteins via the Ras binding domain (see Figure 1.8). Ras binding to p110γ has been demonstrated *in vitro* (Rubio *et al.*, 1997) but activation has not been shown. p110δ interacts with Ras *in vitro* (Vanhaesebroeck *et al.*, 1997) and co-precipitates with Ras from nitric oxide-treated rat PC12 pheochromocytoma cells (Deora et al., 1997) however, PI 3-kinase regulation through Ras has been best studied with the

p110α/p85 complex. GTP-Ras activates PI 3-kinase activity *in vitro* (Rodriguez Viciana *et al.*, 1994) and expression of Ras in rat PC12 cells leads to 10-20 fold increases in PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 on stimulation with EGF and NGF (Rodriguez Viciana *et al.*, 1994). Conversely, cells transfected with a dominant negative Ras mutant show attenuated PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 responses. In addition, co-expression of the p110/85 complex and Ras in COS cells caused a large increase in the levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Rodriguez Viciana *et al.*, 1994). PI 3-K may be a Ras effector but the mechanism is not known but could involve recruitment to the plasma membrane in a similar manner to the Ras-Raf-1 interaction (Leevers *et al.*, 1994).

1.5.4 Signalling downstream of PI 3-kinases

The lipid products of PI 3-kinases are resistant to hydrolysis by PLCs and thus do not constitute signalling precursors that are further metabolised to produce second messengers in a manner analogous to PtdIns(4,5)P_2. Instead, the D-3 phosphoinositides possess intrinsic biological activity and mounting evidence from diverse experimental systems suggests that D-3 phosphoinositides represent the primary output signal from PI 3-kinases. Consistent with this, cellular levels of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 rise rapidly upon agonist stimulation. The hypothesis that down regulation of PI 3-kinase signalling occurs via PI phosphatases (Section 1.6) has been strengthened by the recent finding that the tumour suppressor PTEN is in fact a PtdIns(3,4,5)P_3 3-phosphatase (Maehama and Dixon, 1998). A number of proteins such as nucleotide exchange factors, PLCγ, PKB, and the PH domain containing tyrosine kinases of the Tec family, (discussed in Section 1.3.4 above) are known to lie downstream of PI 3 kinases have been found to contain phosphoinositide interaction domains that specifically recognise D-3 phosphoinositides.

1.5.4.a Activation of protein kinases

One of the first potential effectors of PI 3-kinases to be identified were members of the PKC family of protein serine/threonine kinases (Section 1.8). Atypical PKC (PKC-ζ) and novel PKCs, (PKC-ε and PKC-η) and the PKC-related kinase (PRK1) are known to be activated *in vitro* by phosphoinositides, in particular PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Nakanishi *et al.*, 1993; Palmer *et al.*, 1995; Toker *et al.*, 1994). However, the mechanism for activation is unclear as these proteins do not contain PH domains. Studies in intact cells have provided evidence for activation of PKC-ε and PKC-λ downstream of PI 3-kinase in PDGF-stimulated cells (Akimoto *et al.*, 1996; Moriya *et al.*, 1996) and the phosphorylation of the PKC substrate pleckstrin can be inhibited by wortmannin in stimulated platelets (Toker *et al.*, 1995). More recent work suggests that PI 3-kinase-dependent PKC activation may occur through phosphorylation by phosphoinositide-

dependent protein kinases (Section 1.8.2), which are known to regulate PKC-related protein kinase B isoforms.

The protein serine/threonine kinase protein kinase B (PKB, also known as Akt and RAC-α) is the cellular homologue of v-Akt, a protein encoded in the genome of the transforming retrovirus AKT-8 isolated from a rodent T-cell lymphoma (Bellacosa *et al.*, 1991). So far, three mammalian PKB isoforms (α, β, and γ) have been identified in mammals and one in *D. melanogater*. PKB proteins mediate the transduction of several important biological signals including cell survival (Dudek *et al.*, 1997; Kauffmann Zeh *et al.*, 1997) and glucose uptake/glycogen synthesis (Cross *et al.*, 1995; Kohn *et al.*, 1996; for review see Alessi and Cohen, 1998; Coffer et al, 1998).

All PKB proteins contain an N-terminal PH domain that when mutated blocks the PI 3-kinase-dependent activation of PKB by PDGF (Franke *et al.*, 1997; Franke *et al.*, 1995). PKB or the isolated PH domain binds PtdIns(3,4)P_2 with high affinity and causes dimerisation (Franke *et al.*, 1997) and it has been reported that PtdIns(3,4)P_2, but not PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3, stimulates PKB activity *in vitro* (Franke *et al.*, 1997; Frech *et al.*, 1997; Klippel *et al.*, 1997). However, several other studies have not confirmed direct activation of PKB by these lipids (Alessi *et al.*, 1997; James *et al.*, 1996; Stokoe *et al.*, 1997). The activation of PKBα by IGF-1 leads to the PI 3-kinase-dependent phosphorylation of two residues (T308 and S473, Alessi *et al.*, 1996). Mutation of these residues to neutral amino acids abolishes PKBα activation and conversely, the introduction of acidic residues at these positions produces a constitutively activated molecule. PKBα phosphorylation at T308 is achieved by the 3-phosphoinositide-dependent kinase, PDK1. PDK1 is directly activated by binding PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Alessi *et al.*, 1997) and phosphorylation of PKB by PDK1 is greatly enhanced in the presence of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 micelles (Alessi *et al.*, 1997; Stokoe *et al.*, 1997).

The studies outlined above have led to the following model for activation of PKB through PI 3-kinase: localised production of PtdIns(3,4)P_2 at the plasma membrane recruits PKB and causes it to dimerise; this is thought to alter the conformation of the protein exposing it to phosphorylation by regulatory membrane-bound protein kinases including PDK1. PDK1 phosphorylates a threonine residue in the activation loop of the PKB catalytic domain and once phosphorylated PKB becomes fully active leading to the phosphorylation of multiple substrates. Targets of PKB include glycogen synthase kinase GSK-3 (Cross *et al.*, 1995), phosphofructo kinase PFK-2 (Deprez *et al.*, 1997) which may be important in insulin signalling, BAD (Datta *et al.*, 1997), and other unknown targets that lead to the inhibition of apoptosis (for review see Alessi and Cohen, 1998).

D-3 PIs are central to this model and PI 3-kinase has been implicated in generating the activating PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 by using several experimental approaches. Firstly, mutant PDGF receptors which are unable to activate PI 3-kinase are also unable to

activate PKB (Burgering and Coffer, 1995). As mentioned above, low levels of wortmannin will block PKB activation by growth factors, as will dominant negative PI 3-kinase, whilst constitutively active PI 3-kinase leads to increased PKB activity, independent of growth factor stimulation (Klippel *et al.*, 1996). PtdIns(3,4)P_2 is produced by the action of PtdIns(3,4,5)P_3 (5)-phosphatases in some cell types, however, there is also evidence for another pathway leading to the production of PtdIns(3,4)P_2. Recent studies in platelets suggest that the majority of PtdIns(3,4)P_2 generated following aggregation is synthesised by a route involving the phosphorylation of PtdIns by the sequential actions of a PtdIns 3-kinase and a PtdIns(3)*P* 4-kinase activity (Banfic *et al.*, 1998a; Banfic *et al.*, 1998b). This PtdIns(3)*P* 4-kinase activity could not be immuno-depleted with an anti-PtdIns*P*K II mAb (Banfic *et al.*, 1998a) and thus appears distinct from PtdIns*P*K IIα which can catalyse this reaction *in vitro* (Rameh *et al.*, 1997b; Zhang *et al.*, 1997).

1.5.4.b Guanine nucleotide exchange factors as PtdIns(3,4,5)P_3 targets

Guanine nucleotide exchange factors (GEFs) catalyse the conversion of GTPases from the inactive GDP-bound to form the the active GTP-bound form. PH domains are found in all known GEFs which are specific for the Rho family of small GTPases (Section 1.10.1) and some GEFs specific for the Arf GTPases. In several cases D-3 phosphoinositides have been shown to interact with GEFs and subsequently stimulate their exchange factor activity.

The small GTPase Rac regulates the reorganisation of the actin cytoskeleton (Section 1.10.1) and is known to lie downstream of PI 3-kinase. Activation of PI 3-kinase by PDGF leads to an increase in levels of Rac-GTP (Hawkins *et al.*, 1995) although the precise mechanism is unknown. More recently it has been shown that the PH domain-containing Rac GEF, Vav can be stimulated by PtdIns(3,4,5)P_3 (Han *et al.*, 1998) and thus Vav activation may be at least partially responsible for the effects of PI 3-kinase on GTP-Rac levels (Hawkins *et al.*, 1995).

Regulatory PH domains are also found in the N-terminus of ARNO and related nucleotide exchange factors Grp1 and cytohesin (Chardin *et al.*, 1996; Klarlund *et al.*, 1997; Kolanus *et al.*, 1996). The Grp1 and cytohesin PH domains have high affinities for PtdIns(3,4,5)P_3 and relatively low affinities for PtdIns(3,4,)P_2 and PtdIns(3)*P*. ARNO also binds PtdIns(3,4,5)P_3 *in vitro* and its PH domain translocates to the plasma membrane in a wortmannin-dependent manner (Venkateswarlu *et al.*, 1998), suggesting that *in vivo,* PtdIns(3,4,5)P_3 is responsible for interaction with the PH domain. The finding that PtdIns(4,5)P_2 binding to the PH domain of ARNO and stimulates the exchange of GDP for GTP on ARF-1 (Chardin *et al.*, 1996) may be complicated by the

earlier observation that PtdIns(4,5)P_2 micelles can increase the rate of nucleotide on ARF by fivefold *in vitro* (Terui *et al.*, 1994).

1.5.4.c Interaction of PtdIns(3,4,5)P_3 with C_2 domains

There is compelling evidence for the involvement of phosphoinositide kinases in neurotransmitter release, although the biochemical function of phosphoinositides has yet to be elucidated. The C_2 domain-containing synaptic vesicle membrane protein synaptotagmin is essential for the fast, calcium-dependent phase of neurotransmitter release. The C_2B domain of synaptotagmin I has been shown to bind PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 *in vitro.* Calcium ions switch the specificity of this binding from PtdIns(3,4,5)P_3 (at calcium concentrations found in resting nerve terminals) to PtdIns(4,5)P_2 (at concentrations of calcium required for neurotransmitter release). These observations suggest that synaptotagmin may act as a calcium sensor, switching from PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 during exocytosis (Schiavo *et al.*, 1996).

1.5.4.d A PtdIns(3)*P* interaction motif

The yeast PtdIns 3-kinase Vps34p and its human homologue both exclusively synthesise PtdIns(3)*P*, however, the function of this lipid has been difficult to determine. Most 3-phosphoinositide binding proteins have low affinities for this less highly charged lipid but the Golgi localised adaptin, AP-2 has been identified as a specific 3-phosphoinositide binding protein which binds PtdIns(3)*P* (Rapoport *et al.*, 1997). It is thought that 3-phosphoinositides increase the affinity of AP-2 for endocytic sorting motifs present on the C-terminal tails of cell surface receptors. More recently, a PtdIns(3)*P* binding motif, termed the FYVE domain, has been identified in several yeast and mammalian proteins implicated in vesicle transport. FYVE domains are cysteine-rich zinc-finger-like motifs with a consensus sequence $CX_2CX_{9\text{-}39}CX_{1\text{-}3}(C/H)X_{2\text{-}3}CX_2CX_{4\text{-}48}CX_2C$ that coordinate two zinc atoms and have characteristically basic amino acids surrounding the third cysteine (Stenmark *et al.*, 1996). A FYVE domain is found in the C-terminus of the early endosomal autoantigen EEA1 which associates with endosomal fractions in a wortmannin-dependent manner (Stenmark *et al.*, 1996; Mills *et al.*, 1998). This association with the endosome has since been shown to require a functional FYVE domain which alone is sufficient to direct endosomal localisation (Gaullier *et al.*, 1998; Patki *et al.*, 1998; Simonsen *et al.*, 1998). EEA1 is an essential component of the wortmannin-sensitive endosome fusion apparatus (Mills *et al.*, 1998) which also requires the small GTPase Rab5 which also localises to early endosome. Interestingly, Fab1p, one of the proteins originally identified as having a FYVE domain and implicated in vesicle trafficking, has recently been shown to be a PtdIns(3)*P* 5-kinase (Cooke et al, 1998). The function of the the N-terminal FYVE domain in Fab1p has yet to be determined but it is unlikely that this

motif is responsible for substrate binding, since it lies outside the conserved catalytic core and is not required for complementation (Yamamoto *et al.*, 1995). Instead the FYVE domain may perform a role similar to that proposed for the PtdIns(4,5)P_2-binding PH domain of PLCδ (Section 1.4), by localising to sites of substrate and promoting processive catalysis.

1.6 Phosphoinositide Phosphatases as Regulators of PI Signalling

The interconversion of bioactive phosphoinositides is achieved by PI kinases, PI-PLCs and PI phosphatases (Figure 1.2). *In vivo,* the balance between these competing activities is tightly controlled during signalling events and an understanding of substrate flux through PI pathways requires consideration of the equilibria of these reactions. Empirically, it is often difficult to distinguish cellular increases in a given lipid due to PI-kinase activation from PI-phosphatase down-regulation, thus PI-phosphatases must be considered potential points of regulation. However, despite the identification of many PI phosphatases in recent years, much remains to be learned about their function and regulation. Some bioactive PI species, most notably the 3'-phosphorylated phosphoinositides, can only be degraded by phosphatases. Consequently, these activities have important roles in down-regulating PI 3-kinase signals. The PI 5-phosphatases have the potential to down-regulate PLC signalling and also to generate signalling molecules such as PtdIns(3,4)P_2, which is known to have important second messenger functions. Not surprisingly the PI phosphatases have features in common with PI kinases, in particular the association with receptors and other signalling molecules. Many PI phosphatases are structurally related to inositol polyphosphate phosphatases and some have been shown to dephosphorylate both PI and inositol phosphate substrates. It should also be noted that the substrate specificity of some homologues has yet to be determined.

1.6.1 Phosphoinositide 4-phosphatases

PtdIns 4-phosphatase activities are unknown, however, the existence of PtdIns 4-phosphatases has been suggested by studies of PtdIns(4)P biosynthesis which predict the presence of a rapidly turned over PtdIns-PtdIns(4)P cycle in erythrocytes (King *et al.*, 1989; Muller *et al.*, 1986).

Two mammalian PI 4-phosphatases have been characterised at the molecular level, the so-called type I and type II inositol polyphosphate 4-phosphatase isoforms (Norris *et al.*, 1997a; Norris *et al.*, 1995). The type I inositol polyphosphate 4-phosphatase was originally characterised as an activity which catalysed the hydrolysis of the D-4 phosphate of Ins(3,4)P_2 and Ins(1,3,4)P_3 (Bansal *et al.*, 1990). It was subsequently found that this enzyme preferentially hydrolysed PtdIns(3,4)P_2 with much greater efficiency than the

corresponding inositol substrates (Norris and Majerus, 1994). Antiserum to the type I PtdIns 4-phosphatase depletes 95 % of the total PtdIns(3,4)P_2 4-phosphatase activity in rat brain supernatant suggesting that this is the predominant activity in this tissue (Norris *et al.*, 1995). The regulation of the type I PtdIns 4-phosphatase is of particular interest because of the role of PtdIns(3,4)P_2 in regulating PKB and PDK function (Section 1.5.4.a) and because it is a likely pathway for controlling PtdIns(3,4)P_2 responses. The type I PtdIns 4-phosphatase is not known to be regulated directly by receptor signals, although there is evidence for the regulation of type I PtdIns 4-phosphatase by proteolytic inactivation. PtdIns 4-phosphatase contains PEST sequences found in proteins which are substrates for the calcium-dependent thiol protease calpain, treatment of platelets with calcium ionophores resulted in a 75% decrease in enzyme activity and it has been proposed that calpain proteolysis of type I PtdIns 4-phosphatase contributes to the PtdIns(3,4)P_2 response in thrombin-stimulated platelets (Norris *et al.*, 1997b).

The type II inositol polyphosphate 4-phosphatase has substrate specificities and catalytic properties similar to the type I isoform and is 37% identical at the amino acid level (Norris *et al.*, 1997a). Unlike the type I inositol polyphosphate 4-phosphatase, the type II isoform does not contain PEST sequences. Splice variants containing potential transmembrane segments were also isolated and evidence from northern blots suggests that these may be tissue specific (Norris *et al.*, 1997a).

1.6.2 Phosphoinositide 5-phosphatases

At least 10 distinct eukaryotic genes for inositol polyphosphate 5-phosphatases can be currently identified on the basis of shared sequence homology, however only those with demonstrated activity towards PI substrates will be considered here (Woscholski and Parker, 1997, for review). The 5-phosphatase family includes the Golgi-associated PtdIns(4,5)P_2- and PtdIns(3,4,5)P_3 phosphatase OCRL which is mutated in Lowe's oculocerebrorenal syndrome (Attree *et al.*, 1992; Olivos Glander *et al.*, 1995). OCRL has also been shown to co-purify with the p85α-p110α complex from brain (Hsuan *et al.*, 1994) and an unidentified PI 5-phosphatase activity has been found to associate with the same complex from platelet cytosol (Jackson *et al.*, 1995). How a defective PI 5-phosphatase causes the OCRL disease state is unknown but OCRL$^-$ cell lines derived from the proximal tubules of Lowe's syndrome patients' kidneys accumulate PtdIns(4,5)P_2 (Zhang *et al.*, 1998). Given the many functions of this lipid, it is possible that PtdIns(4,5)P_2 toxicity is responsible for the complex OCRL phenotype.

Evidence for the involvement of PI kinases in synaptic vesicle formation/recycling is mounting (see Section 1.11), and synaptojannin, an amphiphysin and Grb2-binding PI 5-phosphatase which is enriched in brain (David *et al.*, 1996; McPherson *et al.*, 1996; Woscholski *et al.*, 1997) may be involved in such a process. Synaptojannin colocalises

with dynamin in nerve terminals and is rapidly dephosphorylated in parallel with dynamin after the stimulation of neurotransmitter release (Bauerfeind *et al.*, 1997). Whilst the role of synaptojannin is unclear, dynamin is known to function in the fission of clathrin-coated vesicles that participate in the recycling of synaptic vesicle membranes (reviewed in De Camilli *et al.*, 1995).

There is also evidence for PI 5-phosphatase involvement in growth factor receptor signalling. Firstly, as stated above, OCRL associates with p110α/p85α, Secondly, the SH2 domain-containing inositol phosphatase SHIP, which is active against Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3, has been found to accumulate in antiphosphotyrosine complexes in response to growth factors (Osborne *et al.*, 1996). SHIP binds to Grb2 and SHC (Jefferson, 1997; Osborne *et al.*, 1996) probably via SHIP's C-terminal proline-rich motifs and its predicted PTB-domain binding sites (Damen *et al.*, 1996). The function of SHIP in these complexes is unknown, but because SHIP can dephosphorylate both Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3, it may be involved in regulating Ca^{2+} signalling through Ins(1,3,4,5)P_4 or the generation of PtdIns(3,4)P_2 in response to extracellular signals.

1.6.3 Phosphoinositide 3-phosphatases

PTEN (also known as MMAC1), was recently identified as a tumour supressor gene with homology to tyrosine phosphatases that is mutated in a substantial proportion of endometrial tumours and glioblastomas (Duerr *et al.*, 1998; Li *et al.*, 1997; Steck *et al.*, 1997). Poor activity of PTEN towards tyrosine phosphorylated proteins led workers to find that PTEN specifically dephosphorylated PtdIns(3,4,5)P_3 at the D-3 position (Maehama and Dixon, 1998). Expression of PTEN suppresses growth of PTEN$^-$ glioma cells and inhibits cell migration, spreading and focal adhesion formation in fibroblasts (Tamura *et al.*, 1998). Some of these phenotypes are consistent with the known function of PtdIns(3,4,5)P_3: for example, PTEN-defficient mouse embryonic fibroblasts display decreased sensitivity to TNFα, ultraviolet radiation, heat shock and a variety of other apoptotic stimuli, whereas high levels of PTEN can induce cell death (Stambolic, 1998). The PTEN$^-$ cells also show increased activity of endogenous PKB and PTEN-induced apoptosis could be rescued using a constitutively active PKBα mutant (Stambolic, 1998; Section 1.5.4.a). These results suggest that PTEN mutant may contribute to cell transformation by constitutively raising the levels of PtdIns(3,4,5)P_3, implying that the physiological role of PTEN is to down regulate PI 3-kinase signals.

1.7 Phospholipid Transfer Proteins

Cytosolic phospholipid transfer proteins are widespread in eukaryotic systems where they are thought to act as diffusable factors capable of transferring phospholipid monomers between membranes (for review see, Wirtz, 1991; Wirtz, 1997). Of particular interest to phosphoinositide metabolism are the PtdIns transfer proteins (PtdInsTPs) which have emerged as important components of secretory and phosphoinositide signalling pathways.

1.7.1 Mammalian PtdInsTP function

Currently, three homologous mammalian PtdInsTP proteins have been described: PtdInsTPα, PtdInsTPβ and human *RdgB*, a homologue of the Drosophila *RdgB* gene product. All can transfer PtdIns and PtdCho between membranes in *in vitro* transfer assays and PtdInsTPβ has the additional property of being able to transfer sphingomyelin analogues *in vitro* (Westerman *et al.*, 1995), although the *in vivo* significance of this is not understood.

There is long standing evidence for the involvement of PtdIns metabolism in secretory processes (reviewed in Hokin, 1985; De Camilli *et al.*, 1996) and secretion from adrenal chromaffin cells is known to require ATP and PI's (Eberhard *et al.*, 1990). PtdInsTP has been identified as a factor required for the ATP-dependent priming of the Ca^{2+}-stimulated secretion of norepinephrine from cytosol-depleted PC12 cells (Hay and Martin, 1993). Similarly, exocytosis of secretory granules from HL60 cells required PtdInsTP (Fensome *et al.*, 1996). The function of PtdInsTP is still unclear but the additional requirement for PtdIns*P*K suggests that the biosynthesis of PtdIns(4,5)P_2 is needed (Hay *et al.*, 1995).

Similar reconstitution experiments have been used to demonstrate the involvement of PtdInsTP in PLCβ signalling in fMet-Leu-Phe-stimulated HL60 cells (Cunningham *et al.*, 1995; Thomas *et al.*, 1993), PLCγ signalling in EGF-stimulated A431 cells (Kauffmann Zeh *et al.*, 1995), and PI 3-Kγ signalling in fMetLeuPhe-stimulated neutrophils (Kular *et al.*, 1997). Because PtdInsTPs can transfer PtdIns (but not poly-phosphorylated forms) and PtdCho between bilayers *in vitro* (Wirtz, 1991), it was originally suggested that their role in signalling was to replenish PtdIns in the plasma membrane by transporting it from its site of synthesis in the ER (Michell, 1975; Downes and Batty, 1993). This represents the simplest possible model for PtdInsTP function. However, evidence from both secretion and signalling studies suggest that PtdInsTP may play a more complex role than simple substrate resupply (reviewed in Hsuan and Tan, 1997). In brief, cytosol-depleted HL60 cells which have lost their endogenous PtdInsTP are capable of producing a weak but sustained Ins(1,4,5)P_3 increase in response to GTP[γ]S, presumably by utilising PtdIns that is present at the plasma membrane. Furthermore, the addition of exogenous PLCβ to the GTP[γ]S-treated cells increases the rate of PtdIns(4,5)P_2 hydrolysis,

suggesting that the rate of hydrolysis is determined by the amount of PLCβ activity (Cunningham *et al.*, 1995). The addition of exogenous PtdInsTP greatly enhances the initial rate of Ins(1,4,5)P_3 production in permeabilised HL60 cells (Cunningham *et al.*, 1995). These data are inconsistent with the substrate re-supply hypothesis and suggest that PtdInsTP promotes the synthesis of PtdIns(4,5)P_2 and its subsequent hydrolysis by PLC by acting as a cofactor. The role of PtdInsTP in PLC signalling is further supported by the finding that PtdInsTP associates with EGF receptor, a type II PtdIns 4K and PLCγ in an agonist-dependent fashion (Kauffmann Zeh *et al.*, 1995). This has led to the proposal that PtdInsTP, PI kinases, and PLCs are all components of multienzyme signalling complexes formed during G-protein and receptor tyrosine kinase signalling (Cockcroft, 1998; Hsuan, 1993; Hsuan and Tan, 1997). According to this model, PtdInsTP retains and sequentially presents PI species to the lipid kinases and PLCs. This is proposed to allow efficient and rapid signalling but may also compartmentalise agonist-sensitive PtdIns (Section 1.2).

There is currently no universally accepted model for PtdInsTP function and the hypothesis that PtdInsTP transfers PtdIns from its site of synthesis is supported by recent data from studies of turkey erythrocyte ghosts (Currie *et al.*, 1997). It was observed that approximately 15% of the PI pool could be consumed in the absence of PtdInsTP and that in the presence of bovine PtdInsTP, more that 70% was consumed at a linear rate for over 2 hours. Unlike the studies in permeabilised HL60 cells described above, the initial rate was not affected. However, it should be noted that turkey erythrocyte ghosts do not represent a typical eukaryotic cell since erythrocytes are known to synthesise PtdIns in the plasma membrane itself. Furthermore, the use of a heterologous system raises the question of compatability of the avian membranes and bovine PtdInsTP.

1.7.2 Genetic Models of PtdInsTP function

The existence of functional PtdInsTP homologues in organisms amenable to genetic study has given some valuable clues to PtdInsTP function. In *S. cerevisiae*, mutation of the *SEC14* transfer protein gene, leads to a defect in protein transport from the Golgi apparatus and the accumulation of secretory vesicles in the cytosol (Bankaitis *et al.*, 1990; Bankaitis *et al.*, 1989; Rambourg *et al.*, 1996). This is consistent with the ability of PtdInsTPs, including Sec14p to reconstitute vesicle biogenesis at the TGN in mammalian cells (Ohashi *et al.*, 1995). *SEC14* function has been extensively studied in yeast and is believed to play a role in determining the relative levels of PtdIns and PtdCho in the yeast Golgi apparatus by regulating PtdCho biosynthesis. It has been proposed that Sec14p located at the Golgi exists in PtdIns and PtdCho-bound forms in equilibrium with PtdIns and PtdCho in the Golgi membrane. The rate determining enzyme in PtdCho synthesis is choline phosphate cytidyltransferase which is inhibited by Sec14p-PtdCho, thus an increase in Golgi PtdCho leads to an increase in the amount of PtdCho bound to Sec14p

and results in inhibition of PtdCho synthesis (reviewed in Kearns *et al.*, 1998; Wirtz, 1997). At present it is not known how the budding of secretory vesicles from the Golgi is controlled by the PtdIns/PtdCho ratios.

The *Drosophila RdgB* protein is of broad interest because of the identification of a mammalian homologue (Guo and Yu, 1997). Although the function of these recently described homologues is unknown, their existence is surprising because the *Drosophila RdgB* gene product is, unlike the conventional 32-35 kDa PtdInsTPs, a large 116 kDa polypeptide predicted to be an intrinsic membrane protein (Vihtelic *et al.*, 1993; Vihtelic *et al.*, 1991). The unusual structure of *RdgBp* was thought to be due to its specialised function in the *Drosophila* olfactory (Woodard *et al.*, 1992) and phototransduction cascades where mutation causes light-dependent retinal degeneration. Unlike the cAMP-linked visual system of mammals, phototransduction in *Drosophila* is under the control of a light-dependent GPCR pathway which signals via PLCβ. A transfer protein function for *RdgB* was originally suggested by the finding that the PtdInsTP domain of *RdgB* can transfer PtdIns and PtdCho *in vitro* (Milligan *et al.*, 1997) and that expression of the PtdInsTP domain was sufficient to rescue the retinal degeneration phenotype (Milligan *et al.*, 1997). However, the function of *RdgB* now appears far more complex than originally suggested: electrophysiological assays indicate that *RdgBp* is required following the termination of a photostimulus in the recovery phase where photoreceptor cells return to the resting state (Milligan *et al.*, 1997). Despite the ability of the PtdInsTP domain to transfer activity *in vitro*, mutations that do not effect PtdIns or PtdCho transfer activity display a potent dominant-negative phenotype (Milligan *et al.*, 1997). Furthermore, PtdInsTPα cannot rescue *RdgB* null mutants and when the PtdInsTP domain of *RdgB* was swapped with PtdInsTPα and expressed as a chimeric protein, a dominant negative phenotype was obtained (Milligan *et al.*, 1997). This suggests that PtdInsTPs and *RdgB* are not functionally interchangeable *in vivo* as the *in vitro* data would suggest. The function of the mammalian *RdgB* protein is unknown at present, but it is likely that it is involved in very different roles to the signalling PtdInsTPs. It is also notable that unlike the ubiquitous distribution of PtdInsTP, expression the mammalian *RdgB* homologue is limited to neural tissue, with specific expression in retina, olfactory bulb and brain (Guo and Yu, 1997).

A genetic model for PtdInsTP function has recently emerged with the discovery that the mouse mutant *vibrator* is caused by a non-coding mutation in the PtdInsTPα gene which results in a 5-fold reduction in PtdInsTPα expression (Hamilton *et al.*, 1997). The phenotype is characterised by early-onset degeneration of brain stem, spinal cord and dorsal root ganglia neurons. How a reduction in PtdInsTPα levels causes the complex *vibrator* phenotype is unknown, but there are parallels with the degeneration phenotype of the *RdgB* fly. Nevertheless, the identification of the *vibrator* locus using a novel and potentially powerful method of complementation (Hamilton *et al.*, 1997; Smith *et al.*,

1995) means that known genes in the PI cycle can be assayed for their ability to modify the *vibrator* phenotype. The availability of such a system may lead to significant advances in the understanding of neuronal PI metabolism.

1.8 Protein kinase C

Protein kinase C (PKC) was originally identified as a histone protein kinase from rat brain which could be activated by limited proteolysis, Ca^{2+}, phospholipids and tumour-promoting phorbol esters. PKC has numerous physiological substrates and is involved in the control of a large number of cellular processes including receptor signalling and gene expression (for review see Hug and Sarre, 1993). Of particular interest to this thesis is the classical role of PKC downstream of receptor-mediated PtdIns(4,5)P_2 hydrolysis. In addition, certain PKC isoforms are regulated by the products of PI kinases, most notably the PI 3-kinases (Section 1.5.4) and are therefore, potential effectors of PtdIns 4- and PtdIns*P*Ks. Many of the functions of PKC are beyond the scope of this study and are reviewed elsewhere (Hug and Sarre, 1993; Mellor and Parker, 1998; Nishizuka, 1992).

1.8.1 Structure and enzymology

There are currently twelve members of the protein kinase C superfamily which, on the basis of sequence homology and biochemistry, have been grouped into four classes: the conventional PKCs (composed of PKCα, -β1, β2, and -γ), which are activated by Ca^{2+}, DAG, and phorbol esters; the calcium-independent, DAG and phorbol ester-activated novel PKCs (PKCδ, -ε, -η, and θ); and the atypical PKCs (PKCζ and -ι, also known as PKCλ) which are unresponsive to Ca^{2+}, DAG and phorbol esters. The PKC-related kinases (PRKs) form the fourth group and are insensitive to Ca^{2+}, DAG and phorbol esters. PRK1 and -2 have been shown to bind RhoA but this important function of PRK will not be considered here (Mellor and Parker, 1998; Hannun, 1995, for review).

In vitro activation of the conventional PKCs requires mixed vesicles of DAG and PtdSer, the presence of which reduces the Ca^{2+} requirement to the micromolar range (Lee and Bell, 1991). Ca^{2+} ions are not required for the *in vitro* activation of PKC by phorbol esters but the presence of Ca^{2+} lowers the concentration of phorbol ester needed to achieve maximal activation (Ryves *et al.*, 1991). These *in vitro* properties reflect the cellular regulation of conventional PKC isoforms and support the following model. Hydrolysis of PtdIns(4,5)P_2 leads to the generation of Ins(1,4,5)P_3 and, consequently, a rise in cellular Ca^{2+} concentration. PKC binds to the DAG and PtdSer-containing plasma membrane, probably via the C_2 domain which, in the case of PKCβ, has been shown to bind PtdSer in a Ca^{2+}-dependent manner (Fukuda *et al.*, 1996). PtdSer is constitutively present in the plasma membrane and is thought to contribute to activation *in vitro* by acting as a cofactor (Lee and Bell, 1991). The lipophillic phorbol esters mimic the action of DAG and their

persistence in the plasma membrane accounts for the long term activation of PKC by these compounds.

Translocation of PKC isoforms is an important feature of their activation. However, Ca^{2+}-regulated protein-lipid interactions are probably just one aspect of membrane targeting, as a class of proteins has been defined which are receptors for activated PKC, the so-called RACKs. These are not substrates for PKC but appear to be able to interact with PKCs, targeting them to specific membranes. In the case of RACK1, this interaction takes place via part of the C_2 domain in PKCβ and peptides corresponding to this region block PKCβ translocation to the plasma membrane (Ron *et al.*, 1995).

1.8.2 Regulation of PKC by PI pathways

The activation of PKC-ζ, PKC-ε, PKC-η, and the PKC-related PRK1 by the products of PI kinases has already been discussed (Section 1.5.4.a). Although the activation of these PKC isoforms has been demonstrated *in vitro*, it is unclear whether direct interaction of PIs with PKC is responsible for the PI 3-kinase-dependent activation of PKC isoforms observed *in vivo* (Moriya *et al.*, 1996; Toker *et al.*, 1995). Recently a mechanism whereby PI 3-kinase may activate PKCs was suggested by the demonstration that PDK1 can phosphorylate key regulatory sites in the activation loop of PKCδ and PKCζ *in vitro* (Le Good *et al.*, 1998; Chou *et al.*, 1998). This phosphorylation was shown to be dependent on PI 3-kinase activity when PDK1 and PKCδ or PKCζ were co-expressed in human embryonic kidney (HEK) 293 cells. Furthermore, ectopically expressed PKCδ in HEK 293 cells becomes dephosphorylated upon serum starvation and is rephosphorylated in a PI 3-kinase-dependent manner when cells are restimulated with serum (Le Good *et al.*, 1998). It is not yet clear whether binding of PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 to the PH domain of PDK1 is required for these phosphorylation events, nevertheless, the concept that PDK1 acts as a link between PI 3-kinase signals and the activation of protein kinases suggests an interesting research direction.

1.9 Nuclear PI signalling

Evidence for PI signalling in the nucleus has been reviewed elsewhere (Divecha *et al.*, 1993) and therefore will not be discussed in detail here.

When isolated rat liver or Friend cell nuclei are incubated with [γ-^{32}P]ATP, radioactivity becomes incorporated into PtdIns*P*, PtdInsP_2, and PtdOH (Cocco *et al.*, 1987; Smith and Wells, 1984) suggesting the presence of an autonomous PI cycle in nuclei. In support of this, stimulation of quiescent Swiss 3T3 fibroblasts with IGF-1 leads to a specific increase in nuclear DAG and a decrease in nuclear PtdInsP_2 (Divecha, 1991). Furthermore, a number of enzymes in the PI cycle have been found associated with nuclear fractions including PI-kinases, DAG-kinase, PKC, PLCβ1, PLCδ4, and PtdInsTP (Liu *et al.*,

1996; Payrastre *et al.*, 1992; Snoek *et al.*, 1993). However, it should be noted that it is difficult to assess the purity of nuclear preparations used in these studies and it remains a possibility that they may be contaminated with perinuclear or even plasma membranes, both known to contain PI-kinases and PtdInsTPs. In some cases detergents have been used to remove the nuclear membrane but the question of the presence of detergent insoluble membrane rafts (Section 1.2.4) in nuclei has not be specifically addressed.

1.10 Phosphoinositides and Cytoskeletal Reorganisation

PtdIns(4,5)P_2 binds to a variety of actin regulating proteins including profilin, cofilin, gelsolin, CapZ and gCap39, suppressing the function of these proteins (Janmey and Stossel, 1987; Lassing and Lindberg, 1985; Stossel, 1993; Yonezawa *et al.*, 1991; Yu *et al.*, 1990), for review). PtdIns(4,5)P_2 also binds to the actin crosslinking protein α-actinin and promotes actin filament bundling (Fukami *et al.*, 1994; Fukami *et al.*, 1992). It has long been suggested that PtdIns(4,5)P_2 controls actin polymerisation and depolymerisation through its interaction with actin regulatory proteins. This hypothesis was strengthened by the observation that complexes of actin and profilin, an actin monomer-sequestering protein, could be dissociated by PtdIns(4)P and PtdIns(4,5)P_2 (Janmey and Stossel, 1987; Lassing and Lindberg, 1988), and also that the amount of PtdIns(4,5)P_2 bound to α-actinin and vinculin decreases on stimulation of fibroblasts with PDGF and correlates with actin depolymerisation (Fukami *et al.*, 1994).

Despite the many reported effects of PIs on actin organisation, convincing evidence for the regulation of the actin cytoskeleton by phosphoinositides has been scarce, mainly due to the fact that studies on actin de/polymerisation have relied on *in vitro* methods and because of the potential for artefacts in protein-phospholipid interactions. However, the hypothesis that PI signalling is directly linked to reorganisation of the actin cytoskeleton is becoming more compelling. Firstly, there is the finding that PtdIns$P$Ks are regulated by small GTPases Rac and Rho which are known to perform key functions in controlling the actin cytoskeleton (see below). Secondly, actin polymerisation induced by thrombin in platelets has been found to occur through a Rac pathway and actin uncapping and polymerisation was PtdIns(4,5)P_2-dependent (Hartwig *et al.*, 1995). Thirdly, LPA-stimulated stress fibre formation in PAE cells is sensitive to inhibitors of PLD activity (Cross *et al.*, 1996). The product of PLD activity, PtdOH, was able to stimulate stress fibre formation alone when added to cells. PLD is known to be activated by PtdIns(4,5)P_2 which suggests that there is potential for the regulation of PLD activity via a PtdIns$P$K. It is notable that type I PtdIns$P$Ks are known to affect actin structures *in vivo* (see below) and are activated by PtdOH *in vitro* (Section 1.5.2.b). However, it appears that in PAE

cells at least, PLD is acting upstream of Rho since the effects of PtdOH can be blocked by C3 transferase.

Vinculin is a major structural protein located at the cytoplasmic face of cell-substrate and cell-cell interfaces where it has important roles in adhesion (reviewed by Jockusch *et al.*, 1995). Vinculin is a 90 kDa protein which interacts *in vivo* with many cytoskeletal proteins including actin, α-actinin, paxillin, and talin as well as acidic phospholipids. These ligands are known to bind different determinants in a conformation-regulated manner. PtdIns(4,5)P_2 causes vinculin to adopt an open conformation which allows the binding of talin and F-actin, two important components of focal adhesions (Gilmore and Burridge, 1996). Microinjection of serum starved fibroblasts with monoclonal antibodies directed against PtdIns(4,5)P_2 prior to serum stimulation prevented the formation of stress fibres or focal adhesions (Gilmore and Burridge, 1996). The requirement for PtdIns(4,5)P_2 synthesis in the formation of focal adhesions and stress fibres is consistent with the increases in cellular PtdIns(4,5)P_2 seen in the adhesion of fibroblasts to fibronectin (Chong *et al.*, 1994; McNamee *et al.*, 1993) and platelet aggregation (Hinchliffe *et al.*, 1996). Recent evidence suggests that the Rho family of small GTPases may directly regulate phosphoinositide pathways which are important in the control of the actin cytoskeleton.

1.10.1 Rho proteins and the actin cytoskeleton

To date, some thirteen distinct Rho-related proteins have been described: RhoA-E and G, Rac1, 2, and E, Cdc42Hs, TC10, and Rnd1 and 2. Like other GTPases, the Rho proteins cycle between GTP-bound (active) and GDP-bound (inactive) states, and function as molecular switches controlling diverse cellular processes. Activation of Rho proteins occurs by exchange of bound GDP for GTP, a reaction catalysed by a group of activating molecules called guanine nucleotide exchange factors (GEFs). The GTP-bound protein remains active until the intrinsic GTPase activity hydrolyses the nucleotide to the GDP form. Certain proteins can influence activity by either stimulating the rate of hydrolysis (GTPase-activating proteins, GAPs), or by stabilising the inactive state (guanine nucleotide dissociation inhibitors, GDIs). Together these modulatory proteins play important roles in regulating the large number of biological processes controlled by Rho GTPases. At present, only Rac and Rho have been implicated in phosphoinositide pathways involving PtdIns*P*Ks and for reasons of space, only these will be discussed here (for review see Hall, 1998; Tapon and Hall, 1997; Van Aelst and D'Souza Schorey, 1997).

Rho proteins control the actin cytoskeleton in all eukaryotic cells. Activation of Rho in fibroblasts by growth factors has been shown to cause the formation of stress fibres and the clustering of integrins and associated proteins into focal adhesion complexes.

Activation of Rac causes the formation of lamellipodial extensions and membrane ruffles by promoting *de novo* actin polymerisation at the cell periphery. Actin filaments in stress fibres and lamellipodia are associated with integrin adhesion complexes whose dynamic assembly and disassembly provides a mechanism which generates the traction and force required for cell migration.

Small GTPase targets often bind cognate GTPases in a GTP-dependent manner and consequently, affinity purification and yeast two hybrid screening have been used to identify potential regulator and effector proteins which interact with Rac and Rho. These interacting proteins include protein kinases (Amano et al., 1996; Ishizaki *et al.*, 1996; Matsui *et al.*, 1996; Watanabe *et al.*, 1996). Several GEFs and GAPs have also been found to physically interact with Rac and Rho (Van Aelst and D'Souza-Schorey, 1997, for review). Although two hybrid screens have not yielded any interacting PI-kinases, Rac and Rho have been shown to bind PtdIns*P*K and PI 3-kinase activities *in vitro* (see Figure 1.7; Ren *et al.*, 1996; Tolias *et al.*, 1995).

The involvement of a PtdIns*P*K in a Rho pathway was first suggested when it was shown that RhoA was able to stimulate a PtdIns*P*K activity in fibroblast lysates in a GTP-dependent manner (Chong *et al.*, 1994). It has since been found that RhoA binds a 68 kDa type I PtdIns*P*K (Ren *et al.*, 1996). Interestingly, this interaction was independent of GTP, data reminiscent of the results obtained with the Rac-associated PtdIns*P*K (Tolias *et al.*, 1995). However, these results are contrary to those shown in Figure 1.7 and the data obtained from fibroblast lysates (Chong *et al.*, 1994). The discrepancy between these results is probably due to differences in experimental conditions. However, the ability of RhoA to stimulate GTP-dependent PtdIns*P*K activity in lysates but not in precipitated complexes argues for an additional GTP-dependent component (or components) of a complex which mediates the stimulatory interaction with RhoA and Rac1 but not the constitutive physical interaction with these proteins. The GTP-dependent activity shown in Figure 1.7 is likely to be due to the low salt concentration (50 mM) used in the washing buffer in order to maintain weaker interactions. The hypothesis of a complex is consistent with recent findings (Tolias *et al.*, 1998) and the fact that no PtdIns*P*K has been isolated by two hybrid, a method which only detects binary interactions. The link between Rac and PtdIns*P*K was strengthened when it was shown that thrombin-stimulated actin polymerisation in platelets was accompanied by an increase in PtdIns(4,5)P_2 and that the effects of thrombin could be emulated by GTP-Rac1 but not RhoA (Hartwig *et al.*, 1995). This has led the authors to suggest a model in which PtdIns*P*K is activated by Rac on thrombin stimulation and leads to an increase in PtdIns(4,5)P_2. The PtdIns(4,5)P_2 generated leads to the dissociation of actin-capping proteins from actin filaments resulting in rapid polymerisation. The use of intact cells or semi-intact (ie, permeabilised) systems may be important when studying the function of the PtdIns*P*K I-Rac1/RhoA complex

because the potential to interact with membranes is preserved. It is possible that Rac and Rho play some role in targeting the complex to PtdIns-containing membranes.

The cloning of several type I PtdIns*P*Ks has led to two reports showing that overexpression of type I PtdIns*P*Ks leads to dramatic changes in the actin cytoskeleton (Shibasaki *et al.*, 1997; Ishihara *et al.*, 1998) as does the overexpression of synaptojannin, a PI 5-phosphatase (Sakisaka *et al.*, 1997). Interestingly, overexpression of either the 5-kinase or 5-phosphatase caused the same effect; a complete loss of stress fibres and these observations have not been adequately explained. Furthermore, it should be noted that synaptojannin shows activity towards PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Section 1.6.2) and type I PtdIns*P*Ks can generate a number of PIs *in vitro* (Section 1.5.2.b), also, in none of the above studies were the effects of kinase/phosphatase overexpression on cellular (or indeed cytoskeletal) levels of PIs reported.

1.11 Phosphoinositides and Vesicle Traffic

The transport of intracellular vesicles carrying specific membrane and lumenal cargoes underlies a diverse range of cellular processes including, the targeting of lysosomal proteins, peptide hormone and neurotransmitter release, the non-degradative recycling of cell surface receptors, glucose transport and membrane homeostasis. Each of these processes depends on a large number of specific proteins with complex interactions and mechanisms of regulation. The involvement of phosphoinositides in vesicle transport has been acknowledged for many years but understanding the biochemical functions of phosphoinositides has been difficult. Recently, a number of proteins implicated in vesicle traffic have been identified which contain phosphoinositide binding domains which may underlie mechanisms of recruitment and regulation. Furthermore, regulated PI kinase activity may be important in determining the local phospholipid composition of specific subcellular membranes and it is possible that highly acidic phospholipids can determine membrane curvature and the fusogenic properties of transport vesicles (Sheetz and Singer, 1974). PtdIns(4,5)P_2 has been implicated in a variety of vesicle formation reactions because this lipid is important in the function of the ARF family of small GTPases which regulate PLD activity.

Because of the complexity of this field, only vesicular processes relevant to the products of PtdIns 4- and PtdIns*P*Ks will be discussed (for review see De Camilli *et al.*, 1996; Martin, 1997; Schekman and Orci, 1996).

1.11.1 Phosphoinositides and vesicle biogenesis

Vesicles formed at the *trans*-Golgi network (TGN) are destined for several intracellular locations including lysosomes for degradative functions, endosomes for temporary storage, and the plasma membrane for secretion. The formation of vesicles by budding

from the Golgi requires the recruitment and assembly of cytosolic factors on the cytoplasmic leaflet of the TGN.

Evidence for the involvement of PIs in the formation of post-TGN vesicles has been provided by studies using secretory vesicle formation in cell-free TGN preparations from rat PC12 neuroendocrine cells. Two cytosolic factors from bovine adrenal medulla were required to reconstitute secretory vesicle formation. The first, CAST1, was found to contain PtdInsTP, and PtdInsTPs α, β and Sec14p were able to substitute for CAST1 (Ohashi *et al.*, 1995). This requirement for PtdInsTP may explain the finding that PtdInsTPβ localises to the Golgi in Swiss 3T3 fibroblasts (De Vries *et al.*, 1995). The role of PtdInsTP in the TGN is unclear but as with its putative role in PLC signalling (Section 1.7.1), it may be involved in substrate supply and presentation. Indeed, Ohashi *et al.* (1995) suggest that CAST2 may contain phosphoinositide kinase activity and both PtdIns 4Kβ and p230/PtdIns 4Kα have been reported in the Golgi (Section 1.5.1). However, it remains a possibility that PtdInsTP has an analogous function to Sec14p in yeast where the transfer protein controls protein secretion by regulating the relative PtdIns/PtdCho content of the Golgi (Section 1.7.1).

As yet no direct role for a PtdIns 4K or PtdIns*P*K has been demonstrated in vesicle formation. However, PtdIns 3-kinase activities have important roles in the sorting of proteins to the yeast vacuole and the mammalian lysosome. This pathway has been best studied genetically in yeast where the PtdIns 3-kinase Vps34p is required in the TGN to direct newly synthesised vacuolar hydrolases away from the default pathway (leading to the plasma membrane) to the vacuole (Stack *et al.*, 1995, for review). A mammalian homologue of Vps34 exists (Volinia *et al.*, 1995) and one of the effects of PI 3-kinases inhibitors in mammalian cells is to cause specific defects in TGN to prelysosomal compartment trafficking resulting in exocytosis of lysosomal proteins (for review see Shepherd *et al.*, 1996).

1.11.2 Exocytosis

The importance of phosphoinositides in Ca^{2+}-regulated exocytosis was illustrated by the finding that a bacterial PI-PLC was able to inhibit secretion from permeabilised adrenal chromaffin cells (Eberhard *et al.*, 1990). The process of regulated exocytosis can be broken down into several stages: formation of secretory vesicles by budding from the TGN, docking to the plasma membrane, priming, and the Ca^{2+}-triggered fusion with the plasma membrane and subsequent release of the lumenal cargo. The priming of exocytotic vesicles prior to fusion requires ATP (Banerjee *et al.*, 1996). In PC12 cells, this ATP-dependent priming step was found to require three cytosolic protein factors (designated PEPs 1-3). PEP 1 was subsequently identified as a type I PtdIns*P*K (Hay, 1995) and PEP3 as a PtdInsTP (Hay *et al.*, 1995). Furthermore, secretion was inhibited by anti-

PtdIns(4,5)P_2 antibodies and recombinant PLC (Hay *et al.*, 1995), suggesting that PtdIns(4,5)P_2 itself, rather that a metabolic intermediate, is the important factor in ATP-dependent priming. The function of the PtdIns(4,5)P_2 is not known, however, large amounts (>10 mol %) of PtdIns(4,5)P_2 are synthesised on secretory vesicles (Loyet *et al.*, 1998) which may recruit PtdIns(4,5)P_2-binding proteins to the vesicle or activate proteins in the vicinity which facilitate membrane fusion. A model has been proposed in which a type I PtdIns*P*K, PtdInsTP, and a vesicle-associated PtdIns 4K produce PtdIns(4,5)P_2 in a pathway closely linked to PLD (Liscovich *et al.*, 1994). Because PLD and type I PtdIns*P*K are activated by PtdIns(4,5)P_2 and PtdOH, respectively (Liscovitch *et al.*, 1994; Moritz *et al.*, 1992), it has been suggested that when a vesicle containing membrane bound ARF and PtdIns 4K contacts an acceptor membrane containing PLD and a type I PtdIns*P*K, a feedback loop is set up in which PtdOH generated by ARF-activated PLD stimulates the PtdIns*P*K activity. This leads to increased PtdIns(4,5)P_2 production. This would result in a rapid and localised generation of two highly charged phospholipids at the membrane interface leading to Ca^{2+}-triggered membrane fusion. In support of this model a type II PtdIns 4K is associated with secretory granules (Husebye and Flatmark, 1988) and is required for stimulated secretion from chromaffin cells (Wiedemann *et al.*, 1996).

A number of PtdIns(4,5)P_2-binding proteins implicated in membrane fusion have been described including synaptotagmin and the calcium-dependent activator protein for secretion (CAPS). Synaptotagmin exhibits calcium-dependent PtdIns(4,5)P_2 binding *in vitro* (Wiedemann *et al.*, 1996) via a C_2 domain. Interestingly, inositol hexakisphosphate, which competes for PtdIns(4,5)P_2 binding inhibits secretion in neuroendocrine cells (Ohara Imaizumi *et al.*, 1997). The 290 kDa dimeric protein CAPS is associated with secretory vesicles and the plasma membrane in brain and has an essential function in the late Ca^{2+}-triggered stage of exocytosis in PC12 cells. The precise function of CAPS during the late stages of exocytosis is unknown but its biochemical properties suggest that CAPS may be a component of the membrane fusion machinery. CAPS specifically binds PtdIns(4,5)P_2 *in vitro* (Loyet *et al.*, 1998), a property that may be relevent to its function following the priming step. CAPS also binds Ca^{2+} which switches its binding specificity to other phospholipids causing the aggregation of liposomes *in vitro* (Loyet *et al.*, 1998). These properties suggest a possible mechanism for CAPS function in which CAPS is recruited to PtdIns(4,5)P_2-containing vesicles. Increased Ca^{2+} concentration, which specifically inhibits PtdIns(4,5)P_2 binding, may cause a switch from vesicle binding to other phospholipids in the membrane, resulting in membrane fusion.

Regulated secretion requires GTP (Glenn and Burgoyne, 1996) and it is likely that both heterotrimeric and small G-proteins are involved, although the relative contributions of these in the control of secretion has not been determined (Burgoyne and Morgan, 1993). Constitutively active Rac and Rho, proteins known to associate with a type I PtdIns*P*K activity (Section1.5.2.b), were found to stimulate secretion from mast cells (Mariot *et al.*,

1996; Price *et al.*, 1995). The small GTPase ARF1, along with PtdInsTP, is able to stimulate PLD activity and secretion in permeabilised HL60 cells, whilst enhanced PLD activity alone is not sufficient (Fensome *et al.*, 1996). The involvement of a heterotrimeric G-protein in exocytosis was recently suggested by the finding that G_o is associated with secretory granules, along with Rho and the type II PtdIns 4K. Granule-associated PtdIns 4K activity was inhibited by antibodies to the effector region of $G\alpha_o$, the Rho-specific toxin C3, and activated by mastoparan, an activator of $G\alpha$ (Gasman *et al.*, 1998).

1.12 Conclusion

PI kinases and their lipid products are intimately involved in many fundamentally important cellular processes and understanding the complexity of phosphoinositide signalling represents a vast challenge for biology. Several major questions need to be answered; (i) how are levels of biologically active PIs regulated in response to signalling events such as the activation of cell surface receptors and, (ii) how can distinct biological processes achieve specificity when so many systems rely on the same phosphoinositide messengers (that is, how is inappropriate crosstalk between signalling pathways avoided).

At the inception of this project, it was hypothesised that the molecular characterisation of the PI kinases that synthesise PtdIns(4,5)P_2 and the generation of specific reagents such as antibodies, would provide a better understanding of the mechanisms of regulation of PtdIns(4,5)P_2 biosynthesis. The following studies describe the cloning and characterisation of a type II PtdIns*P*K and a PtdIns 4K.

2.0 Experimental Procedures

2.1 Materials

All chemical reagents, unless otherwise indicated, were supplied by BDH (at least AnalaR grade) or Sigma.

Affiniti Bio-Reagents: anti-PtdIns*P*K type II monoclonal antibody.

Amersham International: [γ^{32}P]-ATP (110 TBq/mmol), [α^{32}P]-dCTP (220 TBq/mmol), [^{35}S]-Pro-mix ($\geq$37 TBq/mmol), L-3-phosphatidyl[2-^{3}H]-inositol(4,5)bisphosphate (740 GBq/mmol), *myo*-[2-^{3}H]-inositol (2.96-4.44 TBq/mmol), ECL reagent, Rainbow markers, Hybond-N membranes, anti-mouse IgG HRP conjugate, anti-rabbit IgG HRP conjugate.

Amicon: filter concentrators.

Bio-Rad: AffiGel 10, Bradford reagent, Coomassie stains, SDS PAGE molecular weight markers, Dowex ion exchange resin.

Boeringer-Mannheim: GTP[γ]S, APMSF.

Calbiochem: Okadaic acid, human recombinant casein kinase II, and bovine thrombin.

Clontech: λDR2 human fetal brain library, λDR2 human placental library, and human multiple tissue northern blot, Talon affinity resin.

Costar: Spin-X filter columns.

Eurogentech: Anti-type IIα PtdIns*P*K peptide polyclonal antiserum

FMC BioProducts: NuSeive Genetic Technology Grade Agarose.

Genosys: All oligonucleotides.

Gibco/BRL: 1kb DNA molecular weight marker, 100 bp DNA marker, dNTPs, guanidine isothiocyanate (Ultrapure) glycogen, lipid concentrate, lipofectin, T4 DNA ligase buffer, penicillin-streptomycin, phenol, (Ultrapure), Superscript RT reverse transcriptase, yeast tRNA.

ICN: Inositol-free RPMI and DMEM, cysteine and methionine-free DMEM.

Millipore: PVDF membrane.

New England Biolabs: All restriction enzymes, Klenow DNA polymerase, and T4 DNA ligase.

Novagen Inc: pET 21d, *E.coli* strain BL21, and Ni-NTA agarose resin.

Perkin-Elmer: Dye-Deoxy sequencing kit.

Pharmacia: Calf intestinal alkaline phosphatase, T4 DNA Ligase, T4 polynucleotide kinase, protein A Sepharose 4B, protein G Sepharose 4B, glutathione Sepharose 4B, random hexamer oligonucleotide primer, and oligo-dT random primer.

Pharmingen: BaculoGold.

Promega: Taq DNA polymerase, Poly(A)Tract mRNA purification kit.

Qiagen: Qiaquick gel extraction kit, Tip-5000, Tip-100, and Tip-20 plasmid DNA purification columns.

Research Genetics: (custom peptides) L4 PtdIns*PK* IIα peptide immunogen.

Sigma: Adenosine 5'-triphosphate, ampicillin, aprotinin, benzamidine hydrochloride, benzamidine agarose, bovine serum albumin, epidermal growth factor, ethidium bromide, FSBA, GDP, insulin-transferrin supplement, kanamycin sulphate, leupeptin hydrochloride, *O*-phosphoserine, *O*-phosphothreonine, *O*-phosphotyrosine, phosphatidic acid, phosphatidylinositol(4)monophosphate, phosphatidylinositol, PMSF, polylysine, reduced glutathione, streptomycin sulphate, tetracycline hydrochloride, wortmannin, and yeast extract ultrafiltrate.

Stratagene: λZap II human Jurkat cDNA library, R408 helper phage, oligo dT-cellulose, and PCR Script cloning kit.

Whatman: Silica 60 TLC plates and filter papers.

Miscellaneous: 9E10 anti-myc monoclonal antibody was provided by G. Evan (ICRF), pEFLink was a gift from C. S. Hill (LICR).

2.2 Molecular Biology

All handling and analysis of nucleic acids not detailed here was performed according to standard protocols (Sambrook *et al.*, 1989).

2.2.1 Manipulation of Nucleic Acids

2.2.1.a Purification of plasmid DNA by alkaline lysis

Cultures (2-5 mls) of LB or TB, containing the appropriate antibiotic, were inoculated with bacterial colonies and incubated at 37°C overnight. Bacteria were sedimented at 4000 $\times g$ for 10 min and the pellet resuspended in 300 μl of GTE (50 mM glucose, 25 mM Tris.HCl, pH 8.0, and 10 mM EDTA) and transferred to microcentrifuge tubes. Cells were then treated with 300 μl of lysis solution A (0.2 M NaOH containing 1% SDS) followed by 300 μl of neutralising solution B (3 M potassium acetate, pH 4.8) and placed on ice for 10 min after which they were centrifuged at 12-14000 rpm in a microcentrifuge. The cleared lysate was transferred to fresh microcetrifuge tubes and incubated with 20 μg of RNAse A at room temperature for 20 min. and then extracted with phenol chloroform reagent (H_2O-saturated phenol:chloroform in a 1:1 ratio). The aqueous phase was transferred to a fresh tube and plasmid DNA precipitated by adding 0.7 volumes of isopropanol and centrifuging at 12-14000 rpm for 20 min. The pellet was washed once with 70 % ethanol, air-dried and resuspended in sterile distilled water.

Plasmid DNA for template in dideoxy sequencing reactions was prepared using an identical protocol except that the phenol chloroform reagent was replaced with chlorofom and the final DNA was purified by a second precipitation step with NaCl and polyethyleneglycol as follows: plasmid DNA was resuspended in 32 μl of H_2O to which 8 μl of 4 M NaCl and 40 μl of 13% (w/v) PEG 8000 was added. After incubation on ice for 20 min tubes were centrifuged for 20 min at 12-14000 $\times g$ and the precipitated

plasmid DNA washed in 70% ethanol. After air-drying, the DNA was resuspended to a concentration of 1 mg/ml.

For purification of larger amounts of plasmid (100-500 μg), the alkaline lysis method was scaled up as follows: overnight cultures (100 ml) of TB inoculated with single colonies were sedimented in 50 ml centrifuge tubes at 4000 $\times g$, the supernatants were aspirated and the pellets resuspended in 3 ml of GTE. The bacteria were subsequently lysed in solution A followed by solution B and incubated on ice for 10 min. Lysates were centrifuged in 12 ml polypropylene tubes at 8000 rpm in a Beckman SS34 rotor for 30 min. The supernatants were incubated with RNAse A (10 μg/ml) as before and extracted with phenol chloroform. The aqueous phases were precipitated with 0.7 volumes of isopropanol and centrifuged at 8000 rpm. Pellets were washed as above and resuspended to a concentration of 1 mg/ml.

2.2.1.b Purification of Total RNA

Total RNA was prepared from cultured cells essentially as described (Chomczynski and Sacchi, 1997) with few modifications. Cells were washed free of culture medium with ice cold PBS and lysed in ten volumes of 4M guanidine isothiocyanate containing 25 mM sodium citrate, 0.5% (w/v) sarkosyl, and 100 mM β-mercaptoethanol. After a low speed centrifugation at 2000 rpm for 5 min to eliminate insoluble debris, the following were added: 0.1 volumes of 3M sodium acetate solution (pH 4.0), one volume of H_2O-saturated phenol, and 0.2 volumes of chloroform (chloroform:isoamyl alchohol, 49:1). The mixture was mixed well and placed on ice for 15 min and then centrifuged at 4200 rpm for 40 min. An equal volume of isopropanol was added to the aqueous phase in a fresh 15 ml polypropylene tube. After mixing the tube was incubated at -20°C for 1 h and the precipitated RNA was collected by centrifugation at 8000 rpm in a Beckman SS34 rotor. The pellet was washed in 75% ethanol and dried before resuspension in DEPC-treated H_2O.

2.2.1.c Isolation of poly-(A)$^+$ RNA

Total RNA was enriched for the poly (A)$^+$ fraction by chromatography on oligo-d(T) cellulose. Total RNA was resuspended in DEPC-treated, 0.5 M SSPE and heated to 68°C for 15 min before rapidly cooling on ice. The sample was then applied to oligo-d(T) cellulose, previously equilibrated into 0.5 M SSPE and incubated at room temperature for 20 min, after which the slurry was loaded into Promega Wizard columns and washed four times with 400 μl of 0.5 M SSPE. The poly-(A)$^+$ fraction was eluted in 200 μl of DEPC-treated ddH_2O at 70°C and ethanol precipitated using 2 μg glycogen as carrier.

2.2.1.d First-strand cDNA synthesis

Typically 0.1-1 μg of poly-$(A)^+$ RNA was reverse transcribed in a 50 μl reaction using a mixture of 25 U of AMV and 200 U of MMTLV reverse transcriptases or 200 U of Superscript RT. Final reaction conditions were 10 μg RNA/ml, 350 pmol/ml primer, 100 mM Tris.HCl (pH 7.4), 40 mM KCl, 8 mM $MgCl_2$ 10 mM DTT, 4 mM Na_2HPO_4, 0.8 mM dNTPs and 0.01% Triton X-100. The reaction was heated to 65°C for 10 min and cooled rapidly on ice prior to the addition of reverse transcriptases after which the reaction was incubated at 41°C for 1 h. Random hexamer and oligo (dT)-primed first strand synthesis were used to generate templates which were used directly for PCR.

2.2.1.e Gel purification of DNA

Restriction fragments and PCR products were excised from agarose gels following electrophoresis and purified using the Qiagen Qiaquick gel extraction kit. Alternatively gel slices were placed in a Costar Spin-X filter unit and frozen by placing on dry ice for 10 min before centrifuging at 12-14000 rpm in a microcentrifuge. The filtrate was then extracted once with phenol-chloroform reagent and ethanol precipitated.

2.2.1.f Ligation of DNA

Cohesive ligations of restriction fragments were carried out in 20 μl reactions containing; 60 nmol vector DNA, 200 nmol each insert DNA, (1x BRL ligase buffer) and 2.5 U T4 DNA ligase (BRL) and incubated at 14-22°C for 3 h to overnight before transformation of *E. coli.*

2.2.1.g Blunt-end ligation of PCR products

Prior to ligation, Taq PCR products were gel-purified and phosphorylated at the 5'-termini with T4 polynucleotide kinase (PNK) in 100 μl of 1x PNK buffer (Pharmacia) containing: 1-5 pmol DNA, 10 mM ATP, and 10 U T4 PNK. Reactions were incubated at 37°C for 20 min, after which T4 PNK was inactivated by heating to 65°C for 20 min and purified by direct application to Qiaquick columns. Phosphorylated DNA was subsequently end-filled by incubating 1-5 pmol DNA in T4 DNA polymerase buffer (NEB) containing: 150 μM dNTPs, 5 U T4 DNA polymerase, and 5 U Klenow for 20 min at 37°C. DNAs produced with Vent DNA polymerase, which lacks terminal transferase activity, were treated in the same way but omitting the end-filling step.

The reaction products were gel-purified and cloned into *Sma* I cut vectors under the same conditions as in Section 2.2.1.f above except that a greater concentration of T4 DNA ligase (5 U) and a higher insert to vector ratio of 4:1 were used.

2.2.1.h Preparation and transformation of competent *E. coli*

Overnight LB broth cultures were inoculated with freshly grown *E. coli* strains, 0.5 ml of each starter culture was subsequently used to inoculate a 50 ml culture the next morning. Bacterial growth was monitored by measuring optical density (OD_{600}). When the cultures reached mid-log phase (OD_{600} = 0.4-0.6) the bacteria were pelleted by centrifugation at 4000 $\times g$ and resuspended in storage buffer (100 mM KCl, 50 mM $CaCl_2$, 10 mM potassium acetate and 10 % (v/v) glycerol) and incubated on ice for 10 min after which the tubes were centrifuged at 4°C for 15 min at 1000 rpm and resuspended in 2 ml storage buffer.

Competent *E. coli* (80 µl) was transformed with 10 µl of each ligation reaction by mixing cells and DNA on ice for 20 min and then subjecting to heat shock at 42°C for 90 s before returning to ice. After a further 5 min on ice, 900 ml of LB was added and the bacteria incubated for 30 min at 37°C prior to spreading on agar plates containing the appropriate antibiotic.

2.2.2 PCR methods

2.2.2.a PCR from libraries and first strand cDNA

PCR from high titre libraries (typically greater than 10^9 pfu/ml) and first strand cDNA was used to check cell lines and tissues for expression and isolate probes for cDNA library screening. Conditions were optimised for each set of primers and template. Typically, 50 µl reactions were performed in 1x Taq polymerase buffer containing 100 pmol of each primer, 2-6 mM $MgCl_2$, 0.2 mM dNTPs and 2.5 U Taq or Vent DNA polymerase. The amount of template used to obtain a PCR product visible by EtBr staining was dependent on the abundance of the relevant message but for medium to low abundance messages 10 ng of first strand reaction or greater than 2×10^6 pfu were used per reaction. A typical reaction profile is as follows: 96°C for 20 s. 58°C for 30 s. 72°C for 1-4 min. For 35-40 cycles.

2.2.2.b Rapid amplification of cDNA ends from cDNA libraries.

5'-RACE was used to obtain cDNA ends absent in clones obtained from library screening. In the following protocol, GS1 and GS2 are gene specific antisense primers and +F1 and +F2 are sense primers flanking the multiple cloning site of the library vector. The RACE reaction consisted of two rounds of PCR in which the first extended from GS1 and +F1 primers. A small portion of this reaction was subsequently reamplified with nested primers GS2 and +F2 (see Figure 2.1).

For the first reaction conditions are essentially as in Section 2.2.2.a above but with 400 pmol of +F1 vector primer, 50 pmol of gene-specific primer (GS1), 2-10×10^6 pfu of

library, and a 2 min extension time. An initial denaturation step of 96°C for 3 min was employed to release phage DNA before addition of polymerase. After 25-30 cycles a 1 µl aliquot of each reaction was used in a second PCR with the nested primers +F2 and GS2 for a further 25 cycles. To analyse RACE products, 10 µl of each reaction was separated by agarose gel electrophoresis, transferred to nylon membranes by Southern blotting, and hybridised with an appropriate probe (see Section 2.2.4.c).

2.2.2.c PCR using degenerate primers

Degenerate primers, based on the conserved regions of known PtdIns 4-kinases, were used in an attempt to isolate the corresponding regions of novel PtdIns 4-kinase homologues from Jurkat and A431 first strand cDNA. Primers PIK1; 5'-GGNGAYGAYTGYMGNCARG and PIK2; 5'-RTTNCCRTTKTGNCKRTCYTT (representing GDDCRQD and KDRHNGN, respectively) were used in 50 µl reactions as in 2.2.2.a above, with the following reaction profile: 96°C, for 25 s. 51°C, for 1 min. 72°C, for 45 s. For 35 cycles. Because of the degeneracy, larger amounts of primer (400 pmol) were used.

2.2.2.d PCR screening of phage plaques and bacterial colonies

PCR screening of recombinant bacteria was employed when large numbers of clones were to be analysed or plasmid copy number was too low for adequate plasmid yields using standard plasmid analysis techniques. This method was also used to analyse bacteriophage plaques prior to excision, lambda DNA preparations and mixtures of phage obtained from early rounds of library screening. By using primers complimentary to sequences flanking the multiple cloning site, the size of the insert could be determined. Alternatively, insert-specific primers were used to identify positive clones.

Single colonies or plaques were picked from petri dishes and resuspended in 20 µl of ddH_2O, heated to 100°C for 3 min and centrifuged at 12-14000 $\times g$ for 5 min to pellet any debris. Aliquots (5-10 µl) of the supernatant were subsequently used as template in 20 µl reactions containing 1x Taq DNA ploymerase buffer (Promega), 50 pmol of each primer, 4 mM $MgCl_2$, 0.2 mM dNTPs and 1.0 U Taq DNA polymerase. Typical reaction profile: 96°C, for 20 s. 55°C, for 25 s. 72°C, for 2 min, for 25 cycles. Reaction products (approximately 5 µl) were analysed by agarose gel electrophoresis and where appropriate Southern hybridisation.

2.2.2.e Site-directed mutagenesis using overlap extension

Point mutations were introduced into cDNA sequences using a method adapted from that of (Ho *et al.*, 1989). Oligonucleotide primers (M1f and M1r) containing the sequence to be mutated were used in two separate extension reactions with primers flanking the region

A

B

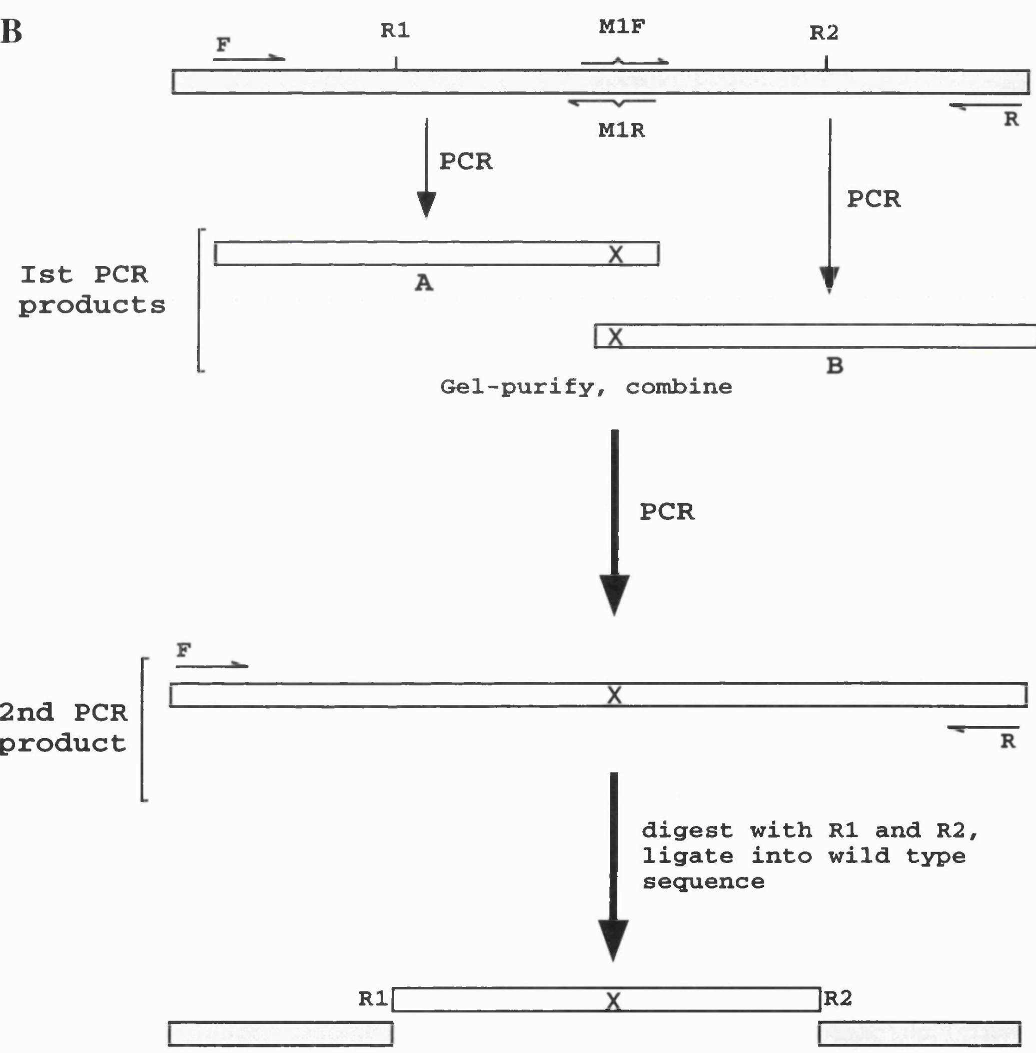


Figure 2.1 PCR methods

Rapid amplification of cDNA ends (RACE) PCR (A) and site-directed mutagenesis by overlap extension (B). See text for details.

to be mutated (F and R) to generate PCR products A and B which were subsequently gel-purified (Section 2.2.1.e, above). These PCR products were subsequently used in a second reaction to generate the mutagenic cassette which, after digestion with the appropriate restriction enzymes (R1 and R2) was used to replace the corresponding wild type sequence in expression constructs (see Figure 2.1). All mutants were extensively sequenced to eliminate clones containing polymerase-induced secondary mutations.

Conditions for the first reaction were as in Section 2.2.2.d above. 25 cycles were performed using 3.5 nmol of plasmid template in the initial reaction. In the second reaction, 15 nmol of each gel-purified PCR product was extended for 25 cycles. In both cases Vent DNA polymerase was employed to minimise secondary mutations.

2.2.2.f Cycle sequencing

Using double stranded plasmid DNA as template, fluorescently-labelled dideoxy-terminated extension products were prepared using the Perkin-Elmer ABI Prism cycle sequencing kit. 10 μl reactions contained 90 nmol of plasmid DNA, 2 pmol of sequencing primer and 4 μl of ABI Prism pre-mix. Reactions were carried out using the following program: 96°C, for 30 s. 50°C, for 30 s. 60°C, for 4 min, for 25 cycles. Extension products were purified by ethanol precipitation and analysed using an Applied Biosystems 373 or 377 automated DNA sequencer. Data was collected and analysed using Perkin-Elmer ABI software.

2.2.3 Radioisotopic Labelling Techniques

2.2.3.a Random primer labelling of double-stranded DNA probes

25 μl reaction contained 15-30 ng of template DNA, 0.7 mM dATP, 0.7 mM dTTP, 0.7 mM dGTP, and 5-10 μCi [$\alpha^{32}P$]-dCTP, 10 pmol random hexamer, 10 mM $MgCl_2$, 2.5 mM DTT, 0.1 mg/ml BSA, and 2 U Klenow DNA polymerase. All reaction components excluding the radiolabel and Klenow were heated to 100°C for 4 min before cooling rapidly on ice, after which the [$\alpha^{32}P$]-dCTP label and Klenow were added and the reactions incubated at 37°C for 45 min. Reactions were stopped by the addition of 75 μl of TEN buffer (containing 10 mM Tris.HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 1mg/ml yeast transfer RNA) and the labelled probe purified by gel filtration through a Sephadex G-50 spin column. The specific activity was determined by Cerenkov counting in a Beckman LS 1801 scintillation counter.

2.2.3.b End-labelling with T4 polynucleotide kinase

Synthetic oligonucleotide probes and restriction fragments previously dephosphorylated with calf intestinal alkaline phosphatase, were labelled by phosphorylation with T4 polynucleotide kinase. Reactions were performed in restriction buffer (70 mM Tris.HCl,

pH 7.6, 10 mM $MgCl_2$, 5 mM DTT) containing 10 pmol oligonucleotide or double-stranded DNA, 10 μCi [$\gamma^{32}P$]-ATP, and 10 U T4 PNK. After incubation at 37°C for 20 min reactions were terminated by the addition of 2 μl of 0.5 M EDTA and the unincorporated label removed by ethanol precipitation.

2.2.4 Hybridisation techniques

2.2.4.a Southern blotting

Restriction fragments and PCR products were transferred from agarose gels following electrophoresis using the method of Southern (Sambrook *et al.*, 1989). Briefly, DNA was denatured in agarose gels by treatment with a solution containing 0.5 M NaOH and 1.5 M NaCl for 20 min. After neutralisation in 1.5 M NaCl and 0.5 M Tris.HCl, The DNA fragments were transferred in 10x SSC to Hybond-N filter membranes by placing the gel face down on a filter paper wick (soaked in 10x SSC) with the nylon membrane on the opposite side of the gel. A stack of paper towels is then placed on top of the membrane such that capillary action drew buffer up from the wick and through the membrane to the towels, transferring the DNA fragments in the process. Transfer was allowed to proceed for 4 h to overnight. When transfer was complete, the positions of the wells were marked and the DNA covalently crosslinked to the membrane by exposure to a dose of 120 mJ of short wave ultraviolet radiation using a Stratagene Stratalinker.

2.2.4.b Northern blotting

Formaldehyde agarose gels were prepared containing 1x MOPS buffer, 18% formaldehyde (from a 37% solution) and 1% (w/v) agarose. Total RNA (50-80 μg) or poly $(A)^+$ RNA (2-10 μg) was ethanol precipitated and resuspended in 20 μl of loading buffer (1x MOPS-EDTA-acetate buffer (Sigma), 48% (v/v) formamide, 18% formaldehyde (37% solution), 5mg/ml ethidium bromide, containing bromophenol blue), heated to 65°C for 15 min, cooled on ice and applied to the formaldehyde-agarose gel. Following electrophoresis in 1x MOPS-EDTA-acetate buffer at 5 V/cm, the RNA was transferred to nylon membranes exactly as in Section 2.2.4.b above.

2.2.4.c Hybridisation with ^{32}P-labelled probes

Hybridisation of oligonucleotide and DNA probes to plaque lifts, Southern, and northern blots was carried out in a solution containing 6x SSC, 5x Denhardts reagent (1% acetylated BSA, 1% Ficoll, 1% polyvinylpyrolidone), and 400 μg/ml sonicated, denatured salmon sperm DNA. After incubation at 68°C for 1 h, denatured probe was added to 1-10 $\times$ 10^5 cpm/ml and allowed to hybridise overnight in a rotary oven. Filters were washed twice in 2x SSC, 0.1% SDS for 15 min at room temperature, followed by once at 45°C and then monitored to determine the background. Further washes were

carried out in 1x SSC, 0.1% SDS at 65°C until an acceptable background was obtained. The filters were then exposed to X-ray film.

Hybridisation to northern blots occasionally employed formamide in a hybridisation solution containing 6x SSPE, 50% formamide, 10x Denhardts reagent, and 400 mg/ml sonicated and denatured salmon sperm DNA. These hybridisations were carried out at 42°C.

2.2.5 Screening lambda cDNA libraries

Commercially obtained DNA libraries in λ-ZAP Express (Stratagene) and λ-DR2 (Clontech) were plated on 245 x 245 mm bioassay dishes at a maximum density of 750,000 plaques per dish. Filter replicas were made by overlaying carefully orientated, 220 x 220 mm Hybond-N membranes. After 1 minute (2 min for duplicates) membranes were removed and left, DNA side up, in a denaturing solution (0.5 M NaOH and 1.5 M NaCl) for 2 min before neutralisation for 6 min in 1.5 M NaCl and 0.5 M Tris.HCl. Filters were then washed in 2x SSC and crosslinked by exposure to ultraviolet light. Filters were hybridised to ^{32}P-labelled, double stranded DNA probes as described in 2.2.3 and washed at high stringency before autoradiography.

Positive plaques on these primary plates were revealed by placing the correctly orientated autoradiograph underneath the relevant plate and removing a plug of agarose with a 1000 µl pipette. Plaques picked in this manner were resuspended in 1 ml of 10 mM $MgCl_2$ containing one drop of chloroform and vortexed to release the phage particles. Positives were subsequently purified by repeating the screening procedure on 150 mm plates. When a homogenous population of positive phage was obtained, plaques were picked and the plasmids rescued by *in vivo* excision.

2.2.5.a Plasmid rescue from λ-ZAP Express

Plasmid rescue of pBK-CMV from the λ-ZAP system required infection of the non-suppressor *E.coli* strain XLOLR, which is permissive for helper phage infection, yet prevents replication of the ExAssist helper phage genome which contains an amber mutation.

E.coli MRF' (which expresses both the lambda receptor and the F-pilus) was used to inoculate a culture of LB and incubated at 37°C until the OD_{600} reached 0.2-0.5. Bacteria were resuspended in 10 mM $MgSO_4$ and 200 µl of this suspension was incubated with 1×10^5 pfu of plaque-purified phage stock and 1×10^6 pfu of ExAssist helper phage for 15 min at 37°C. After infection, 3 mls of LB broth was added and the culture incubated at 37°C for 2 hours. The tube was then heated to 70°C for 15 min and centrifuged at 4000 $\times g$ for 15 min. 100 µl of the supernatant containing the excised phagemid was then used to infect 200 µl of freshly grown XLOLR cells by incubating the two at 37°C for 15 min followed by the addition of 300 µl of LB and a further incubation for 45 min. The

recombinant XLOLR containing the pBK-CMV plasmid was recovered by spreading this culture on LB-kanamycin agar plates (50 μg/ml).

2.2.5.b Plasmid rescue from λ-DR2.

In order to convert the λ-DR2 to a pDR2 plasmid, the purified phage had to infect a permissive *E. coli* host (such as AM1) which expresses *cre* recombinase. *In vivo* excision is then achieved by virtue of the two phage *loxP* sites which undergo a site-specific recombination catalysed by the recombinase. Recombinant bacteria containing the plasmid were then selected on ampicillin containing plates.

Plaques picked in Section 2.2.1 above, were used to infect AM1 cells by incubating 2 ml of an overnight culture of AM1 with 15 μl of phage stock for 30 min at 32°C. The infected AM1 cells were further incubated with 50 μl of 2x YT broth for 1 h at 32°C after which IPTG was added to 0.3 mM to induce replication of the plasmid and the culture incubated for a further 1 h. Citric acid and carbenicillin was added to a final concentration of 12 mM and 60 μg/ml, respectively and incubated for two hours before spreading on LB agar and growing overnight at 37°C.

Rescued plasmids were analysed by restriction digestion and Southern blotting prior to sequencing.

2.3 Expression of Recombinant Proteins

2.3.1 Expression of Bacterial Fusion Proteins

In all cases bacterial expression required optimisation of expression and purification parameters for each construct. Specific conditions are not given.

2.3.1.a Purification of GST fusion proteins from bacteria

Overnight cultures of *E. coli* containing cDNA constructs in the pGex series of vectors were used as starter cultures to inoculate larger flasks at a dilution of 1/10-1/20. The cultures were expanded for 90 min before induction with IPTG at a final concentration of 0.1-0.2 mM. Flasks were incubated at 25-37°C for 2- 4 hours before harvesting bacteria by centrifugation at 4000 $\times g$ for 15 min. Bacterial pellets were subsequentlty resuspended in ice-cold lysis buffer containing protease inhibitors and sonicated for 10 s 3-6 times with cooling on ice between each sonication. For larger scale preparations (1-500 ml of culture), the resuspended bacterial pellet was incubated with 50 μg/ml lysozyme for 15 min on ice prior to sonication. Lysates were cleared by centrifugation at 14-30,000 $\times g$ and the supernatants incubated at 4°C for 30 min with glutathione-Sepharose previously equilibrated into lysis buffer. Unbound protein was removed by washing 4-6 times in lysis buffer containing 200 mM NaCl.

2.3.1.b Thrombin cleavage of GST fusion proteins

GST-tagged proteins on glutathione (GSH)-Sepharose beads were washed extensively in cleavage buffer (50 mM Tris.HCl, pH 8.0, 100 mM KCl, and 2.5 mM $CaCl_2$) to remove any residual protease inhibitors, and incubated with 25 U of bovine thrombin (Calbiochem) per mg of fusion protein. The cleaved protein was collected by centrifugation or by filtration through glass wool. The amount of thrombin, temperature and duration of the incubation were determined empirically by carrying out time courses at 4°C, room temperature, 30°C, and 37°C to obtain the best conditions for cleavage.

2.3.1.c Purification of $(H)_6$ fusion proteins from bacteria

E. coli BL21 (DE3) were transformed with pET vectors and single colonies used to inoculate 2 ml cultures of LB broth which were grown overnight. The following morning cultures were expanded and induced as in 2.3.1.a above. Cell lysates were prepared in the same way except that strong chelators and reducing agents were omitted from the lysis buffers. Recombinant proteins were purified on activated Ni-NTA agarose previously equilibarated into binding buffer (20 mM Tris.HCl, pH 7.9, 50 mM NaCl, 5 mM imidazole) and washed four times in wash buffer (20 mM Tris.HCl, pH 7.9, 50 mM NaCl, 60 mM imidazole and 0.1% Triton X-100). Bound protein was eluted by chelation of Ni^{2+} ions with EDTA or in elution buffer (20 mM Tris.HCl, pH 7.9, 1M imidazole, 500 mM NaCl, and 1 mM β-mercaptoethanol).

2.3.2 Expression of GST fusion proteins using baculovirus

Spodoptera frugiperda Sf9 cells were cultured in IPL41 medium supplimented with 10% (v/v) FCS, 2% (v/v) yeast extract ultrafiltrate, and 1% (v/v) lipid concentrate and incubated at 27°C. The same medium was used for the culture of Sf9 cells both in monolayers, spinner flasks and roller bottles. Cells were passaged by dilution between 1:4 and 1:12.

2.3.2.a Transfection of Sf9 cells

cDNAs were subcloned into the baculovirus transfer vectors pACG-2T and pBac4 and sterile, supercoiled plasmid DNA was co-transfected with BaculoGold DNA into Sf9 cells using lipofectin as follows; approximately 3×10^6 cells were seeded in 25 ml flasks and allowed to adhere. 8 µl of lipofectin, 4 µl of sterile distilled water, 0.5 µg of BaculoGold DNA, and 2 µg of plasmid DNA were mixed in polystyrene tubes and incubated for 15 min at room temperature. Meanwhile, the Sf9 monolayers were washed once in serum-free medium and brought to a volume of 1.5 ml of serum-free medium to which the DNA mixture was subsequently added. After incubating overnight, the medium was replaced with 5 ml of complete medium and incubated for a further 72 h.

2.3.2.b Generating high titre baculovirus stocks

After 72 h, the first culture supernatants (S1 fractions, containing recombinant baculovirus) were collected by centrifugation at 1000 $\times g$ for 5 min and 4 ml was used to infect a 5 ml flask containing 1 $\times$ 10^6 mid-log phase Sf9 cells, this was subsequently incubated for 96 h and the S2 fraction collected. This amplification was repeated until 0.5 ml of the S4 fraction containing high-titre virus, was used to infect three 175 ml flasks to produce a stock of high titre virus. These were stored at 4°C and frozen in 1 ml aliquots at -70°C. The pelleted cells from each amplification (P1-4) were stored at -20°C for analysis of protein expression.

2.3.2.c Large scale purification of recombinant GST fusion proteins from Sf9 cells

50 ml of Sf9 cells from logarithmic phase, spinner flask cultures were used to inoculate 250 ml flasks of medium and grown overnight. The following day each of these was infected with 800 µl of virus stock and incubated with constant stirring. At 96 h post infection, the cells were collected by centrifugation at 1000 $\times g$ for 10 min and the pellets lysed on ice, in 25 ml of ice cold lysis buffer (100 mM Tris.HCl, pH 7.5, 1.5 % Triton X-100, 150 mM KCl, 0.1% (v/v) β-mercaptoethanol, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1 µg/ml aprotinin and 1 µg/ml leupeptin). The lysates were then centrifuged at 4000 $\times g$ for 10 min and the supernatants recentrifuged at 40,000 $\times g$ for 20 min to remove remaining debris. GST-tagged proteins was purified by incubating cleared lysates with 2 ml of GSH-Sepharose 4B (Pharmacia) with tumbling for 30 min at 4°C. The GSH-Sepharose was washed four times in wash buffer A (100 mM Tris.HCl, pH 7.5, 1 % Triton X-100, 250 mM KCl, 0.1% (v/v) β-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1 µg/ml aprotinin and 1 µg/ml leupeptin.) and twice in wash buffer containing 100 mM Tris.HCl, pH 7.5, 100 mM KCl, 0.1% (v/v) β-mercaptoethanol).

2.3.4 Mammalian Expression

2.3.3.a Electroporation

Cells to be transfected were split one day prior to electroporation. The folowing day cells were pelleted at 1000 $\times g$ for 5 min before resuspension in 500 µl of sterile electroporation buffer (EPB: 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4, 6 mM glucose, 21 mM Hepes, pH 7.1). Approximately 1 $\times$ 10^7 cells were mixed in electroporation cuvettes (Biorad) with 10-20 µg of plasmid DNA dissolved in 500 µl of EPB and incubated at room temperature for 10 min with gentle agitation. Cells were electroporated using a Biorad Gene-Pulser controller at 260-300 V and 960 µF before

plating in complete medium. Cells were harvested 36-48 hours post-electroporation for analysis of expressed proteins.

2.3.3.b Lipid-mediated transfection of mammalian cells

Lipid mediated transfections using Lipofectin (BRL) were performed in 60 mm or 90 mm tissue culture dishes. The amount of plasmid DNA and lipofectin used was optimised for each cell type used. Typically cell monolayers at 40-60% confluence were washed once in serum-free medium and incubated for 6-12 h with a mixture containing 2 μg plasmid DNA, 4 μl lipofectin reagent and serum-free medium (for 90 mm plates the amounts of DNA and lipofectin were multiplied 2.5-fold). After incubation, the medium was aspirated, the cells washed once, and incubated in complete medium. Cells were harvested after 12-48 hours for analysis of expressed proteins.

2.3.3.c Microinjection

Plasmid DNA was diluted to between 1-500 μg/ml in PBS (without Ca^{2+} or Mg^{2+}) and centrifuged to remove particulate matter. Cells for microinjection were grown on coverslips for at least 48 h prior to microinjection. DNA solutions (approximately 2×10^{-11} ml) were injected directly into the nucleus of each cell using a Zeiss motorised microinjection workstation equipped with a temperature-controlled incubator jacket. After 6 h cells were fixed and stained with appropriate antibodies or rhodamine-labelled phalloidin to stain for actin as in Section 2.8.3 below.

2.4 Cell Culture Techniques

2.4.1 Cryostorage of cell lines

Adherent cells were trypsinised and gently resuspended in a mixture of 60% culture medium, 20% serum and 20% DMSO (freezing medium) and place on ice in a cryovial. An equal volume of freezing medium was added dropwise to the cell suspension. Vials were allowed to freeze slowly at -70°C and were transferred to liquid nitrogen the following day.

Vials were thawed rapidly in a 37°C waterbath and the cells transferred to a 15 ml conical tube to which culture medium was added dropwise to 10 ml. Approximately 5 ml of the cell suspension was plated per 90 mm dish.

2.4.2 Metabolic labelling with [^{35}S]-amino acids

Cultured cells were washed free of medium with PBS and incubated with labelling medium containing 90% cysteine- and methionine-free medium, 10% normal medium, 2% dialysed FCS, and 50-100 mCi/ml Pro-mix (containing approximately 70% [^{35}S]-

methionine and 30% [^{35}S]-cysteine). After incubation for at least 24 h, cells were washed twice in ice-cold PBS prior to preparation of lysates.

2.4.3 Metabolic labelling with *myo*-[^{3}H]-inositol and analysis of metabolites

Cells in 60 mm dishes (at 30-50% confluence) were washed free of culture medium and labelled for 36-48 h in inositol-free medium containing 10% dialysed FCS and 10 µCi/ml of *myo*-[^{3}H]-inositol after which they were washed with PBS and lysed in 100 µl 1M HCl containing 10 mM LiCl and scraped into Eppindorf tubes. Each tube was extracted with 600 µl of chloroform:methanol:1 M HCl reagent (15:5:5) by vortexing for 30 s. After phase separation, the aqueous phase containing inositol and inositol phosphates was removed to a separate tube and the organic phase, containing the inositol phospholipids was re-extracted twice with methanol:1 M HCL (1:1). The organic phase was dried in a stream of nitrogen and analysed by thin-layer chromatography (TLC, see 2.6). The aqueous phase was purified by chromatography on Dowex 1-X8 ion exchange resin as follows: each sample was neutralised by adding a few drops of 10 M NaOH and applied to a 0.5 ml bed volume of Dowex 1-X8 resin which was washed with 6 ml ddH_2O to elute free inositol. Glycero-Ins was eluted in 6 ml buffer containing 5 mM sodium tetraborate and 5 mM sodium formate. For elution of specific inositol phosphates the following were used; Ins*P*; 3 ml 0.2 M ammonium formate and 0.1 M formic acid. InsP_2; 3 ml 0.4 M ammonium formate and 0.1M formic acid. InsP_3; 3 ml 1 M ammonium formate and 0.1 M formic acid. For the analysis of total inositol phosphates, elution in 3 ml buffer containing 1 M ammonium formate and 0.1 M formic acid was used following the water and borate washes. In each case eluates were collected directly into scintillation vials containing 5 ml of Packard Ultima-Flow liquid scintillant for counting.

2.5 Protein Chemistry

2.5.1.a SDS-PAGE

All protein electrophoresis was performed according to Laemmli (1970).

2.5.1.b Silver staining of polyacrylamide gels

SDS PAGE gels were fixed by washing in 10% (v/v) acetic acid and 40% (v/v) methanol for 1 h. This solution was removed and the gels washed once for 5 min in ddH_2O, followed by 12.5 % (v/v) glutaraldehyde solution for 7 min. Sensitised gels were then washed free of remaining glutaraldehyde with two 5 min washes of water and two 5 min washes with 20% (v/v) ethanol. Gels was subsequently stained in a solution containing 20% (v/v) ethanol, 0.16% (w/w) NaOH, 2.5% (v/v) .880 ammonia solution and 2% (w/v) $AgNO_3$ for 15 min and then washed twice in 20% (v/v) ethanol for 5 min. Gels

were developed by shaking in a solution of 20% (v/v) ethanol, 0.037 % formaldehyde and 0.6 mM citric acid.

2.5.2 Biochemical Methods

All biochemical assays were performed in duplicate and data presented in figures is the product of at least two experiments unless otherwise indicated.

2.5.2.a PtdIns 4-kinase assay

PtdIns 4-kinase assays were performed in a 50-100 µl final volume containing 50 mM Tris.HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM phosphatidylinositol, 1mM β-mercaptoethanol, 0.4% Triton X-100, 0.1 mM EGTA and 100 µM [γ^{32}P]-ATP (1-10 µCi). After incubation at 37°C for 10-20 min, reactions were stopped with an equal volume of 1M HCl and each organic phase extracted with chloroform:methanol reagent. The organic phase was further extracted with methanol:1M HCl (1:1), dried under inert gas and the lipid products analysed by TLC (see Section 2.6).

2.5.2.b PtdIns*P* kinase assay

PtdIns*P*K assays were carried out in a 50-100 µl volume in 50 mM Tris.HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1mM β-mercaptoethanol, 500 µM phosphatidylinositol(4)phosphate (Sigma), and 50-100 µM [γ^{32}P]-ATP (1-10 µCi). Lipid products were extracted and analysed as above.

2.5.2.c Protein kinase assay

In vitro protein kinase assays were performed in a 50-100 µl volume containing 50 mM Tris.HCl, pH 7.5, 100 mM NaCl, 10 mM divalent cation (Mg^{2+}, Mn^{2+} or Ca^{2+}), 50 mM β-glycerophosphate, 1mM β-mercaptoethanol, 0.1 µg/ml okadaic acid and 1-10 µCi [γ^{32}P]-ATP. After incubation at 37°C for 20 min reactions were stopped in Laemmli loading buffer and the samples analysed by electrophoresis and autoradiography.

2.5.2.d Immobilisation of synthetic peptides on AffiGel

Approximately 200 µl of Affi-Gel 10 was washed twice in 1 ml of cold isopropanol. 1 mg of peptide dissolved in DMSO was incubated with the Affi-Gel suspension, with constant mixing, for 4 h at room temperature. The suspension was centrifuged at 1000 ×*g* for 1 min and the supernatant containing unbound peptide removed. Remaining activated groups were blocked by incubating the gel in 1 ml of 1 M ethanolamine.HCl, pH 8.0, for 5 min. The gel was washed extensively in PBS prior to use as an affinity matrix for the purification of polyclonal anti-sera.

2.5.2.e Preparation of A431 membrane fractions

Membrane fractions were prepared as described previously (Thom *et al.*, 1977). Briefly, A431 cells grown in 150 mm dishes were washed once in cold PBS and scraped into 3 ml of harvesting buffer (50 mM boric acid, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM EDTA, 2.5 mM benzamidine, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and centrifuged at 450 $\times g$ for 10 min before discarding the supernatant. The pellet was resuspended in 2 volumes of harvesting buffer and added, dropwise and with constant stirring, to a beaker containing 100 volumes of ice-cold extraction buffer (as extraction buffer but with 20 mM boric acid). The sample was further stirred for 10 min until a ball of debris was observed after which 8 pellet volumes of borate buffer (0.5 M boric acid, pH 10.2) was added and the suspension stirred for a further 5 min. The suspension was filtered through two layers of clean nylon gauze and centrifuged at 450 $\times g$ for 10 min. The supernatant was centrifuged at 12,000 $\times g$ for 30 min at 4°C and the pellet resuspended in 25 mM Tris.HCl, pH 7.5, 50% (v/v) glycerol containing protease inhibitors before storage at -20°C.

2.5.2.f Analysis of PtdIns*P* kinase activities bound to Rho family proteins

To determine which small GTPases were capable of binding PtdIns*P*K activity in cell lysates GST Rac, Rho and CDC42 were purified from *E. coli* as described in Section 2.3.1.a above.

The purified fusion proteins (approximately 10μg) immobilised on GSH-Sepharose, were loaded with either GDP or GTP[γ]S by incubating in 100 mM Tris.HCl, pH 8.0, 50mM NaCl, 0.5mM $MgCl_2$, 2mM EDTA, 1mM DTT (and the appropriate nucleotide at 1mM concentration). After incubation for 20 min at 20°C, 200 μl of 100 mM Tris.HCl, pH 8.0, 20 mM $MgCl_2$, 1mM DTT was added followed by a lysate of Swiss 3T3 cells (approximately half a 90 mm plate per assay, prepared as described in Section 2.8.2) and incubated with the fusion protein for 40 min at 4°C. The GSH-Sepharose was washed 4 times in 1 ml of wash buffer (50 mM Tris.HCl, pH 7.5, 50 mM NaCl and 0.1% Triton X-100). Bound PtdIns*P*K activitiy was assayed as described in Section 2.2.5.b above.

2.6 Separation of phospholipids by thin-layer chromatography

All TLC was carried out on Whatman silica-60 plates soaked in 1% (w/v) potassium oxalate and 1 mM EDTA and activated by heating to 110°C for 10 min prior to loading samples by spotting with a micropipettor at the origin.

A solvent system consisting of chloroform:methanol: 4M ammonia solution (225:175:50) was used to separate PtdIns monophosphates, PtdIns bisphosphates, and

phosphatidic acid. For separations of PtdIns monophosphates, bisphosphates, and trisphosphates, an acidic system consisting of propan-1-ol:glacial aceatic acid (65:35) and 1% 5 M H_3PO_4 was used.

For the separation of PtdIns(3)*P* from PtdIns(4)*P*, a borate solvent system was used (Walsh *et al.*, 1991). Plates were treated with a solution of 4.5 % CDTA before heating to 110°C for 10 min. After the samples were applied, the plates were developed in a solvent mixture made by stirring 75 ml methanol with 60 ml chloroform and 45 ml pyridine. To this was added 12 g boric acid followed by 7.5 ml ddH_2O, 3 ml 88% (v/v) formic acid, 0.375 g 2,6-di-tert-butyl-4-methylphenol and 75 µl technical grade ethoxyquin.

2.7 Analysis of phosphoproteins

2.7.1 Preparation of proteolytic digests for Edman micro-sequencing MALDI-TOF mass spectrometry

Phosphoproteins labelled with ^{32}P were TCA precipitated and separated using SDS-PAGE. Gels were stained with Coomassie Brilliant Blue and protein bands carefully excised with a clean scalpel, washed once in HPLC grade water and then macerated and destained in 50% acetonitrile. After vacuum drying the gel pieces were rehydrated in 10 mM Tris.HCl, pH 7.5-8.5, containing the appropriate protease at an enzyme to substrate ratio of 1:50 and digested overnight at 37°C. Peptides were extracted three times in 50% acetonitrile containing 5% trifluoroacetic acid (TFA), concentrated in a Speedvac and applied to a 1 x 10 mm C8 reverse phase HPLC column. Peptides were collected over a linear gradient of 0-60% acetonitrile, 0.08% TFA and their radioactivity determined by Cerenkov counting. The composition of fractions corresponding to peaks of activity was determined by mass analysis using a Finnigan MALDI-TOF Lasermat instrument. Fractions containing mixtures of peptides were further separated on a C18 HPLC column and re-analysed by MALDI-TOF mass spectrometry.

2.7.2 Phosphoamino acid analysis

HPLC fractions containing ^{32}P-labelled phosphopeptides were dried and subjected to acid hydrolysis in 6M HCl at 110°C for 75 min. Hydrolysates were dried overnight in a Speedvac and phosphoamino acids were analysed by one dimensional thin layer electrophoresis as follows: samples were resuspended in 10 µl of electrophoresis buffer (pyridine:acetic acid:H_2O, 10:100:1890) containing 10 mM *O*-phosphoserine, *O*-phosphothreonine, and *O*-phosphotyrosine standards and spotted onto a Kodak cellulose chromatogram sheet which was then sprayed evenly with electrophoresis buffer. Electrophoresis was performed at 1000 V, 50 mA for 45 min with cooling, after which the plate was dried, stained with ninhydrin spray and developed for 1 minute at 110°C. The chromatogram was exposed to X-ray film and the positions of ^{32}P labelled phosphoamino acids correlated with the ninhydrin stained standards.

2.8 Immunological techniques

Immunological methods not detailed here were performed according to Harlow and Lane (1988). Unless otherwise indicated in figure legends, data obtained using immunological techniques is representative of at least two such experiments.

2.8.1 Affinity purification of polyclonal antisera

Polyclonal antiserum was batch-purified on the L4 PtdIns*P*K IIα peptide immunogen immobilised on Affigel (see Section 2.5.2.d). After washing the affinity matrix in PBS, 1.5 ml antiserum was applied to a 150 μl bed volume of affinity matrix and incubated for 30 min at 4°C. The solid phase was pelleted by centrifugation and then washed extensively in PBS. Bound antibodies were eluted in 200 μl glycine.HCl, pH 2.5 which was rapidly neutralised with NaOH.

2.8.2 Western blotting

Proteins separated on SDS-PAGE gels were transferred to pre-wetted PVDF membranes (Immobilon-P, Millipore) by electrophoresis in transfer buffer (25 mM Tris.HCl, 192 mM glycine, and 20% (v/v) methanol) using an LKB Multiphor II electrophoresis apparatus. Typically, proteins were transferred for 1 h at a charge density of 0.8 mA/cm^2.

After transfer the membrane was blocked for 1 h in PBST (1x phosphate buffered saline containing 0.1% Tween 20) containing 5% (w/v) skimmed milk powder. After washing three times for 10 min in PBST, the membrane was incubated for 1 h in PBST containing 0.5% skimmed milk powder and the primary antibody at the appropriate dilution. Following this step the membrane was washed again and incubated for 1 h with the secondary horse radish peroxidase (HRP)-conjugated antibody as above. The unbound secondary antibody was washed away in PBST and specifically bound antibody detected using the Enhanced Chemiluminescence (ECL) detection reagent (Amersham).

2.8.2 Immunoprecipitation

Cells (1-10 × 10^7) in suspension cultures or monolayers were washed free of serum with cold PBS before lysis on ice in 1 ml of 100 mM Tris.HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Detergent insoluble material was sedimented by centrifugation at 18,000 ×*g* for 10 min and the supernatant incubated with protein A Sepharose or protein G-Sepharose (Pharmacia) for 30 min at 4°C. After this pre-clearing step, the supernatant was taken to fresh tubes containing 1-2 μg of the relevant antibody immobilised on protein A or -G Sepharose and incubated for 1 h at 4°C. The Sepharose beads were washed at least 4 times in lysis buffer before analysis by SDS-PAGE or assayed for lipid kinase activities directly.

2.8.3 Indirect Immunofluorescence

Cells grown on coverslips were fixed in PBS (without Ca^{2+} or Mg^{2+}) containing 3.7% formaldehyde for 10 min, permeabilised with PBS (with Ca^{2+} or Mg^{2+}) containing 0.2% Triton X-100 for 5 min and then washed twice with PBS. Coverslips were incubated for 45 min inverted on parafilm with 15 μl primary antibody at 1:500 dilution (or otherwise diluted approriately) in PBS containing 0.1% (w/v) BSA. After washing 6 times in PBS, the coverslips were incubated in a similar way with FITC-labelled secondary antibody at a 1:400 dilution, then washed six times and mounted on slides for viewing. For co-staining of the actin cytoskeleton, 0.1 μg/ml rhodamine-labelled phalloidin (Molecular Probes) was included with the secondary antibody.

3.0 Cloning and Expression of the Type IIα PtdIns*P* Kinase

3.1 Introduction

PtdIns*P*K activities have been purified from a wide variety of eukaryotic cells and tissues including, erythrocytes (Bazenet *et al.*, 1990; Ling *et al.*, 1989) platelets (Divecha *et al.*, 1995), brain (Van Dongen *et al.*, 1984; Cochet and Chambaz, 1986; Moritz *et al.*, 1990; Divecha *et al.*, 1992; Smith *et al.*, 1992), adrenal medulla (Husebye and Flatmark, 1989), and liver (Urumow and Wieland, 1990). These activities can be distinguished by *in vitro* biochemical and immunological methods and have been classified as either type I or type II activities (Section 1.5.2 and Table 3.1 below). Cellular PtdIns*P*K activity is predominantly cytosolic (Ling *et al.*, 1989; Bazenet *et al.*, 1990; Moritz *et al.*, 1990; Jenkins *et al.*, 1994) but is also found extrinsically associated with the plasma membrane in a variety of different cells and tissues (Ling *et al.*, 1989; Smith and Chang, 1989; Urumow and Wieland, 1990; Bazenet *et al.*, 1990; Jenkins *et al.*, 1994). PtdIns*P*K is found in cytoskeletal fractions along with PtdIns 4K, PLC, and PI 3-kinase (Grondin *et al.*, 1991; Payrastre *et al.*, 1991; Brooksbank *et al.*, 1993; Hinchliffe *et al.*, 1996), the endoplasmic reticulum (Helms *et al.*, 1991) and in nuclear fractions (Helms *et al.*, 1991; Payrastre *et al.*, 1992; Tran *et al.*, 1993). In all of these cases, it is not known what factors determine subcellular localisation of PtdIns*P*K isoforms.

There is substantial evidence that mammalian PtdIns*P*Ks are important regulatory targets for RTK, GPCR, and small GTPase signalling. Firstly, PtdIns*P*K is recruited to the EGF receptor in an agonist-dependent manner (Cochet *et al.*, 1991). Secondly, the GPCR-agonist FMLP stimulates a PtdIns*P*K in neutrophils (Stephens *et al.*, 1993). Thirdly, PtdIns(4,5)P_2 synthesis in fibroblasts is inhibited by the Rho inhibitor C3 transferase and posttranslationally modified Rho stimulates PtdIns*P*K activity in fibroblast lysates (Chong *et al.*, 1994). Furthermore, Rac1 and RhoA have been found to interact with type I PtdIns*P*Ks (Tolias *et al.*, 1995; Tolias *et al.*, 1998; Ren *et al.*, 1996). In addition, type I PtdIns*P*Ks have been strongly implicated in the priming of exocytotic vesicles (Hay *et al.*, 1995) and the downregulation of the CSF-1 receptor, reportedly via an endocytic pathway (Davis *et al.*, 1997). Importantly, in only one of these studies (Davis *et al.*, 1997) was it possible to identify a specific isoform, and it should be noted that this was an ectopically overexpressed fragment. In other experiments it has been impossible to unequivocally identify the isoform involved because of the heterogeneity of PtdIns*P*K isozymes and a lack of specific inhibitors and immunological probes (discussed in detail in Chapter 5).

	Type I*	Type II	Reference
Mass (SDS-PAGE)	68-98	53	see text
Degradation product¶	53	47	see text
Km PtdIns(4)*P* micelles	6 μM	60-250 μM	Bezenet *et al.*, 1991
Km PtdIns(4)*P* liposomes	1.4 μM	40 μM	Bezenet *et al.*, 1991
Activity towards PtdIns*P* in erythrocyte membranes	1.2 μM	not active towards membranes	Bezenet *et al.*, 1991
Effect of PtdOH	activates	no effect	Moritz *et al.*, 1992; Jenkins *et al.*, 1994
Effect of heparin	stimulates	inhibits	Cochet & Chambaz, 1986
Effect of spermine	stimulates	no effect	Bezenet *et al.*, 1991
Effect of PtdIns(4,5)P_2	inhibits	inhibits	Ling *et al*, 1989
Effect of Rac- and Rho-GTP	activates†	no actvation	see Chapter 1
PtdIns(5)*P* 4-K activity	not active	active	Tolias, 1997
Km ATP	25 μM	<5 μM	Ling *et al.*, 1989
Km PtdIns(3)*P*	65 μM (recombinant Iα) 5 μM (recombinant Iβ)	120 μM (recombinant IIα)	Zhang *et al.*, 1997
Km PtdIns(3,4)P_2	80 μM (recombinant Iα) 262 μM (recombinant Iβ)	not active towards PtdIns(3,4)P_2	Zhang *et al.*, 1997
Subcelluar localization	cytosol, membranes	plasma membrane, cytosol, cytoskeleton, nucleus	see text

Table 3.1 Biochemical properties of the PtdIns*P*-kinases

†, Isotype-specific activation has not been studied. *, the high molecular mass type I enzyme may correspond to the recently cloned type Iγ isoform (Ishihara *et al.*, 1998) or to one of the STM7 splice variants. ¶, these degradation products may in fact correspond to the type IIα isoform (53 kDa) and the recently discovered 47 kDa type IIβ and -γ isoforms (Castellino *et al.*, 1997; Itoh *et al*, 1998) which may copurify with the type I and type IIα respectively, and have the potential to cross-react with polyclonal antisera.

An abundant mammalian Ptd*Ins*PK, the 53 kDa type IIα isoform, was the first to be purified to homogeneity in microgram amounts (Divecha *et al.*, 1992) and was therefore considered the best candidate for peptide sequencing. In this chapter the type IIα PtdIns*P*K is cloned and characterised with the aim of defining the PtdIns*P*K family and generating specific immunological reagents for the study of endogenous PtdIns*P*Ks.

3.2 Results

3.2.1 Cloning and sequence analysis

Amino acid sequence data obtained from microsequencing a purified porcine platelet PtdIns*P*K (Divecha *et al.*, 1995) allowed the identification a human expressed sequence tag (EST) by searching the Genbank database. A 350 bp partial cDNA clone (Genbank ID z20468) was obtained and sequenced and found to contain other peptides. The insert contained a short open reading frame, a stop codon and a 3'-untranslated region, but no poly-$(A)^+$ tail. Previous studies of the 53 kDa PtdIns*P*K expression suggested that the enzyme was relatively abundant in Jurkat human T-cell line (Brooksbank *et al.*, 1993). The entire insert was used to screen approximately 1×10^6 plaques of a random-primed, Jurkat cDNA library from which 2 positive clones were obtained. One of these was discarded due to the presence of a portion of the human adenosine deaminase gene. The remaining clone (A5) was fully sequenced and found to contain an open reading frame of 860 nucleotides with no initiating methionine or termination signal. Attempts to isolate the 5'-coding sequence by degenerate PCR using a primer based on N-terminal sequence data proved unsuccessful and it was later discovered that the N-terminal sequence was derived from a contaminant and not the p53 PtdIns*P*K (see Table 8.2.1). The sequence of A5 was extended 182 bp in the 5'-direction using rapid amplification of cDNA ends (RACE) PCR. After cloning and sequencing, these PCR products were found to contain a Kozak consensus sequence (Kozak, 1991) containing an initiating methionine.

The full sequence contained an open reading frame encoding a 406 amino acid protein with a predicted molecular mass of 46.1 kDa. All of the peptides obtained by Edman microsequencing could be aligned with the predicted amino acid sequence (Figure 3.1). Suprisingly, analysis of the derived amino acid sequence indicated little homology to any known lipid, protein or nucleotide kinase with respect to nucleotide or substrate binding sites. Also, with the exception of two short proline-rich regions proximal to the C-terminus, the sequence contained no predictable molecular interaction domains or sites of posttranslational modifications as determined by extensive MOTIFS searching (GCG, University of Wisconsin, WI). Hydrophobicity analysis using PEPPLOT (GCG) and the

```
         10                  30                  50
          .         .         .         .         .         .
gaggggacataggaggcggccatggcgacccccggcaacctagggtcctctgtcctggcg
ctcccctgtatcctccgccggtaccgctgggggccgttggatcccaggagacaggaccgc
     *              >>>M  A  T  P  G  N  L  G  S  S  V  L  A    13

         70                  90                 110
          .         .         .         .         .         .
agcaagaccaagaccaagaagaagcacttcgtagcgcagaaagtgaagctgtttcgggcc
tcgttctggttctggttcttcttcgtgaagcatcgcgtctttcacttcgacaaagcccgg
S  K  T  K  T  K  K  K  H  F  V  A  Q  K  V  K  L  F  R  A      33

        130                 150                 170
          .         .         .         .         .         .
agcgacccgctgctcagcgtcctcatgtgggggtaaaccactcgatcaatgaactgagc
tcgctgggcgacgagtcgcaggagtacacccccatttggtgagctagttacttgactcg
S  D  P  L  L  S  V  L  M  W  G  V  N  H  S  I  N  E  L  S      53

        190                 210                 230
          .         .         .         .         .         .
catgttcaaatccctgttatgttgatgccagatgacttcaaagcctattcaaaaataaag
gtacaagtttagggacaatacaactacggtctactgaagtttcggataagtttttatttc
H  V  Q  I  P  V  M  L  M  P  D  D  F  K  A  Y  S  K  I  K      73

        250                 270                 290
          .         .         .         .         .         .
gtggacaatcaccttttttaacaaagaaaacatgccgagccatttcaagtttaaggaatac
cacctgttagtggaaaaattgtttcttttgtacggctcggtaaagttcaaattccttatg
V  D  N  H  L  F  N  K  E  N  M  P  S  H  F  K  F  K  E  Y      93

        310                 330                 350
          .         .         .         .         .         .
tgcccgatggtcttccgtaacctgcgggagaggtttggaattgatgatcaagatttccag
acgggctaccagaaggcattggacgccctctccaaaccttaactactagttctaaaggtc
C  P  M  V  F  R  N  L  R  E  R  F  G  I  D  D  Q  D  F  Q     113

        370                 390                 410
          .         .         .         .         .         .
aattccctgaccaggagcgcacccctccccaacgactcccaggcccgcagtggagctcgt
ttaagggactggtcctcgcgtggggaggggttgctgagggtccgggcgtcacctcgagca
N  S  L  T  R  S  A  P  L  P  N  D  S  Q  A  R  S  G  A  R     133

        430                 450                 470
          .         .         .         .         .         .
tttcacacttcctacgacaaaagatacatcatcaagactattaccagtgaagacgtggcc
aaagtgtgaaggatgctgttttctatgtagtagttctgataatggtcacttctgcaccgg
F  H  T  S  Y  D  K  R  Y  I  I  K  T  I  T  S  E  D  V  A     153

        490                 510                 530
          .         .         .         .         .         .
gaaatgcacaacatcctgaagaaataccaccagtacatagtggaatgtcatgggatcacc
ctttacgtgttgtaggacttctttatggtggtcatgtatcaccttacagtaccctagtgg
E  M  H  N  I  L  K  K  Y  H  Q  Y  I  V  E  C  H  G  I  T     173
```

```
        550                 570                 590
         .         .         .         .         .         .
cttcttccccagttcttgggcatgtaccggcttaatgttgatggagttgaaatatatgtg
gaagaaggggtcaagaacccgtacatggccgaattacaactacctcaactttatatacac
L  L  P  Q  F  L  G  M  Y  R  L  N  V  D  G  V  E  I  Y  V     193
        610                 630                 650
         .         .         .         .         .         .
atagttacaagaaatgtattcagccaccgtttgtctgtgtataggaaatacgacttaaag
tatcaatgttctttacataagtcggtggcaaacagacacatatcctttatgctgaatttc
I  V  T  R  N  V  F  S  H  R  L  S  V  Y  R  K  Y  D  L  K     213

        670                 690                 710
         .         .         .         .         .         .
ggctctacagtggctagagaagctagtgacaaagaaaaggccaaagaactgccaactctg
ccgagatgtcaccgatctcttcgatcactgtttcttttccggtttcttgacggttgagac
G  S  T  V  A  R  E  A  S  D  K  E  K  A  K  E  L  P  T  L     233

        730                 750                 770
         .         .         .         .         .         .
aaagataatgatttcattaatgagggccaaaagatttatattgatgacaacaacaagaag
tttctattactaaagtaattactcccggttttctaaatataactactgttgttgttcttc
K  D  N  D  F  I  N  E  G  Q  K  I  Y  I  D  D  N  N  K  K     253

        790                 810                 830
         .         .         .         .         .         .
gtcttcctggaaaaactaaaaaaggatgttgagtttctggcccagctgaagctcatggac
cagaaggacctttttgatttttttcctacaactcaaagaccgggtcgacttcgagtacctg
V  F  L  E  K  L  K  K  D  V  E  F  L  A  Q  L  K  L  M  D     271

        850                 870                 890
         .         .         .         .         .         .
tacagtctgctggtgggaattcatgatgtggagagagccgaacaggaggaagtggagtgt
atgtcagacgaccacccttaagtactacacctctctcggcttgtcctccttcacctcaca
Y  S  L  L  V  G  I  H  D  V  E  R  A  E  Q  E  E  V  E  C     293

        910                 930                 950
         .         .         .         .         .         .
gaggagaacgatggggaggaggagggcgagagcgatggcacccacccggtgggaacccccc
ctcctcttgctacccctcctcctcccgctctcgctaccgtgggtgggccacccttgggggg
E  E  N  D  G  E  E  E  G  E  S  D  G  T  H  P  V  G  T  P     313

        970                 990                 1010
         .   <-  GS2  .         .         .         . <- GS1
ccagatagccccgggaatacactgaacagctcaccacccctggctcccggggagttcgag
ggtctatcggggccttatgtgacttgtcgagtggtggggaccgagggccccctcaagctc
P  D  S  P  G  N  T  L  N  S  S  P  P  L  A  P  G  E  F  E     333

       1030                1050                1070
         .         .         .         .         .         .
ccgaacatcgacgtctatggaattaagtgccatgaaaactcgcctaggaaggaggtgtac
ggcttgtagctgcagataccttaattcacggtacttttgagcggatccttcctccacatg
P  N  I  D  V  Y  G  I  K  C  H  E  N  S  P  R  K  E  V  Y     353
```

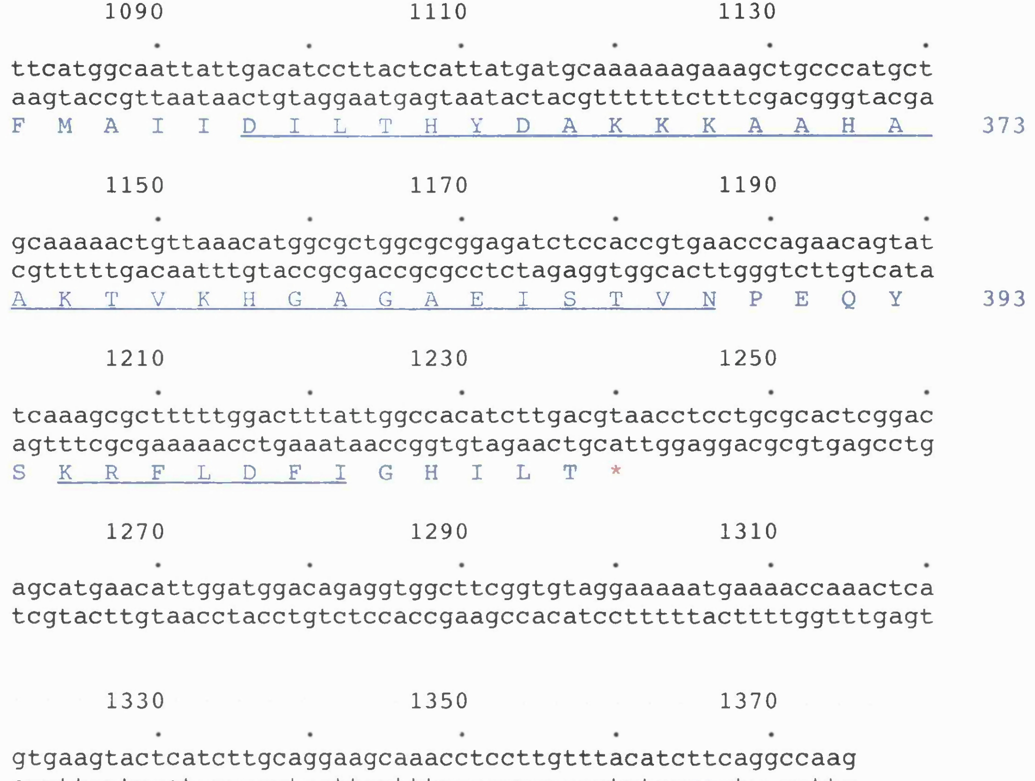


Figure 3.1 Nucleotide and predicted amino acid sequence of PtdIns*P*K IIα

Peptide sequences obtained by Edman microsequencing are underlined. The sequence of the L4 antigenic peptide is shown in italics. The positions of antisense RACE primers GS1 and GS2 are underlined. Lysine residues K91, K145, and K213, targetted by site-directed mutagenesis are highlighted in red. Red asterix indicates a stop codon and >>>; initiation codon.

```
Fabl       ..THSQANLDTLQELEKIMTKKTATHLRYQFEEGLTVMSCKIFFTEHFDVFRK..ICDCQENFIQSLSRCVKWD..SNGGKSGSGFLKTLDDRFIIKELS
CeORF1     ...SSSLKFSQMDDGENLASSQ...HLEVEFEDESASYYVKMLYAEKFRKLRELLIAEGEETFIRSLSNSTFWT..PQGGKSGSFFYRTQDDRFVVKQMS
At 1       ........MFLRATVENRIRYSTKHIKHLPPGSITEFDWKDYCPVGFGLIQELEGIDHDD.YLLSICTDETLKKISS.GKIGNVFHISNDNRFLIKILR
At 4       .MFTREITAKDVKATEKNRIRYSSKHIKHLPPGTITEFEWKDYCPLGFRLIQELEDINHDE.YMKSICNDETLRKLST.SKVGNMFLLSKDDRFLIKILR
At 2       .SIVRDLKQTDFDPKEKFWTRFPPEGTKTTPPHQSVDFRWKDYCPLVFRRLRELFQVDPAK.YMLAICGNDALRELSSPGKSGSFFYLTQDDRFMIKTVK
At 3       ...RREVRASDFGKNARTKMFFPRDGSNFTPPHKSVDFSWKDYCPMVFRNLRQMFKLDAAE.YMMSICGDDGLTEISSPGKSGSIFYLSHDDRFVIKTLK
HsIbeta    ...ERDVLMQDFYVVESIFFPSE..GSNLTPAHHYNDFRFKTYAPVAFRYFRELFGIRPDD.YLYSLC.SEPLIELCSSGASGSLFYVSSDDEFIIKTVQ
MmIgamma   ...ERDVLMQDFYVMESIFFPSE..GSNFTPAHHFQDFRFKTYAPVAFRYFRELFGIRPDD.YLYSLC.NEPLIELSNPGASGSVFYVTSDDEFIIKTVM
HsSTM7     ...ERDVLMQDFYVVESVFLPSE..GSNLTPAHHYPDFRFKTYAPLAFRYFRELFGIKPDD.YLYSIC.SEPLIELSNPGASGSLFFVTSDDEFIIKTVQ
CeORF 2    ...NRDVLLQDFEKVDIVAFPAA..GSTITPSHSFGDFRFRTYAPIAFRYFRNLFHIKPAD.FLRSIC.TEPLKELSNAGASGSIFYVSQDDQFIIKTVQ
Dm 1       ...KRDLLMNDFWEMETISFPPD..GSSITPAHHYNDFRFKVYAPIAFRYFRDLFGIAPDD.FLMSMC.ASPLRELSNPGASGSIFYLTTDDEFIIKTVQ
HsIIalpha  ..IPVMLMPDDFKAYSKIKVDNHLFNKENMPSH....FKFKEYCPMVFRNLRERFGIDDQD.FQNSLTRSAPLPN.DSQARSGARFHTSYDKRYIIKTIT
HsIIbeta   VPVPVMLMPDDFKAYSKIKVDNHLFNKENLPSR....FKFKEYCPMVFRNLRERFGIDDQD.YQNSVTRSAPINS.DSQGRCGTRFLTTYDRRFVIKTVS
RnIIgamma  ..PPVMLLPDDFKASSKIKVNNHLFHRENLPSH....FKFKEYCPQVFRNLRDRFAIDDHD.YLVSLTRSPPSET.EG...SDGRFLISYDRTLVIKEVS
Mss4       ...MKPLTPADFRFTKKLAFDYH..GNELTPSSQYA.FKFKDYCPEVFRELRALFGLDPAD.YLVSLTSKYILSELNSPGKSGSFFYYSRDYKYIIKTIH
                                                                                             I          II

Fabl       HAEL.................EAFIKFAPSYFEYMAQAMFHDLPTTLAKVFGFYQIQVKSSISS.......SKSYKMDVIIMENLFYEK.KTTRIFDLKG
CeORF1     RFEI.................QSFVKFAPNYFDYLTTSATESKLTTLCKVYGVFRIGYK...SK.......TTTLKVDILVMEYLFYNH.NVSQVWDLKG
At 1       KSEIKCVYMFHIESFIENNLVKVTLEMLPRYYRHINY....HRSSLFTRIFGAHSVKPLGGVKVTRIIYIYHSIIRTYFAVMSNMLHSTIFVNKLYDLKG
At 4       KSEI.................KVILEMLPGYFRHIHK....YRSTLLSKNYGAHSVKPIGGVK.............TYFVVMSNILQSDVFMNKVYDLKG
At 2       KSEV.................KVLLRMLPSYYKHVCQ....YENSLVTRFYGVHCVKPVGGQKTR............FIVMGNLFCSEYRIQRRFDLKG
At 3       KSEL.................QVLLRMLPKYYEHVGD....HENTLITKFFGVHRITLKWGKKVR............FVVMGNMFCTELKIHRRYDLKG
HsIbeta    HKEA.................EFLQKLLPGYYMNLNQ....NPRTLLPKFYGLYCV........Q......AGGKNIRIVVMNNLLPRSVKMHIKYDLKG
MmIgamma   HKEA.................EFLQKLLPGYYMNLNQ....NPRTLLPKFYGLYCV........Q......SGGKNIRVVVMNNVLPRVVKMHLKFDLKG
HsSTM7     HKEA.................EFLQKLLPGYYMNLNQ....NPRTLLPKFYGLYCM........Q......SGGINIRIVVMNNVLPRSMRMHFTYDLKG
CeORF 2    HKEA.................DFLQKLLPGYYMNLNQ....NPRTLLPKFFGLFCY........Q......SLGKNIRLLVMNNLLPQTVTMHEKYDMKG
Dm 1       KKEC.................EFLQKLWPGYYMNLSQ....NPRTLLPKFFGLYCF........H......YNSKNVRLVAMNNLLPSDIKMHCKYDLKG
HsIIalpha  SEDV.................AEMHNILKKYHQYIVEC...HGITLLPQFLGMYRL........N......VDGVEIYVIVTRNVFSHRLSVYRKYDLKG
HsIIbeta   SEDV.................AEMHNILKKYHQFIVEC...HGNTLLPQFLGMYRL........T......VDGVETYMVVTRNVFSHRLTVHRKYDLKG
RnIIgamma  SEDI.................ADMHSNLSNYHQYIVKC...HGNTLLPQFLGMYRV........S......VENEDSYMLVMRNMFSHRLPVHRKYDLKG
Mss4       HSEH.................IHLRKHIQEYYNHVRD....NPNTLICQFYGLHRVKMPISFQNK......IKHRKIYFLVMNNLFPPHLDIHITYDLKG
                                                                                                    VIb

Fabl       SMRNRHVEQTGKANE.......VLLDENMVEYIYESPIHVREYDKKLLRASVWNDTLFLAKMNVMDYSLVIGI..........................
CeORF1     SLRNRLASTGKSANEM......VLLDENFVKDLWNQQLYVLPHSKAAMNQAISNDSHFLSSQYIMDYSLLVGV..........................
At 1       SPKGRSNKKI..EVRNT....TVLKDIDF.....DFCFYVDPLARQRIIKQTKLDCELLEEEGIMDYSLLVGLQSKGSCQGSLDGLNPVYGSFAPPSSFK
At 4       SSQGRTNKKI..KVRDK....TILKDIDL.....DFCFYVDSLARHRLIKQTKLDCELLEDEGIMDYSLMLGLQVKGSCHGSIDELIPVYDSFTSRGSVD
At 2       SSHGRSTAKPEGEIDET....TTLKDLDL.....NFSFRLQRNWYQELMKQIKRDCEFLEAERIMDYSLLVGVHFRD.............DNTGEKMGLS
At 3       STQGRFTEKI..KIQEK....TTLKDLDL.....AYEFHMDKLLREALFKQIYLDCSFLESLNIIDYSLLLGLHFRAP..GQLNDILEPPNAMSDQESVS
HsIbeta    STYKRRASQKEREKP.....LPTFKDLDFLQDI.PDGLFLDADMYNALCKTLQRDCLVLQSFKIMDYSLLMSIH..........................
MmIgamma   STYKRRASKKEKEKS.....LPTYKDLDFMQDM.PEGLLLDSDTFGALVKTLQRDCLVLESFKIMDYSLLLGVH..........................
HsSTM7     STYKRRASRKEREKS.....NPTFKDLDFLQDM.HEGLYFDTETYNALMKTLQRDCRVLESFKIMDYSLLLGIH..........................
CeORF 2    STYKRMANKAERAKP.....HPTLKDLDFLENH.KDGIFIDPTALEALMKSISRDCLVMESFKIMDYSLLVGIH..........................
Dm1        SSFRRKASKAERQKA.....SPTFKDLDFAEHH.PNGIFLETDKYNSIMSTIKRDCMVLESFQIMDYSLLVGIH..........................
HsIIalpha  STVAREAS.DKEK.AKE...LPTLKDNDFINE..GQKIYIDDNNKKVFLEKLKKDVEFLAQLKLMDYSLLVGIH..........................
HsIIbeta   STVAREAS.DKEK.AKD...LPTFKDNDFLNE..GQKLHVGEESKKNFLEKLKRDVEFLAQLKIMDYSLLVGIH..........................
RnIIgamma  SLVSREAS.DKEK.VKE...LPTLKDMDFLNK..NQKVYIGEEEKKVFLEKLKRDVEFLVQLKIMDYSLLLGIH..........................
Mss4       STWGRFTNLDKERLAKDRSYRPVMKDLNWLEE..GQKIKLGPLKKKTFLTQLKKDVELLAKLNTMDYSLLIGIH..........................
                                                                          VII

Fabl       ....................................................................................................
CeORF1     ....................................................................................................
At 1       SNSTKSMKTASSSPDR........SSVAMYSCSPDRDSVENEMSMTIQSVTSNSASS..........ETNILATTLSDLFHNSSNINFGMKIPARARRVT
At 4       SNSSKFMKTASNSPDR........SSSTMYSCTPSRNSVDSENSVNIQSVASISPSPAQTNASDSPYESLVSKTNLTNIFQNSSSTNFGMKIPGRARRVG
At 2       PFVLRSGRIDSYQNEKFM.................RGCRF..LEAELQDMDRILAGR.KP........................SIRLGANMPAKAERMA
At 3       SVDVGLTQEHSIPPKGLLLVTHEPNSVNTAPGPHIRGSTLRAFSVGEQEVDLILPGTARL........................RVQLGVNMPAQAHH..
HsIbeta    ..NIDHAQREPLSS..................ETQYSVDTRRPAPQKAL..........................YSTAME...SIQGEARRGG
MmIgamma   ..NIDQQERERQAE..................GAQSKADEKRPVAQKAL..........................YSTAME...SIQGGAARGE
HsSTM7     ..FLDHSLKEKEEE..................TPQNVPDAKRTGMQKVL..........................YSTAME...SIQGPGKSGD
CeORF 2    ..NVELGIKERAEQ..................NRESHPA..PSTSSDTF..........................SGEKAE...NHKVLQEKFS
Dm1        ..NLDLAAKEKREERILNARAKLQRKESAAQGPPSLNPDDDAPEADQNQIQAVASYASIPGTSAGAALNRTRSMNRQRLVAHSTALE...SITADM...D
HsIIalpha  ..DVERAEQEEVECEENDWG.....G...GGRDGWHPPGGTPPDSPGNTL....................................NSSPPL
HsIIbeta   ..DVDRAEQEEMEVEERAEDEECEND...GVGGNLLCSYGTPPDSPGNLL....................................SFPRFF
RnIIgamma  ..DIIRGSEPEEEGPVREEESEWDGDCNLTGPPALVGSYGTSPEGIGGYI....................................HSHRPL
Mss4       ..DINKAKEDDLQLADTASIEE.....QPQTQGPIRTGTGTVVRHFFREF....................................EGGIR.

Fabl       ...................DNEGYTLTVGIIDFIRTFTWDKKLESWVKEKGLVGGASVIKQPTVVTPRQYKKRFREAMERYILMVPDPWYREGN......
CeORF1     ...................DDDNGELILGIVDYMRTYTLDKKLESWVKIVAIPGA....HLPTILSPEMYCARFSEAIDSYFPVVPDQWTGLGSIRSY..
At 1       R..E..........TGEEEWYNVVLYIGIVDTFQDYGMKKRIEHCYKSIQYNSNS.I....STVHPKIYSSRFQDFVSNIFLPHDDDLSSKY.......
At 4       R..GESGSVVGKQSREGGEEWYDVILYLGIIDIFQDYGVRKRLEHCYKSIQHSSKT.I....SAVHPKMYSSRFQDFVSQIFLPDEDPSH.........
At 2       RRSDFDQYSSGGASYPSHGEMYEVVLYFGVIDILQDYDITKKIEHAYKSLQADPAS.I....SAVDPKLYSKRFRDFISRIFIEEG.............
At 3       ...KLIEDKEESATIELF.EVYDVVVYMGIIDILQEYNTKKKVEHTCKSLQYDPMT.I....SVTEPSTYSKRFVNFLHKVFPEER.............
HsIbeta2   TMETD..D.HMGGIPARNSKGERLLLYIGIIDILQSYRFVKKLEHSWKALVHDGDT.V....SVHRPGFYAERFQRFMCNTVFKK.I..PLKPSPSKKFR
MmIgamma   AIETD..D.TMGGIPAVNGRGERLLLHIGIIDILQSYRFIKKLEHTWKALVHDGDT.V....SVHRPSFYAERFFKFMSSTVFRKSS..SLKSSPSKKGR
HsSTM7     GIITENPD.TMGGIPAKSHRGEKLLLFMGIIDILQSYRLMKKLEHSWKALVYDGDT.V....SVHRPSFYADRFLKFMNSRVFKKIQ..ALKASPSKKRC
CeORF 2    VWDTGDGDVPHGGVPARNSNGDRLVLYLGIIDILQNYRLLKKMEHTWKAILHDGDT.I....SVHNPNFYASRFLTFMTEKVFKKGT..ALKQSPSRRR.
dm1        VPLEEDEDVPAGGIPARSENDERLILYIGIIDILQSYRLEKKLEHTFKSILYNGDT.V....SVCRPSFYAKRFQDAMGKQVFKKTPTFPLKHSPSKRKT
HsIIalpha  APGEFDPNIDVYGIKCHENSPRKEVYFMAIIDILTHYDAKKKAAHAAKTVKHGC.AEI....STVNPEQYSKRFLDFIGHILT................
HsIIbeta   GPGEFDPSVDVYAMKSHESSPKKEVYFMAIIDILTPYDTKKKAAHAAKTVKHGAGAEI....STVNPEQYSKRFNEFMSNILT................
RnIIgamma  GPGEFESFIDVYAIRSAEGAPEGGVFH.GLIDILTQYDAKKKAAHAAKTVKHGAGAEI....STVHPEQYAKRFLDFISNIFA................
Mss4       ASDQFNNDVDL.............IYYVGIIDFLTNYSVMKKLETFWRSLRHDTKL.V....SAIPPRDYANRFYEFIEDSVDPLPQKKTQSSYRDDPNQ
```

Figure 3.2 Alignment of PtdIns*P*K calaytic domains

Sequence alignment was generated using the PILEUP program (GCG, University of Wisconsin, Madison, WI). Sequence identity is shown in red and conservation in blue. Roman numerals refer to regions proposed to correspond to the subdomain organisation of the protein kinases (for details see Chapter 5). For definitions of sequences and acession numbers see Figure 1.6. Pairwise similarity scores from this alignment are given in Table 8.2.

lack of any discernable transmembrane segments indicated that this protein was likely to be soluble (features the amino acid sequence are discussed in more detail in Chapter 4). In consideration of the expression and activity data detailed below, this cDNA and its product is subsequently referred to as PtdIns*P*K IIα (PtdIns*P*K IIα).

3.2.2 Antipeptide antibodies recognise a 53 kDa protein in Jurkat cell lysates

Rabbit polyclonal antiserum was raised to a synthetic peptide (L4) with a sequence corresponding to amino acids 101-114 (LRERFGIDDQDFQ). The specificity of immune serum was tested by western blotting against recombinant PtdIns*P*K IIα and Jurkat cell lysates (Figure 3.3 A). Whilst immune serum recognised the recombinant PtdIns*P*K IIα, non-specific background was observed when both immune and pre-immune sera was used to probe Jurkat lysates (Figure 3.3 A). The antiserum was affinity-purified against immobilised L4 peptide immunogen and subsequently used to probe a western blot of a Jurkat cell lysate where a major 53 kDa band was observed (Figure 3.3 B). The identities of the minor proteins visible above and below the 53 kDa band are unknown, however it is possible that the latter represents cross reactivity with the 47 kDa PtdIns*P*K IIβ isoform because the sequence of the L4 antigenic peptide is conserved between these closely related isoforms. This antiserum was unable to immunoprecipitate significant amounts of lipid kinase activity from Jurkat cell lysates (results not shown).

3.2.3 Bacterial expression

The full open reading frame was assembled by ligating together the 5' RACE product, the A5 Jurkat library clone and the 3'-end from the z20468 probe. PCR was then used to incorporate a 5' Bam HI restriction site into the full length PtdIns*P*K IIα cDNA. The open reading frame was fully sequenced and expressed as an N-terminal GST fusion protein in pGEX-2T. Affinity-purified recombinant proteins were analysed by SDS-PAGE. The GST fusion protein had an apparent molecular mass of 79 kDa, comprising the 46 kDa kinase and the 26 kDa GST domain (Figure 3.4). As shown in Figure 3.4, the majority of GST::PtdIns*P*K IIα was insoluble. As well as the major 79 kDa protein, preparations were also found to contain a number of lower molecular mass proteins likely to be fragments of the fusion protein.

3.2.4 Activity

Purified GST fusion proteins were eluted with free glutathione and assayed for kinase activity against PtdIns*P*. The products of this reaction comigrated with [^{3}H]-PtdIns(4,5)P_2

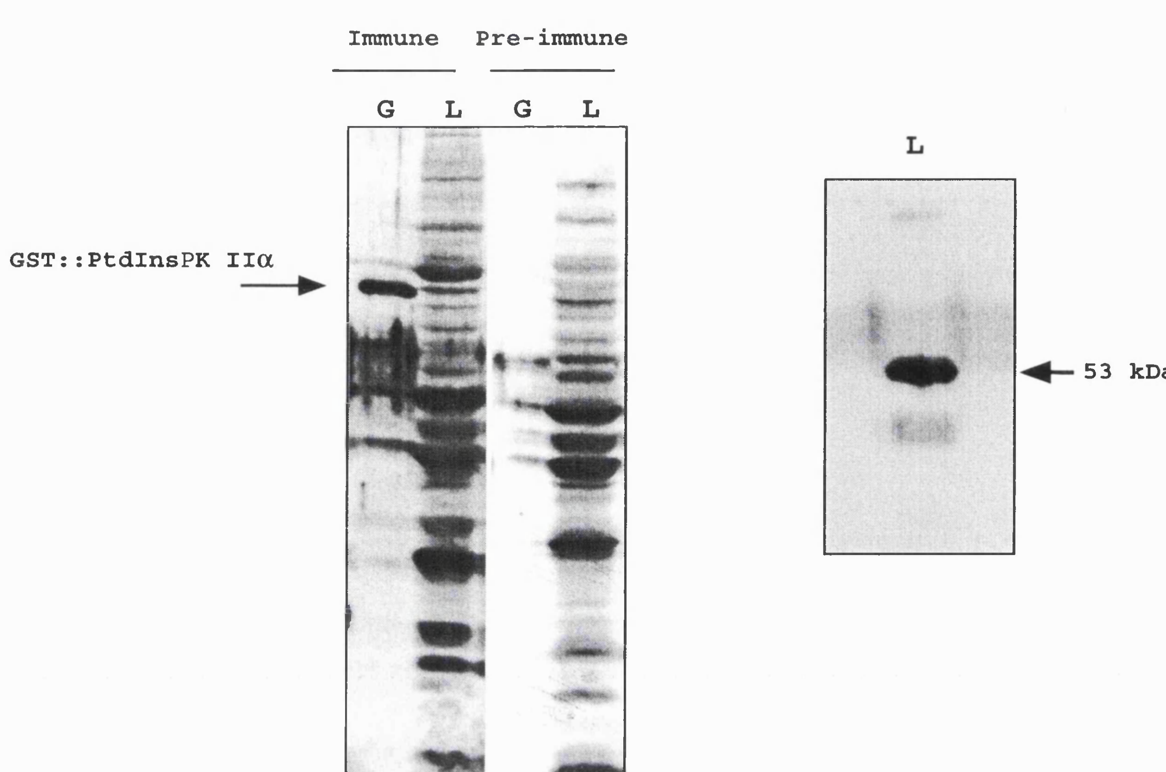


Figure 3.3 Antiserum raised to synthetic peptide detects a 53 kDa protein in cell lysates

A. Serum from a rabbit immunised with the synthetic peptide L4, or pre-immune serum from the same rabbit was used to probe identical western blots containing recombinant GST::PtdIns*P*K IIα (G) or approximately 50 μg of a total Jurkat cell lysate (L). B Immune serum was subsequently affinity purified on the peptide immunogen immobilised on Affigel resin (Bio Rad, see Section 2.5.2.d) and used to probe Jurkat cell lysates.

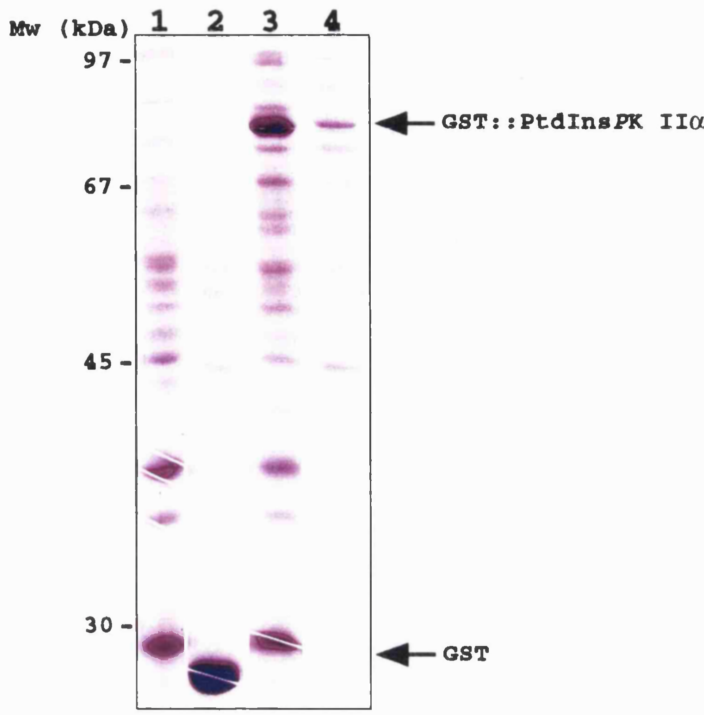
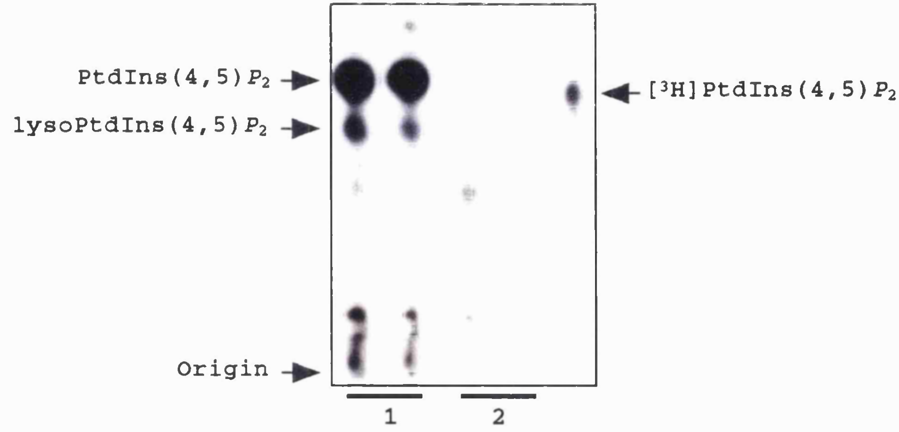


Figure 3.4 Recombinant expression of PtdIns*P*K IIα in *E. coli*.

The full length PtdIns*P*K IIα cDNA was cloned into pGEX-2T (Pharmacia) and expressed as an N-terminal fusion protein in *E.coli* XL1-Blue. 2 ml logarithmic-phase cultures containing pGEX-2T::PtdIns*P*KIIα or parental vector (control) were induced with 0.1 mM IPTG for 3 h at 30°C. Cells were collected by centrifugation and sonicated in lysis buffer (100 mM Tris.HCl, pH 7.4, 100 mM KCl, 1% Triton X-100, 10 mM β-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 1μg/ml aprotinin, 1μg/ml leupetin and 1μg/ml poly(L)lysine). Lysates were cleared at 18,000 *xg* and the supernatant applied to GSH-Sepharose resin (Pharmacia) previously equilibrated in lysis buffer, and incubated for 20 min at room temperature. After washing three times in lysis buffer the affinity-purified proteins were analysed by SDS-PAGE. Lane 1; pGEX-2T soluble fraction, lane 2; pGEX-2T insoluble fraction, lane 3; pGEX-2T::PtdIns*P*KIIα insoluble protein, lane 4; pGEX-2T::PtdIns*P*KIIα affinity-purified protein.

A



B

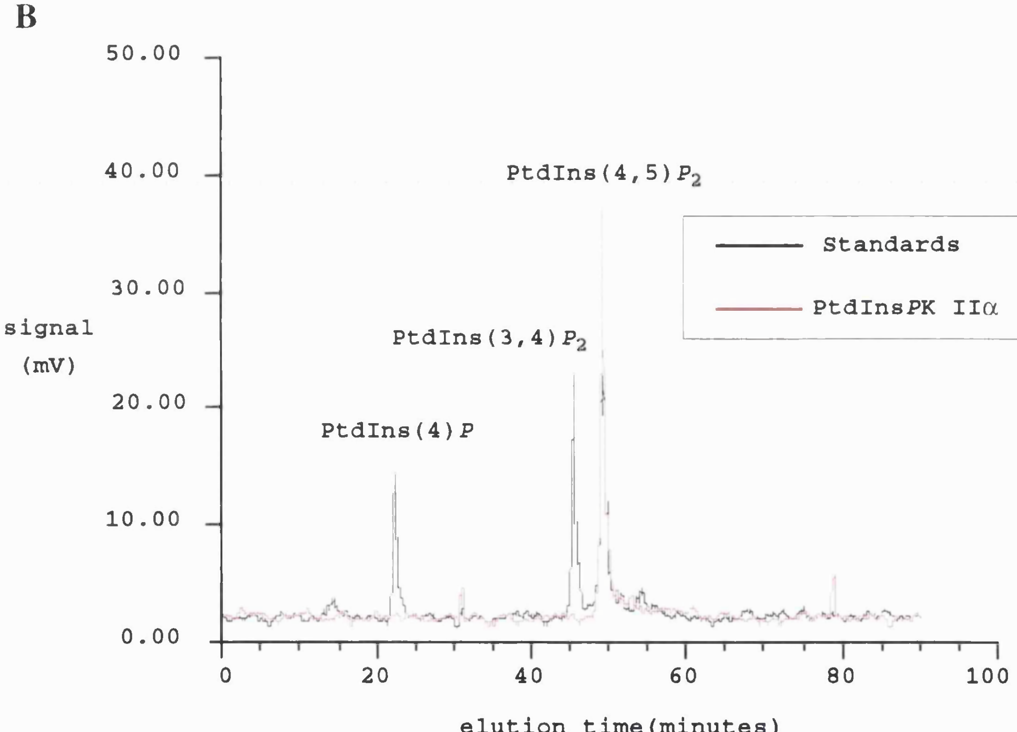


Figure 3.5 The isolated cDNA encodes a PtdIns*P* kinase

Approximately 100 ng of GST fusion protein was eluted from GSH-Sepharose with 10 mM glutathione and assayed for PtdIns*P*K activity. Reactions were performed in 50 mM Tris.HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 50 mM PtdIns*P* (Sigma), 20 μM ATP and 5 μCi of γ[^{32}P]ATP. Assays were started by the addition of ATP, incubated for 15 min at 37°C and terminated by adding HCl to 500 mM. The organic phase was extracted and the lipid products separated by TLC as described in Chapter 2. The PtdInsP_2 spots were scraped from the TLC plate and analysed by deacylation and HPLC as described previously (Serunian *et al.*, 1991).

A. Autoradiogram of the reaction products chromatographed alongside a [^{3}H]-PtdIns(4,5)P_2 standard (1, GST::PtdIns*P*KIIα reaction; 2, GST control). B. HPLC headgroup analysis of the deacylated reaction products.

when separated by TLC. Deacylation and headgroup analysis by HPLC revealed a single product which coeluted with a *Gro*PtdIns(4,5)P_2 standard (Figure 3.5), thereby confirming that the ORF encoded a PtdIns*P*K. GST::PtdIns*P*K IIα activity was not stimulated by PtdOH under conditions that activated recombinant human PtdIns*P*K Iα/STM7 (data not shown).

3.2.5 Recombinant expression in *S. frugiperda* cells

Expression of PtdIns*P*K IIα in bacteria yielded a population of partially truncated proteins which are most likely the result of proteolysis and/or premature translational termination due to overexpression of rare codons in *E. coli* (Figure 3.6, note that this phenomenom is clearer in other experiments, e.g. Figure 4.6). In order to overcome this problem and to improve the yield, solubility, and specific activity of recombinant protein for later structural studies, the PtdIns*P*K IIα ORF was cloned into the baculovirus transfer vector pAcG-2T. Sf9 cells were co-transfected with the transfer vector harboring the PtdIns*P*K IIα ORF and Baculovirus DNA (BaculoGold, Invitrogen) prior to the generation of a high titre stock (Section 2.3.2).

A time course study of the expression of GST::PtdIns*P*K IIα in baculovirus-infected Sf9 cells was used to determine that the optimum yield of soluble protein occurred at 72 h post infection (data not shown). Purification was also analysed by western immunoblotting (Figure 3.6) using affinity-purified αL4 antisera in order to evaluate the efficiency of the purification method, the purity of the protein and the extent of proteolytic degradation. Importantly, baculovirus-expressed protein typically exhibited a higher specific activity over its bacterial counterpart (data not shown), a phenomenom that may result from the presence of inhibitory fragments or a lack of a post-translational modification in the *E. coli* preparations. As expected, yields of protein from insect cells greatly exceeded those from *E.coli* preparations (typically 20 mg/l and 200 μg/l, respectively) and very little degradation was apparent in the Sf9 preparations (Figure 3.6).

3.2.6 Recombinant expression in mammalian cells

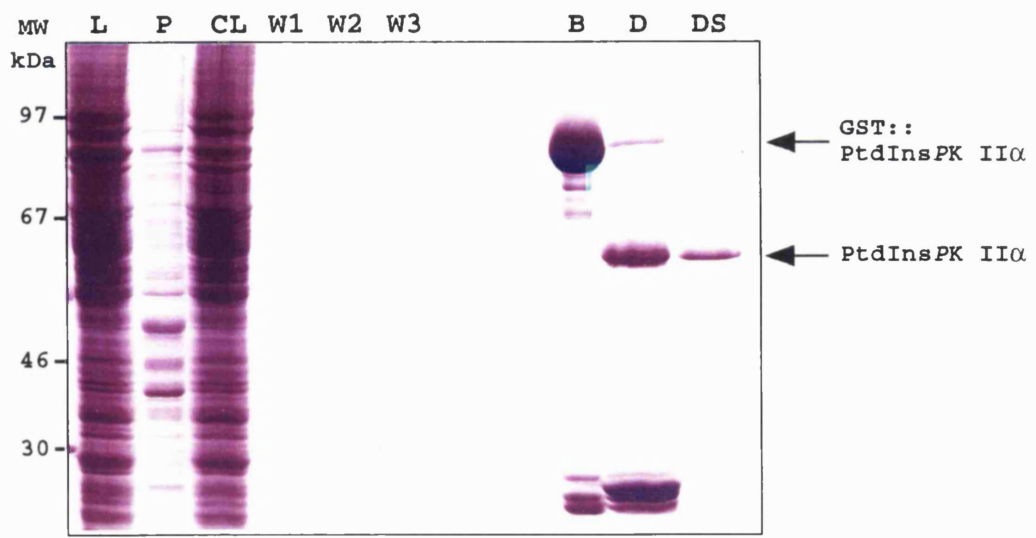
In order to study the *in vivo* properties of PtdIns*P*K IIα such as its effects on cell morphology, levels of phosphoinositide and subcellular localisation by immunofluorescence, the PtdIns*P*K IIα ORF was cloned into a modified pEF-BOS vector (Mizushima and Nagata, 1990) and expressed with an N-terminal myc epitope tag. Western blotting of total cell lysates from transfected human Jurkat, A431 and simian COS cells with 9E10 detected a single band of 53 kDa which was not present in mock transfected cells (Figure 3.7). Anti-myc immunoprecipitates from these cells contained

Figure 3.6 Expression of PtdIns*P*KIIα in Sf9 cells

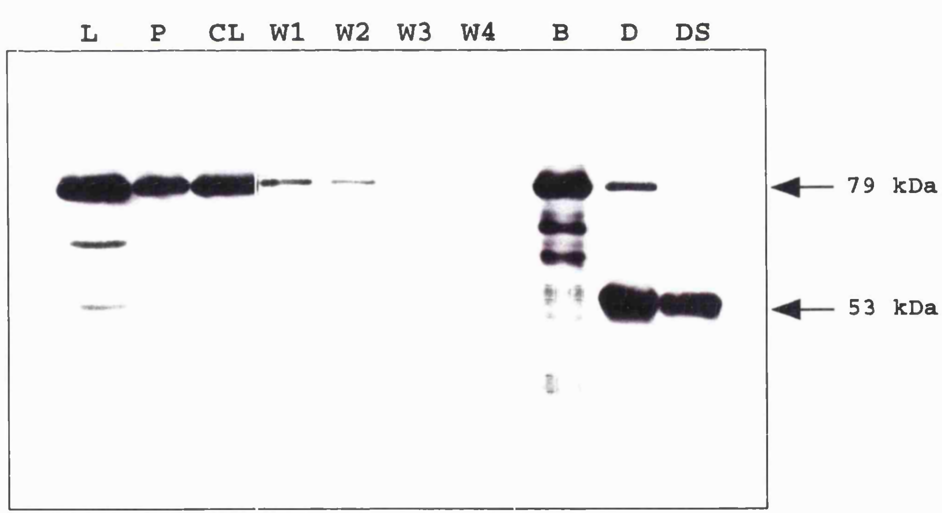
150 ml spinner cultures of Sf9 cells (2.0 $\times$ 10^6/ml) were infected with 300 μl of high-titre recombinant baculovirus expressing GST::PtdIns*P*K IIα. After growth for 72 h, a period previously determined to be optimal for expression of this fusion protein, cells were collected by low speed centrifugation. Cells were lysed in 15 ml of buffer containing 100 mM Tris.HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 10 mM β-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1μg/ml aprotinin, 1μg/ml leupetin and 1μg/ml poly(L)lysine. Total cell lysate was cleared at 30,000 *xg* and the supernatant incubated with GSH-Sepharose for 45 min at 4°C. The beads were washed four times in lysis buffer containing 200 mM NaCl prior to cleavage with thrombin. Thrombin cleavage was carried out by incubating the beads in ten bed volumes of cleavage buffer (50 mM Tris.HCl, pH 8.0, 80 mM NaCl and 5 mM β-mercaptoethanol) with 7.5 units of bovine thrombin (Calbiochem) for 20 minutes at room temperature after which the reaction was stopped by the addition of 0.1 mM APMSF (Boeringer Manneheim).

All steps in the purification protocol were analysed by SDS-PAGE (A) and western blotting with affinity-purified αL4. Lanes are as follows: L; Triton X-100 lysate, P; Triton X-100-insoluble pellet, CL; lysate after incubation with GSH-speharose, W1-W4; supernatants from washing steps 1-4 (note W4 is not shown in 3.7 A), B; washed GSH-Sepharose beads, D; thrombin digestion reaction, DS; supernatant from the thrombin digest.

A



B



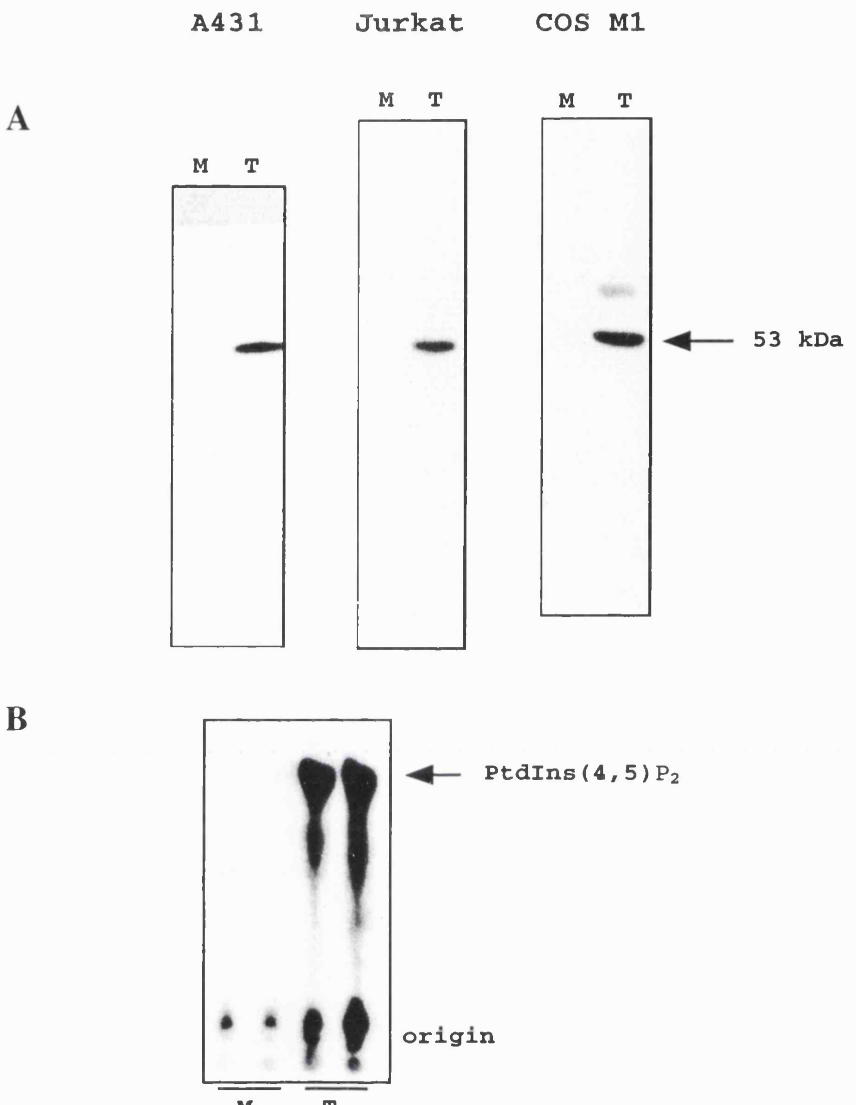


Figure 3.7 Expression of PtdIns*P*KIIα in mammalian cells

The mammalian expression vector pEF-BOS containing the PtdIns*P*K IIα ORF fused to an N-terminal myc epitope tag (see text), was introduced into A431 cells by lipid-mediated transfection and into Jurkat and COS M1 cells by electroporation (Section 2.3.3.a).

A. Total cell lysates from transfected (T) or mock-transfected (M, control) cells were probed with the 9E10 anti-myc monoclonal antibody.

B. Triton X-100 cell lysates from transfected or mock-transfected Jurkat cells were also immunoprecipitated with the same antibody, washed extensively, and assayed for PtdIns*P*K activity. The products of the PtdIns*P*K reactions were analysed by TLC .

PtdIns*P*K activity (Figure 3.6). Immunoprecipitation from transfected Jurkat cells, metabolically-labelled with [^{35}S]-cysteine and methionine, failed to reveal any associated proteins (not shown).

3.2.7 Subcellular localisation of PtdIns*P*K IIα in MDCK cells

Intranuclear microinjection was used to introduce the epitope-tagged mammalian expression construct into MDCK cells. After fixing and staining with 9E10 followed by goat anti-mouse IgG antibodies conjugated to FITC, cells were examined using confocal microscopy (Figure 3.8). No visible changes in the morphology of injected cells were observed. Protein was predominantly cytoplasmic and appeared to be completely absent in the nucleus. A ring was also observed around the nucleus indicating a possible association with the nuclear membrane. The punctate pattern seen in the cytoplasm is probably due to the exclusion of PtdIns*P*K IIα from small subcellular structures such as organelles and vesicles.

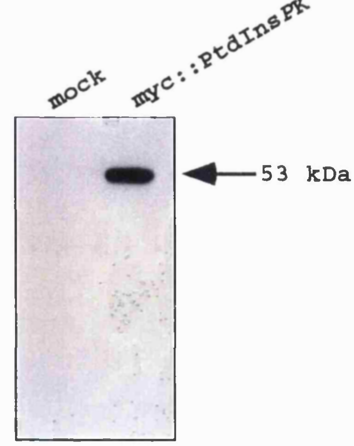
3.2.8 Thrombin cleavage of GST::PtdIns*P*K IIα and FPLC purification

Thrombin cleavage of GST::PtdIns*P*K IIα produced in Sf9 cells typically gave undegraded preparations of the 53 kDa enzyme as judged by SDS-PAGE and western blotting with the αL4 polyclonal antibody (Figure 3.6). For some applications the presence of residual thrombin can be problematic, for example in reconstitution experiments using permeabilised cells which are responsive to thrombin such as platelets and neutrophils. Thrombin-free preparations are also desirable for crystalisation trials, where the highest protein purity is often required. With the aim of producing highly pure PtdIns*P*K IIα, an FPLC protocol was developed to be used as a final purification step when necessary. GST::PtdIns*P*K IIα was eluted from GSH-Sepharose and cleaved in solution with thrombin. The reaction mixture containing native PtdIns*P*K IIα, thrombin and a mixture of recombinant and insect glutathione-S-transferases was purified by anion exchange chromatography on a Mono Q column based on a method described previously (Divecha *et al.*, 1992). As shown in Figure 3.9, this procedure removed the bulk of thrombin and GST from the major PtdIns*P*K IIα-containing fractions

3.2.9 Evidence that PtdIns*P*K IIα does not associate with the EGF receptor

An uncharacterised PtdIns*P*K activity had previously been reported to associate with the juxtamembrane region (JMR) of the EGF receptor (Cochet *et al.*, 1991). To determine whether PtdIns*P*K IIα could associate with the EGF receptor JMR *in vitro*, recombinant

A



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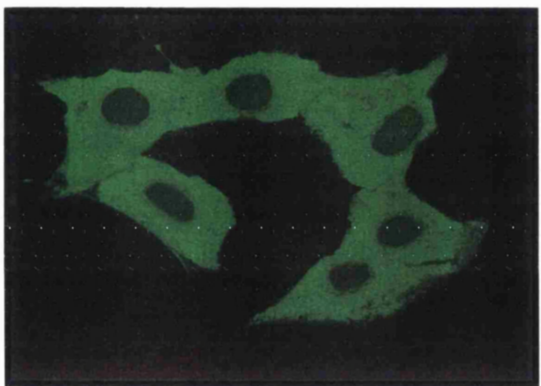


Figure 3.8 subcellular localisation of epitope-tagged PtdIns*P*K IIα

A. expression of the 53 kDa PtdIns*P*K IIα protein in MDCK cells (the expression construct was introduced into MDCK cells using lipid-mediated transfection, see Section 2.3.3.b).

B. confocal micrograph of MDCK cells microinjected with the construct expressing epitope-tagged PtdIns*P*K IIα.

Figure 3.9 Mono-Q chromatography of the thrombin cleavage reaction

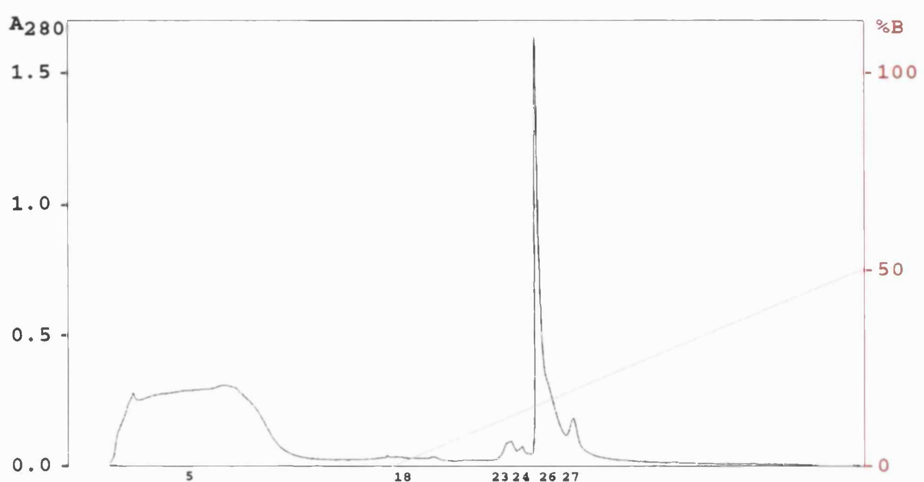
Approximately 50 μg of GST::PtdIns*P*K IIα was proteolytically digested in a 100 μl volume containing 25 mM Tris.HCl, pH 7.4, 80 mM NaCl, 10 mM reduced glutathione, 5 mM β-mercaptoethanol, and 40 units of bovine thrombin (Calbiochem) for 20 min at 30°C. The reaction was stopped by adding APMSF to 0.1 mM and four volumes of Buffer A (20 mM Tris.HCl, pH 7.4, 0.5 mM EGTA, 0.5 mM EDTA, 2 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.5 mM PMSF).

The reaction mixture was diluted fourfold in Buffer A and immediately fractionated on a Mono-Q column using a Smart system (Pharmacia). 100 μl fractions were collected at 1 min intervals using a linear salt gradient of 0-1.0 M NaCl in Buffer A and a 100 μl/min flow rate.

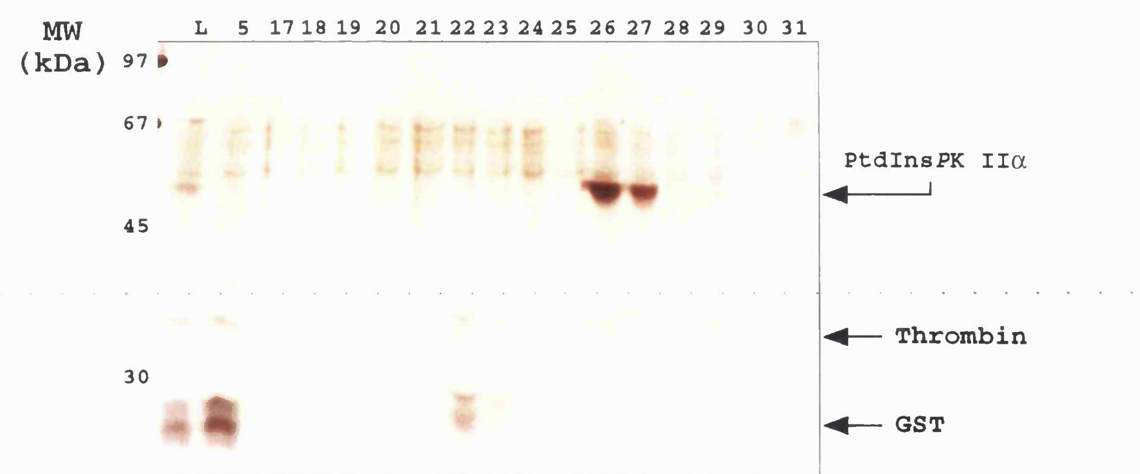
5 μl aliquots of each faction were denatured in 100 mM Tris.HCl, pH 8.1, containing 8 M urea by incubating at 50°C for 30 min after which 10 mM DTT was added and the reaction incubated for a further 2 hours. Cysteine residues were then acetylated by adding iodoacetamide to a final concentration of 20 mM and incubating at room temperature for 30 min in the dark, after which the reaction was terminated by adding 10% β-mercaptoethanol and 4% SDS (final concentrations) and heating to 50°C for 10 min prior to electrophoresis by SDS-PAGE.

A. Chromatogram. B. Analysis of column fractions by SDS-PAGE (silver stained). The 50-70 kDa bands present in all lanes are due to contaminating keratins which result from handling the fractions.

A



B



PtdIns*P*K IIα was incubated with a GST::JMR fusion protein (consisting of human EGFR residues 642-669) bound to GSH-Sepharose beads. After extensive washing the GST::JMR complexes were analysed by SDS-PAGE. PtdIns*P*K IIα was found to associate with GST::JMR but not GST, suggesting a specific interaction (Figure 3.10). However, this result must be treated with caution because, as detailed in Section 1.5.1.a, the JMR sequence contains a high proportion of basic residues which could promote non-specific ionic interactions. A polybasic peptide coupled to agarose beads was used as a control for non-specific binding and was found to bind PtdIns*P*K IIα at pH 7.4 and 150 mM NaCl. In order to test whether the observed association could be reproduced using full-length receptor, serum-starved A431 cells were used to investigate association of endogenous PtdIns*P*K. EGF receptors were immunoprecipitated from cells treated with or without either EGF or 10% foetal bovine serum and immune complexes were analysed by western blotting with affinity-purified αL4 antibodies. Although small amounts of EGF- and serum-dependent PtdIns*P*K activity could be detected in anti-EGF receptor immunoprecipitates (Figure 3.10), no PtdIns*P*K IIα could be detected by western blotting (results not shown). Similar results were obtained by blotting for EGF receptor when epitope-tagged PtdIns*P*K IIα (see Section 3.2.6) was overexpressed and immunoprecipitated from A431 cells (results not shown). Furthermore, attempts to characterise the PtdIns*P*K activity in EGF receptor immunoprecipitates using the type I-specific activator, PtdOH, led to a substantial decrease in PtdIns*P*K activity, possibly because of the activation of PLC by PtdOH (Jones and Carpenter, 1993, not shown).

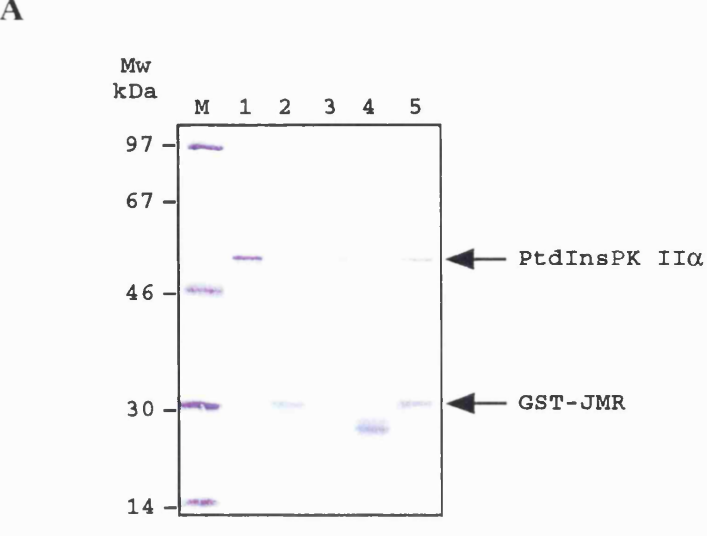
Finally, *myo*-[^{3}H]-inositol metabolically-labelled A431 cells overexpressing PtdIns*P*K IIα displayed no increased Ins(1,4,5)P_3 response or increased cellular levels of PtdIns(4,5)P_2 when stimulated with EGF (results not shown).

3.3 Discussion

The complete open reading frame of PtdIns*P*K IIα predicted a 406 amino acid protein which, despite having no clear homology to known PI-kinases, had PtdIns*P*K activity when expressed in bacterial, insect and mammalian systems. The identification of a PI-kinase distinct from the PI3/4-kinase family was unexpected, and raised the possibility that the PtdIns*P*Ks were related to the PI3/4-kinases by a common protein fold which could not be predicted from the primary structure of PtdIns*P*K IIα (this question is dicussed in detail in Chapter 4).

Purified recombinant proteins migrated at 53 kDa on SDS-PAGE and western blotting with antibodies raised to a synthetic peptide derived from the predicted sequence of the A5 clone, detected a 53 kDa protein in Jurkat cell lysates. All peptides sequenced, some 234 amino acids in total, were present in the asembled ORF. These results are entirely

A



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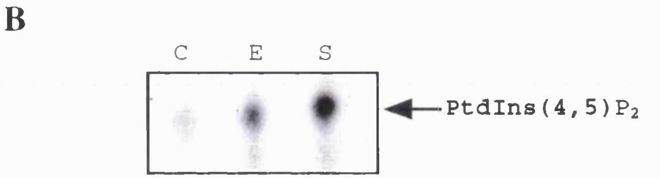


Figure 3.10 Recombinant PtdIns*P*K IIα binds GST-JMR *in vitro* and the EGF-receptor binds PtdIns*P*K activity in an agonist-dependent manner

A. The juxtamembrane region (JMR) of the human EGF receptor (residues 642-669) was amplified by PCR and cloned into the pGEX-2T expression vector (M. Mackinnon and J. J. Hsuan, unpublished). GST fusion protein was expressed and purified essentially as described in Chapter 2 except that a lysis buffer containing 100 mM CAPS, pH 11.0, was used. Approximately 2 µg of GST::JMR fusion protein, GST-alone, or poly(L)lysine agarose was incubated with 2 µg of baculovirus-expressed PtdIns*P*K IIα and incubated for 20 min at 4°C. After extensive, washing in buffer containing 50 mM Tris.HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 and 10 mM β-mercaptoethanol (IPB), GST::JMR complexes were analysed by SDS-PAGE. Lanes: M, molecular mass marker; 1, PtdIns*P*K IIα; 2, GST::JMR; 3, PtdIns*P*K IIα + poly(L)lysine-agarose; 4, GST + PtdIns*P*K IIα; 5, PtdIns*P*K IIα + GST::JMR. Note that this experiment was performed only once and the results should be treated as preliminary data.

B. A431 cells were grown to 40-60% confluence on 90 mm dishes and serum starved as detailed in Chapter 2. Cell monolayers were then stimulated with or without 100 nM EGF or 10% foetal bovine serum for 1 min at 37°C, after which Triton X-100 lysates were prepared. EGF receptors were immunoprecipitated with EGFR1 mAb (Waterfield *et al.*, 1982) bound to protein-G Sepharose by incubation at 4°C for 1 h. After extensive washing in IPB, equal amounts of EGF receptor were assayed for PtdIns*P*K activity.

consistent with the properties of a type II PtdIns*P*K and this protein was named PtdIns*P*K IIα. The properties of PtdIns*P*K IIα are also consistent with those of the PtdIns*P*K C isoform from which the sequence data was obtained (Divecha *et al.*, 1995) indicating that type II and isoform C are products of the same gene.

A high level of conservation between porcine and human PtdIns*P*K II isoforms was apparent from the fact that only three conservative differences existed in the peptide sequencing data. The porcine cDNA has now been characterised and is 98 % identical at the protein level (GenBank U96135).

PtdIns*P*K IIα has been cloned independently (Boronenkov and Anderson, 1995; Divecha *et al.*, 1995). The former sequence differs between residues 297 and 311 and peptide data covering this region (see Figure 3.1 and Table 7.1) indicates that the sequence of Borononkov and Anderson (1995) may contain nucleotide sequencing errors that introduce a frame shift. Both groups investigated the tissue distribution of the PtdIns*P*K IIα mRNA, and have found a single 4.1 kb transcript broadly expressed in human tissues with highest levels in brain (Divecha *et al.*, 1995). Relatively high levels of PtdIns*P*K IIα mRNA have also been found in peripheral blood cells and spleen (Divecha *et al.*, 1995), results consistent with the finding that a 53 kDa protein is present in abundance in haematopoietic cells (Brooksbank *et al.*, 1993; Chapter 5).

While this project was in progress, two further human type II isozymes, PtdIns*P*K IIβ and PtdIns*P*K IIγ, with 77.4% and 64% amino acid sequence identity respectively with PtdIns*P*K IIα, were characterised at the molecular level (Castellino *et al.*, 1997; Itoh *et al.*, 1998), also see Figure 1.6). The existence of these 47 kDa isoforms may explain the discrepencies in mobility of type II enzymes on SDS-PAGE frequently observed in the literature and raises the possibility that these closely related enzymes co-purify and that antibodies cross react. It should be noted however that on storage at 4°C, recombinant PtdIns*P* K IIα slowly degraded to an approximately 47 kDa fragment (data not shown) and could also account for the presence of a protein of 47 kDa derived from PtdIns*P*K IIα in some purified preparations (Loijens *et al.*, 1996). More specific immunological reagents will be required to resolve these questions (see Chapter 5).

Overexpression of myc-tagged PtdIns*P*K IIα by plasmid microinjection into MDCK cells led to no gross structural changes of cells when examined by phase contrast light microscopy. MDCK cells microinjected with the same construct or purified recombinant PtdIns*P*K IIα were also stained with rhodamine-labelled phalloidin to ascertain whether PtdIns*P*K IIα overexpression could cause changes in the actin cytoskeleton, possibly through a Rho or Rac protein (Section 1.10.1). It was also considered possible that overexpression of a PtdIns*P*K could cause cytoskeletal changes through elevated levels of a phosphoinositide since phosphoinositides are known to modulate the functions of actin

regulatory proteins *in vitro* (see Section 1.10). However, microinjected cells were morphologically indistinguisable from non-injected cells. The cytoplasmic localisation of PtdIns*P*K IIα (Figure 3.8) is consistent with previous cell fractionation studies (Bazenet *et al.*, 1990; Jenkins *et al.*, 1994; Ling *et al.*, 1989; Moritz *et al.*, 1990) and also with the absence of any identifiable membrane localisation signals. A fraction of type II activity has been found extrinsically associated with the plasma membrane of various cell types (Krisjansson et al, 1982; Bazenet *et al.*, 1990; Jenkins *et al.*, 1994; Ling *et al.*, 1989; Smith and Chang, 1989; Urumow and Wieland, 1990) and although plasma membrane staining was not observed in this study, it is possible that it is being obscured by the high level of cytoplasmic staining. Further experiments will be necessary to determine whether the membrane-associated type II activity is due to PtdIns*P*K IIα, particularly in view of recent data suggesting that PtdIns*P*K IIβ associates with the TNF-α membrane receptor (Castellino *et al.*, 1997).

Investigators have found PtdIns*P*K activities in other cell fractions (Helms *et al.*, 1991; Payrastre *et al.*, 1991; Tran *et al.*, 1993) but the data presented here suggests that PtdIns*P*K IIα is not responsible for these observations.

The question of whether either overexpression of PtdIns*P*K IIα in MDCK cells or the myc epitope tag interferes with targeting to specific subcellular compartments can be addressed by the use of a specific antibody to endogenous PtdIns*P*K IIα. Indeed, preliminary experiments using two independent monoclonal antibodies suggests that PtdIns*P*K II localises to focal contacts and membrane ruffles in fibroblasts. This is discussed in further detail in Chapter 5.

Bacterial expression of PtdIns*P*K IIα produced a protein which was prone to forming insoluble inclusions *in vivo*. Soluble material was present only in poor yields and preparations contained a large proportion of partially proteolysed or incompletely translated polypeptides resulting in low specific activity. Baculovirus expression was used to provide material suitable for biochemical characterisation, microinjection, crystalisation and reconstitution experiments. The protein obtained in this way was essentially free from truncated products and could be produced in high yields (typically 20 mg/l). In addition it was exceptionally stable and could be stored at 4°C for up to 3 weeks and in 50% glycerol at -20°C for over 30 months with only a minimal drop in activity. The yield and stability of baculovirus-expressed PtdIns*P*K IIα, the archetypal member of the PtdIns*P*K family, suggested that this protein was suitable for crystallography trials. Consequently, to further purify the enzyme to homogeneity, a chromatography step was developed which was capable of producing highly pure preparations of PtdIns*P*K IIα which were free of contaminating proteins and residual thrombin from the cleavage step.

Although PtdIns*P*K activity associates with activated EGF receptors (Cochet *et al.*, 1991; Figure 3.10) and recombinant PtdIns*P*K IIα binds GST::JMR *in vitro* (Figure 3.10), it was not possible to demonstrate the involvement of PtdIns*P*K IIα in EGF receptor signalling despite using a number of experimental approaches. Receptor immunoprecipitates were probed with anti-PtdIns*P*K IIα antiserum, and anti-myc immunoprecipitates from transfected cells were western blotted with anti-EGF receptor antiserum. Failure to detect an association between the EGF receptor and PtdIns*P*K IIα by these means does not rule out an interaction because it is possible that the associated enzyme was below detection levels, this is consistent with the finding that no known PtdIns*P*Ks contain high-affinity protein-protein interaction domains and argues that a distinct and possibly indirect interaction with the EGF receptor occurs. It was also not possible to determine whether the bound activity was a type I or type II PtdIns*P*K by using PtdOH, possibly due to stimulation of the tightly associated PLCγ activity which rapidly hydrolyses the PtdIns(4,5)P_2 generated in the assay (Jones and Carpenter, 1993). However, the recent discovery that type II PtdIns*P*Ks can catalyse the D-4 phosphorylation of PtdIns(5)*P*, a property not shared by the type I class, combined with the recent availability of chemically pure substrates, suggests that it may now be possible to determine which class of enzyme is recruited to the activated EGF receptor.

4.0 Structure-Function Analysis of Type IIα PtdIns*P* Kinase

4.1 Introduction

Sequence analysis of the PI 3-kinases and the PtdIns 4Ks indicates that the catalytic subunits of PI kinases share significant homology with protein kinases (Hunter, 1995 and Figure 4.1). The PI/protein kinase superfamily is now one of the largest and includes PI 3-kinases with intrinsic serine/threonine kinase activity (Section 1.5.3), PIK-related homologues with protein kinase activity but no detectable lipid kinase activity, and those with no known activity (Figure 4.1 and Zvelebil *et al.*, 1996).

It is generally assumed that all phosphotransferases have either evolved from a common ancestor or have converged on a similar structure. The paradigm for protein kinase structure and function is the catalytic subunit of cyclic AMP-dependent protein kinase (PKA) which has been well studied biochemically and the crystal structure solved (Knighton *et al.*, 1991). Eleven regions (subdomains) can be identified within protein kinases which are highly conserved and important for activity (Hanks *et al.*, 1988). Four of these subdomains (I, II, VIB and VII) map onto similar sequences in PI 3-kinase and its homologues where they are expected to be equally important for activity. Significantly, in protein kinases, these subdomains are involved in nucleotide binding, catalysis and maintaining the integrity of the active site, whereas subdomains implicated in peptide substrate recognition are absent in the lipid kinases.

A very large number of proteins bind nucleotides such as ATP. A glycine-rich phosphate binding structure known as the P-loop is found in many nucleotide-binding proteins such as adenylate kinases, GTP-binding proteins, myosin heavy chains, and phosphoglycerate kinases (reviewed in Saraste *et al.*, 1990). In the protein kinases, the well conserved P-loop (subdomain I) has the consensus sequence GXGXXG and is in close proximity to the non-transferable α- and β-phosphates of MgATP in the crystal structure of PKA (Knighton *et al.*, 1991). It is important to note however, that although many nucleotide binding proteins share this local region of similarity, it is not present in all nucleotide binding domains (Saraste *et al.*, 1990).

Subdomain II, containing an invariant lysine residue, has also been implicated in nucleotide binding (Zoller *et al.*, 1981) and there is also evidence for a more direct role in catalysis (Carrera et al., 1993). The crystal structure of PKA suggests that this residue lies close to the γ-phosphate of MgATP (Knighton *et al.*, 1991) and probably forms a non covalent bond with D184 of the highly conserved sequence DFG.

The first aspartic acid residue in subdomain VIb is responsible for hydrogen bonding to the acceptor hydroxyl group and is the proposed catalytic base. The invariant asparagine residue in subdomain VIb helps stabilise the catalytic loop through hydrogen bonding. The aspartic acid residue in subdomain VII also lies in a loop region and chelates a Mg^{2+}

PIK1		-IA**K**-	-QVK**D**RHNG**N**IMI	DNEGHVSHI**D**FGFM	*
PtdIns 4Kα		-IF**K**-	-QIK**D**RHNG**N**IML	DKKGHIIHI**D**FGFM	*
PtdIns 4Kβ		-IV**K**-	-QVK**D**RHNG**N**ILL	DAEGHIIHI**D**FGFI	*
p110α		-IF**K**-	-GIG**D**RHSD**N**IMVK	KTGQLFHI**D**FGHI	*†
p110γ		-IF**K**-	-GIG**D**RHND**N**IMIT	ETGNLFHI**D**FGHI	*†
Vps34		-IF**K**-	-GVG**D**RHLD**N**LLVT	PDGHFFHA**D**FGYI	*†
FRAP		-LL**K**-	-GLG**D**RHPS**N**LMLDRLSGKILHI**D**FGDC		¶
Tor1		-VL**K**-	-GLG**D**RHPS**N**LMLDRITGKVIHI**D**FGDC		¶
Tor2		-VL**K**-	-GLG**D**RHPS**N**LMLDRITGKVIHI**D**FGDC		¶
Mec1		-MC**K**-	-GLG**D**RHCE**N**ILLDIQTGKVLHV**D**FDCL		¶
ATM		-LV**K**-	-GLG**D**RHVQ**N**ILINEQSAELVHI**D**LGVA		¶
DNA-PK		-LV**K**-	-GLG**D**RHLN**N**FMVAMETGGVIGI**D**FGHA		†
c-Src	GCGCFGEVW-	-AI**K**-	-VHR**D**LRAA**N**ILV	GENLVCKVA**D**FGLA	†
PKCα	GKGSFGKVM-	-AV**K**-	-IYR**D**LKLD**N**VML	DSEGHIKIA**D**FGMC	†
cAPK	GTGSFGRVH-	-AM**K**-	-IYR**D**LKPE**N**LLI	DQQGYIQVT**D**FGFA	†
	glycine rich		catalytic loop	Mg^{2+} binding	
subdomain:	**I**	**II**	**VIb**	**VII**	

Figure 4.1 The subdomain orgainsation of protein and PI kinase catalytic domains.

To illustrate the structural relationship between the different subfamilies of PI and protein kinases, three representative members of the PtdIns 4-kinases, PI 3-kinase families are shown. Residues shown in bold are invariant and conservative mutation of these leads to loss of activity (Hunter, 1995). For definitions see Figure 1.4 and Section 1.3.5. Key: * - lipid kinase activity, † - protein kinase or autokinase activity, ¶ - no demonstrated activity although some may autophosphorylate .

ion that bridges the β- and γ-phosphates of the ATP molecule and helps to position the γ-phosphate for transfer. Conservative mutation of any of these residues (shown in bold in Figure 4.1) abolishes kinase activity.

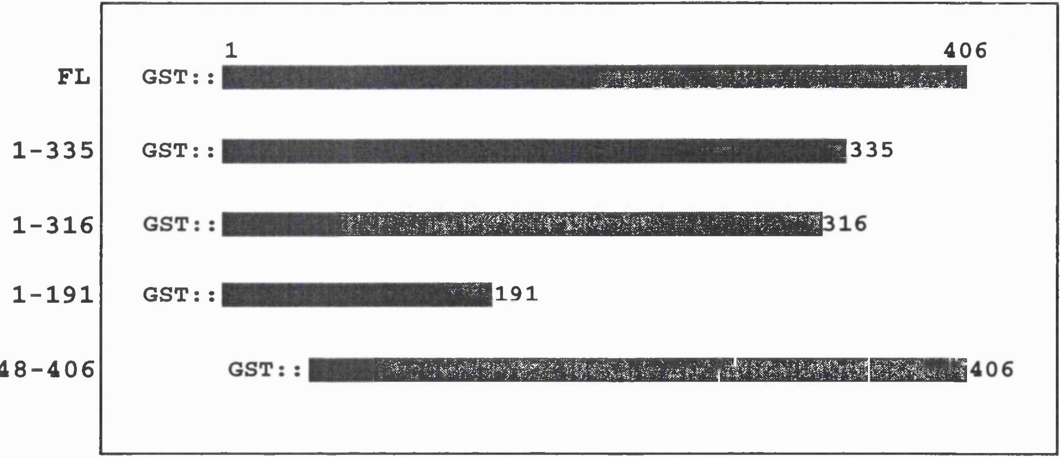
The cloning of PtdIns*P*K IIα, a molecule with little homology to any known kinase (Chapter 3) led to the suggestion that a branch of PI kinases has completely novel structures and furthermore, that these kinases may possess a catalytic mechanism distinct from other phosphotransferases (Boronenkov and Anderson, 1995). Alignment of the PtdIns*P*Ks with protein kinase sequences fails to identify all but the most basic local similarities (Yamamoto *et al.*, 1995; S. Minogue and M. J. Zvelebil, unpublished) and although PtdIns*P*Ks contain a number of conserved lysines which are potential nucleotide binding residues, none of these can be predicted with certainty. I hypothesised that if the nucleotide binding residue could be identified using biochemical means, it would become possible to align this residue with the corresponding subdomain of PKA and to predict other important residues in the PtdIns*P*K IIα sequence whose function could be tested by site-directed mutagenesis. With this information it should be possible to determine the structural relationship between PtdIns*P*Ks and the protein kinases and to produce a rational model of the PtdIns*P*K catalytic core based on the structure of PKA.

4.2 Results

4.2.1Truncation mutants

Initially I sought to define a minimal region that could support PtdIns*P*K activity and thus delineate the catalytic domain of the protein. It was also considered that an active minimal fragment could prove useful for crystallography since small soluble protein domains are frequently better expressed in *E. coli.* and produce better crystals. The boundries to the protein fragments were chosen by aligning all available PtdIns*P*K homolgues (Figure 3.2). Potential domain boundries were identified by looking for regions of flexibility such as regions predicted to be loops by using secondary structural programs NNPREDICT and PEPPLOT (GCG, University of Wisconsin, WI). To this end, N- and C-terminal truncated cDNAs were synthesised by PCR using primers positioned at predicted domain boundries. After sequencing, these fragments were expressed as GST-fusion proteins in *E. coli* and the recombinant proteins assayed for PtdIns*P*K activity. All the constructs shown in Figure 4.2 produced soluble protein. Surprisingly, all mutant proteins were inactive: deletion of just 48 residues from the N-terminus or 71 residues from the C-terminus was sufficient to completely destroy kinase activity. It was clear from these results that a different approach was needed to identify the catalytic core of this novel kinase structure.

A



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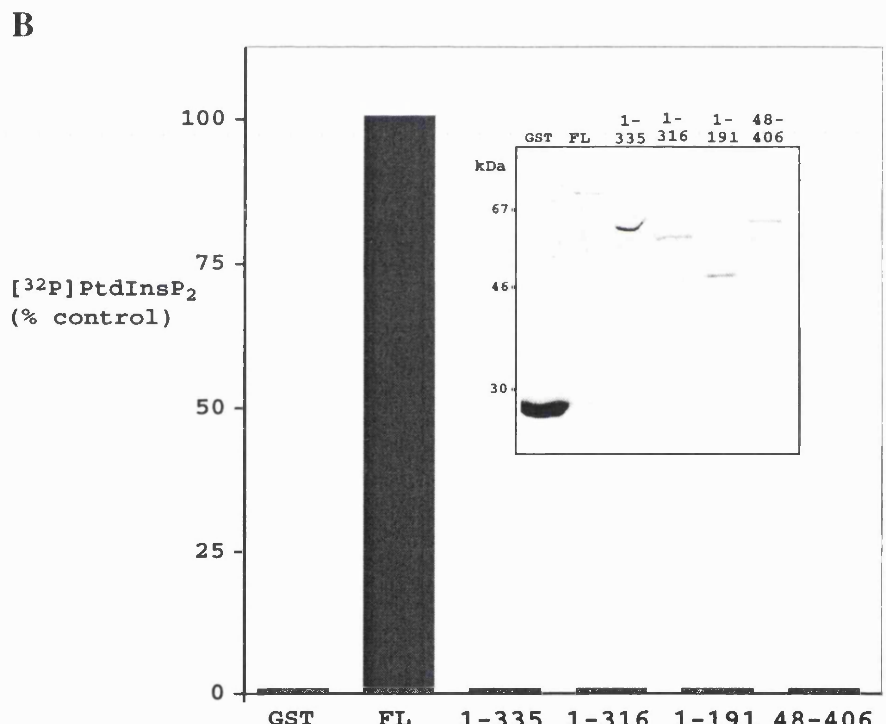


Figure 4.2 Activity of truncated PtdIns*P*K IIα

A. Truncated forms of PtdIns*P*K IIα were generated by PCR.

B. Truncated proteins were expressed as GST fusion proteins. Similar amounts of protein were assayed for PtdIns*P*K activity as described (Section 2.5.2.b). The results of these assays are expressed as a percentage of the activity of full-length protein.

4.2.2 Identification of a nucleotide binding residue

Affinity labelling with the ATP analogue 5'-[*p*-(fluorosulphonyl)benzoyl]adenosine (FSBA), has been used to identify the nucleotide binding residue in a number of phosphotransferases including PKA (Zoller *et al.*, 1981; Zoller and Taylor, 1979) and the EGFR (Russo *et al.*, 1985). FSBA shares the adenosyl moiety with ATP but contains carboxybenzoylsulphonyl fluoride in place of the trisphosphate chain (Figure 4.3). The reactive sulphonyl fluoride is situated in a position analogous to the transferable γ-phosphate of ATP where it can react with the the nucleotide binding lysine residue to form the stable lysine derivative (carboxybenzenesulphonyl)lysine ((CBS)-lysine).

4.2.2.a Effect of FSBA on PtdIns*PK* IIα activity

To demonstrate that FSBA could inhibit PtdIns*P*K IIα, baculovirus-expressed protein was incubated with 1 mM FSBA or vehicle alone at 30°C for various times. It was found that PtdIns*P*K activity was inhibited by 60% after 20 min incubation, while 20 min incubation with vehicle only resulted in a minimal drop in activity (Figure 4.4). This behaviour was very similar to that reported for PKA (Zoller and Taylor, 1979).

4.2.2.b Mass analysis of FSBA-labelled peptides

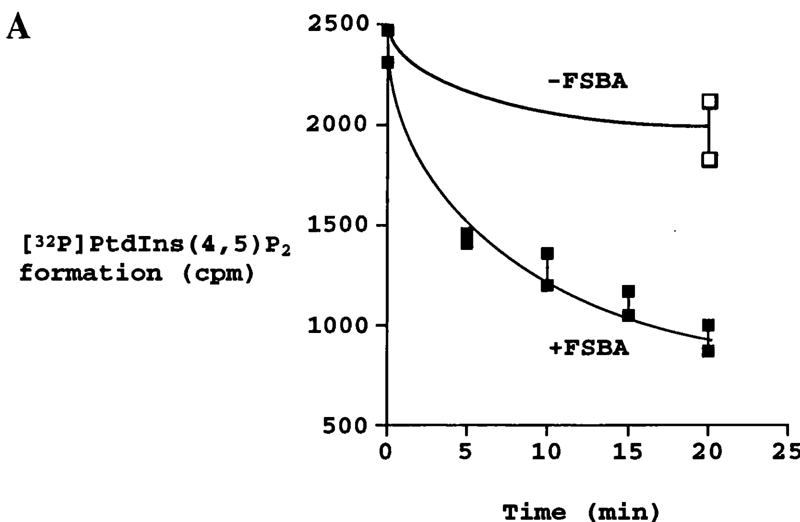
Having established the labelling conditions, FSBA-treated and untreated samples were prepared by incubating 8 μg of thrombin-cleaved PtdIns*P*K IIα in buffer containing 50 mM Tris.HCl, pH 8.0, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% β-mercaptoethanol, and 1 mM FSBA or DMSO for 30 min at 30°C. Protein was TCA-precipitated and digested overnight in 10 mM Tris.HCl, pH 7.4, 1 mM EDTA containing 1 μg of sequencing grade trypsin (Promega). FSBA-labelled and -unlabelled (control) tryptic peptides were analysed by matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry using a Finnegan Lasermat spectrometer. This instrument was unable to detect all the predicted tryptic peptides but as shown in Figure 4.4b the major difference detected between these samples was the loss of a peptide of mass 1702 which corresponds with the mass of peptide 24, (residues 145-160). This was presumed to be due to modification of lysine residue K145 which is a substrate for trypsin in the control sample but not the FSBA-labelled sample because modification of K145 prevents its cleavage by trypsin. However, the fact that the modified peptide was not detected in mass spectra could be due to a combination of factors: firstly, sub-stoichiometric labelling could result in a low yield of (CBS)-peptide adduct. Indeed this is suggested by the observation that PtdIns*P*K activity was not totally inhibited (Figure 4.4a). Secondly, the CBS-group is labile and is hydrolysed at neutral and alkaline pH (Zoller and Taylor, 1979). Finally, additional heterogeneity can arise through side reactions of FSBA because the reactive fluorosulphonyl group can react with several residues in proteins

5'-[*p*-(fluorosulphonyl)benzoyl]adenosine (FSBA)

adenosine 5'-trisphosphate (ATP)

Figure 4.3 The structure of ATP and FSBA

A



B

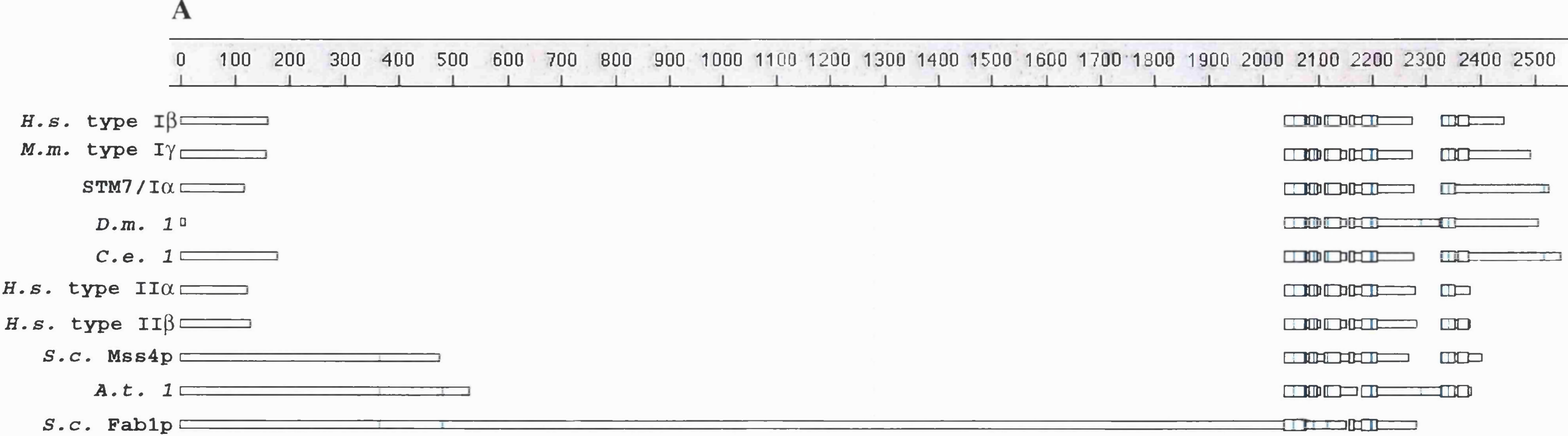
Peptide	Expected M/z	Observed (Control)	Observed (FSBA)
43	1062	1063	1063
19	1155	1057	1157
37	1179	1080	1178
18	1656	1657	1656
24	1701	1702	-
44	1760	1762	1761
54	1788	1789	1789

Figure 4.4 Inhibition of PtdIns*P*K IIα and mass analysis

A. FSBA inactivates PtdIns*P*K IIα. Baculovirus expressed PtdIns*P*K IIα was treated with FSBA for various times as indicated, after which the reaction was stopped by the addition of excess DTT. PtdIns*P* kinase activity was then assayed.

B. MALDI-TOF mass spectrometry of FSBA-labelled peptides. Control and FSBA-treated PtdIns*P*K IIα were digested with trypsin and analysed using a Lasermat spectrometer. As shown in the table, the major differences between the two spectra was the disapearence of peptide 24 whose sequence is predicted to contain lysine 145.

A



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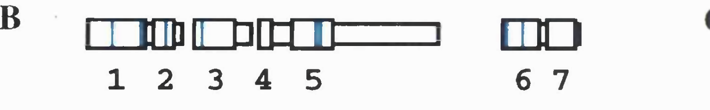


C

1. PKA(I)	G.SGS.F GT-G-SF	P-Loop
2. PKA(II)	.IK AMK	ATP-binding
3. PKA(VIb)	DLKGS DLK	Catalytic loop
4. PKA(VII)	MDYSL DFG	Mg^{2+}-binding

Figure 4.5 Alignment of PtdIns*P* kinases with PKA

A. Alignment of PtdInsPK homologues. B. Seven local regions of homology were identified. C. K145, a putative PtdIns*P*K IIα nucleotide-binding residue suggested by FSBA-labelling and site-directed mutagenesis, can be aligned with the nucleotide binding residue of PKA. Alignment of K145 with PKA residue K72 has been used to identify the putative P-loop, the catalytic loop and the Mg^{2+}-binding loop, which are arranged in a topologically similar manner to subdomains I, II, VIb and VII of protein kinases. Note that for clarity not all PtdIns*P*K homologues are shown in this schematic. For an alignment of PtdIns*P*K catalytic domains see Figure 3.2.

PKA motif (subdomain)	PKA Residue	Corresponding residue in PtdInsPK IIα	Putative function	Reference
P-loop (I)	**G**50 T51 **G**52 S53 F54 G55 R56	 A128 R129 S130 G131 A132	Phosphate anchor: G55 binds to β-phosphate of ATP	Knighton *et al.*, (1991)
ATP binding site (II)	 A70 M71 K72	 I143 I144 K145	ATP/FSBA binding site: K72 ion pairs with α- and β-phosphates and forms salt bridges with D166 and D184	Zoller *et al.*, (1981) Knighton *et al.*, (1991) Zheng *et al.*, (1993)
Catalytic loop (VIb)	R165 **D**166 L167 **K**168 P169 E170 **N**171	 D212 L213 K214	R_{165} ion pairs with (P)T97. D166 proposed catalytic base. K168 ion pairs with γ-phosphate. N171 H-bonds to N166 and also ligates to Mg^{2+} in the active site.	Knighton *et al.*, (1991) Zheng *et al.*, (1993)
Mg^{2+} binding loop (VII)	**D**184 F185 G186	D273 Y274 S275	D184 chelates Mg^{2+}	Knighton *et al.*, (1991) Zheng *et al.*, (1993)

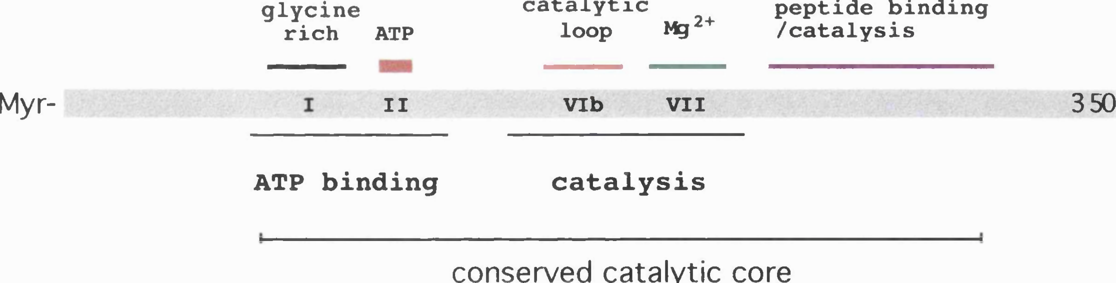


Table 4.1 Comparison of the function of PKA and PtdIns*P*K IIα residues

Residues in bold type are highly conserved amongst members of the protein and PI kinase superfamily and many other phosphotransferases, those in red are conserved between PKA and the PtdIns*P*-kinases. Shown below is a schematic representation of the primary structure of PKA, illustrating the subdomain organisation.

resulting in the modification of tyrosine, cysteine and histidine residues (Bullough and Allison, 1986; Esch and Allison, 1979; Likos and Colman, 1981; Zoller and Taylor, 1979) and perhaps serine residues (Colman, 1983). Competition with MgATP has been used to demonstrate the labelling specificity of nucleotide analogues (Zoller and Taylor, 1979), however complete protection with ATP was not observed in this study (data not shown) suggesting that enzymatic activity can be destroyed by modification of other sites in the kinase core. Examination of the PtdIns*P*K IIα sequence indicates the presence of several highly conserved lysine and tyrosine residues which are potential targets for FSBA and could account for this phenomenom. Finally, the predicted size of the (CBS)-peptide lies outside the optimum range of the Lasermat instrument.

4.2.3 Site-directed mutagenesis

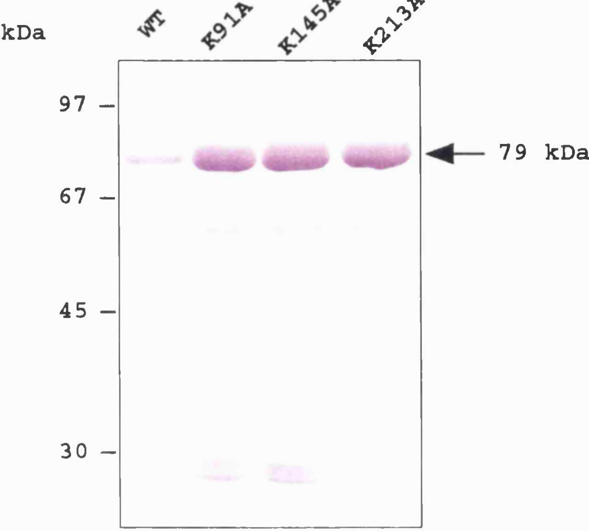
In order to demonstrate the functional importance of K145, the putative FSBA and nucleotide-binding residue, PCR mutagenesis (Section 2.2.2.e) was used to construct a mutagenic cassette containing a K145A point mutation. After digestion with the appropriate restriction enzymes, these PCR products were ligated into wild type PtdIns*P*K IIα in pUC-18 and sequenced on both strands. Clones which were free of secondary (PCR-induced) mutations were subcloned into pGEX-KG (Guan and Dixon, 1991) for bacterial expression as GST fusion proteins (Figure 4.6a). Fusion proteins were eluted as described in Section 2.3.2.1 and assayed for PtdIns*P*K activity (Figure 4.6b). Mutation of K145 resulted in a complete loss of activity, indicating that this residue was essential for catalysis. This result supported the proposition that K145 represents a nucleotide-binding residue.

K145 was aligned with subdomain II of PKA and, by using the subdomain organisation of PKA, it was possible to map conserved regions of PtdIns*P*Ks onto similar regions of local homology in PKA corresponding to subdomains I, II, VIb and VII as shown in Figure 4.5. It was proposed that if this model was accurate, mutation of other conserved residues in these putative subdomains would abolish activity. To this end, K91 (a conserved lysine residue lying outside the proposed PtdIns*P*K catalytic core) and K213 (a conserved lysine residue predicted to be in subdomain VIb) were mutated to alanine residues as above. It was found that K91A produced a protein with reduced activity (approximately 80% of the wild type), whereas the K213A mutation resulted in a complete abrogation of activity (Figure 4.6b).

4.2.4 Potential SH3-binding sites

Two, short, proline-rich sequences were identified in the divergent insert region of PtdIns*P*K IIα (amino acids 313-328 in Figure 3.1) which had the potential to act as ligands for SH3 domains. As discussed in Section 1.3.3, SH3 domains are found in a large number of PI signalling molecules including PLCγ and heterodimeric PI 3-kinases. PtdIns*P*K IIα is a predominantly cytosolic enzyme which must, presumably, be

A



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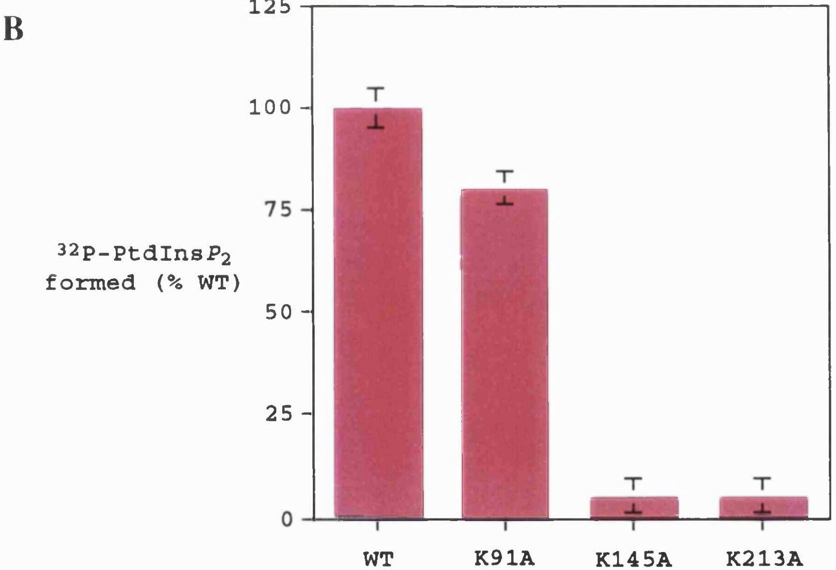
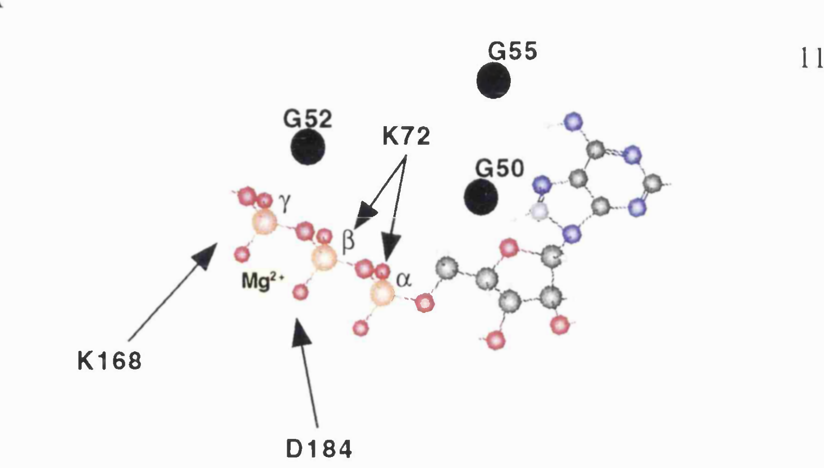


Figure 4.6 Expression and assay of mutant PtdIns*P*K IIα proteins

A. GST fusion proteins, containing lysine to alanine mutations as indicated, were eluted from a GSH-Sepharose affinity matrix and analysed by SDS-PAGE. B. Eluted proteins were assayed in duplicate for PtdIns*P*K activity.

A



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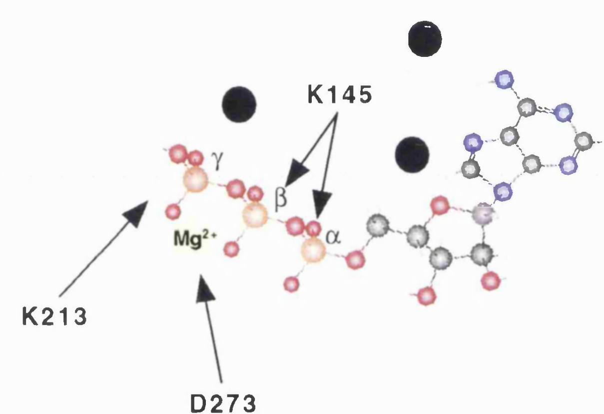


Figure 4.7 The putative ATP binding site of PKA and PtdIns*P*K IIα

A. Biochemical and crystallographic data has been used to model of the ATP binding site of PKA (Buechler and Taylor, 1988; Knighton *et al.*, 1991; Zheng *et al.*, 1993; Zoller *et al.*, 1981). A number of interactions contribute to ATP binding and positioning of the nucleotide γ-phosphate for transfer. The adenine ring is held in a hydrophobic pocket and is further anchored by hydrogen bonding through the N6 and N7 nitrogens. The glycine-rich loop (P-loop) stabilises the β-phosphate by hydrogen bonding to several backbone amides. Residue K72 (PtdIns*P*K IIα K145) is fixed by an ionic interaction with E91. In the presence of MgATP, K91 (PtdIns*P*K IIα K145) ion pairs with the α- and β-phosphates of ATP. In this ternary complex, K168 (PtdIns*P*K IIα K213) interacts with the γ-phosphate of ATP and D184 (PtdIns*P*K IIα D273) chelates the Mg^{2+}-ion.

B. The proposed structure of the PtdIns*P*K IIα ATP-binding site modelled on the structure of PKA is shown indicating the positions of residues conserved with PKA.

Carbon atoms are shown in grey, nitrogen in blue, oxygen in red, and phosphate in brown. Black circles represent the conserved glycine residues of the PKA P-loop in (A). In (B) it is not known which residues of the putative PtdIns*P*K IIα P-loop correspond to those of PKA (see text).

recruited to a membrane compartment in order to gain access to lipid substrate. The mechanism of PtdIns*P*K IIα regulation and membrane localisation is currently unknown but interaction with an SH3 domain-containing membrane protein is an attractive proposition for several reasons: firstly, by promoting access to compartmentalsied substrate, recruitment may be a primary regulatory event. Secondly, SH3 domains are present in cytoskeletal proteins and there is evidence that one or more type II PtdIns*P*Ks are recruited to the cytoskeleton in thrombin-stimulated platelets (Hinchliffe *et al.*, 1996). Finally, protein-protein interactions are important in the formation of multienzyme signalling complexes and the SH3 domain-containing PLCγ and heterodimeric PI 3-Ks preferentially utilise PtdIns(4,5)P_2 as substrate.

Initially, the sequence of the two putative SH3-binding regions of PtdIns*P*K IIα were compared with known binding partners of SH3 domains. These generally have the consensus sequences PXLPPXP or XPPLPXR (Chen *et al.*, 1993; Yu *et al.*, 1994) which correspond only weakly to the PtdIns*P*K IIα sequences PPDSPG and PPLAPG. However, both sequences contain the critical proline residues identified in Src SH3 binding peptides (Feng *et al.*, 1994). Using the structure of the Src SH3 domain (Feng *et al.*, 1994), putative ligands PPDSPG and PPLAPG were docked onto the Src SH3 ligand binding site in both possible orientations (Figure 4.9). Both PPDSPG and PPLAPG were found to fit in the amino to carboxyl orientation only, with the critical proline residues occupying equivalent positions to the Src peptides (Feng *et al.*, 1994).

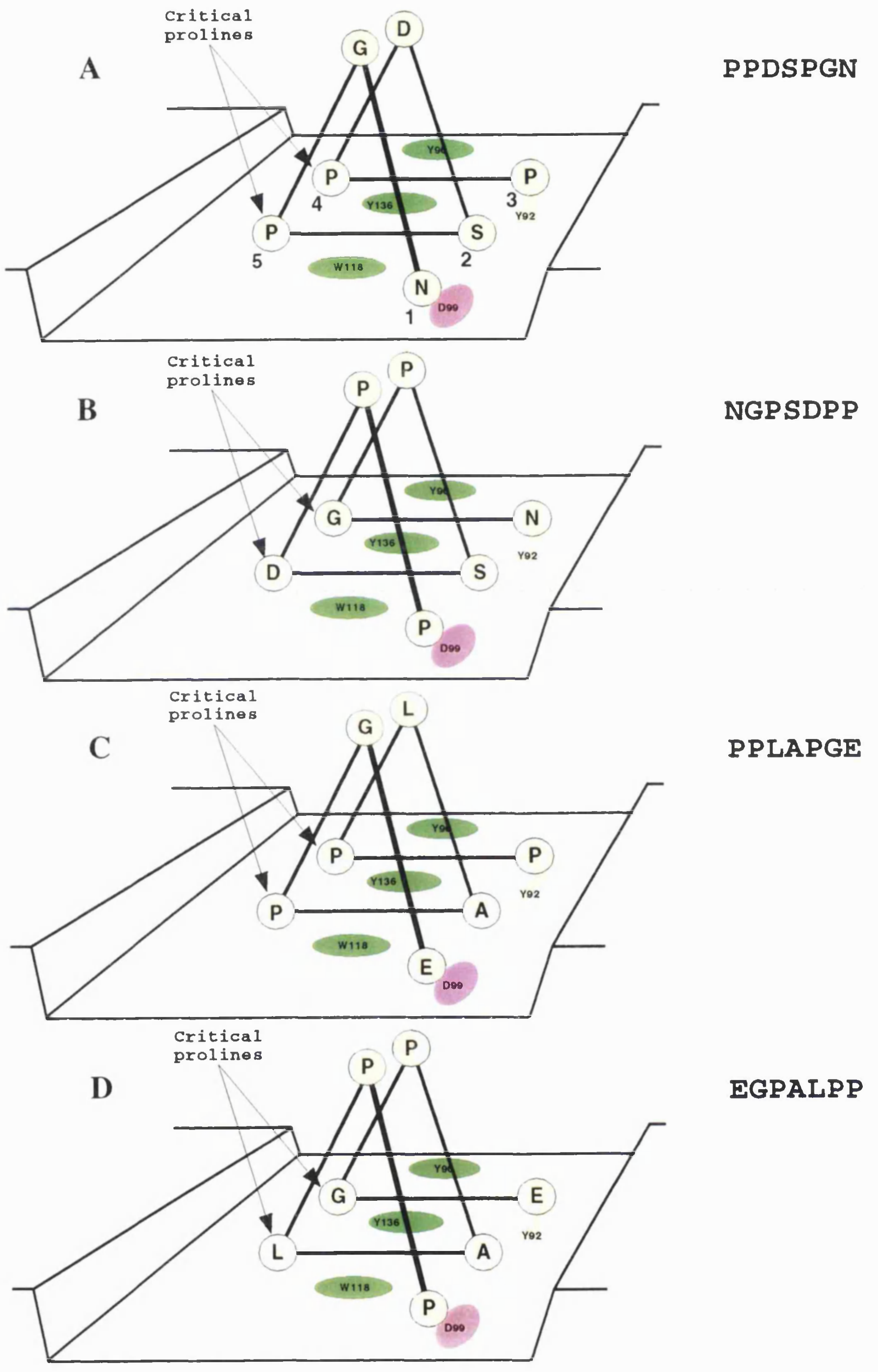
SH3 ligands have been identified by affinity purification using immobilised recombinant SH3 domains (Gout *et al.*, 1993). In order to investigate whether PtdIns*P*K IIα could interact with known SH3 domains including those of PLCγ and p85, bacterially expressed GST fusion proteins of SH3 domains from p85, GAP, PLCγ, Src, Crk, Fgr, spectrin, the N-terminus and C-terminus of Grb2, Fyn, Csk, and Nck or the corresponding full-length GST-fusions (Gout *et al.*, 1993) were incubated with recombinant PtdIns*P*K IIα. After washing, GST-SH3 complexes were analysed by SDS-PAGE (Figure 4.10) and also by the more sensitive PtdIns*P*K assay (not shown). In both tests PtdIns*P*K IIα was not found to interact with the panel of SH3 domains.

4.3 Discussion

Extensive sequence analysis of PtdIns*P*K IIα revealed surprisingly little homology with any known class of phosphotransferase enzyme. It subsequently became apparent that a family of proteins existed which were more closely related to PtdIns*P*K IIα than other lipid, sugar, or protein kinases (Boronenkov and Anderson, 1995; Carvajal *et al.*, 1995; Castellino *et al.*, 1997; Ishihara *et al.*, 1996; Itoh *et al.*, 1998; Loijens and Anderson, 1996; Yamamoto *et al.*, 1995; Yoshida *et al.*, 1994). This finding was intriguing because many phosphotransferase enzymes share structural features in their kinase domains and it was therefore expected that PtdIns*P*Ks would be based on a similar structure. Although we and others noted weak similarities with subdomains of the PKA

Figure 4.9 Modelling the putative SH3 ligands PPDSPG and PPLAPG on the Src SH3 domain

Structure-function analyses of Src SH3 peptide ligand interactions have been used to establish a general model for SH3-ligand interactions (Feng *et al.*, 1994). In this model two critical proline residues (representing the conserved prolines in the PXXP consensus sequence) must be positioned such that they contact the SH3 domain binding pocket (positions 4 and 5). Assuming that the PtdIns*P*K IIα peptides PPDSPGN and PPLAPGE adopt a type II poly-proline helix, the model of Feng *et al.* (1994) predicts that both peptides can be accomodated in the N-C orientation with the critical prolines in positions 4 and 5 (A and C) but not in the reverse orientations (B and D). See text for details.

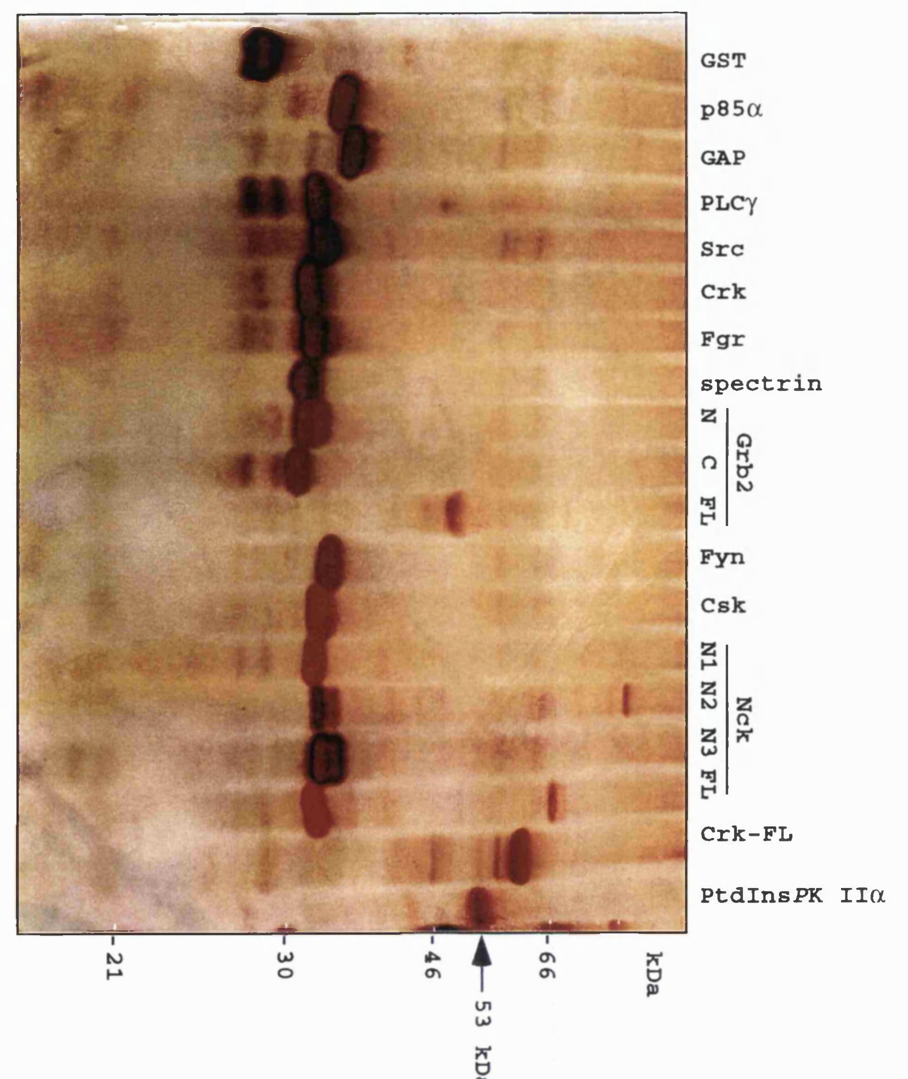


Critical prolines
A
PPDSPGN
G
D
P
P
4
3
Y136
Y92
P
S
5
2
W118
N
D99
1
Critical prolines
B
NGPSDPP
P
P
G
N
Y136
Y92
D
S
W118
P
D99
Critical prolines
C
PPLAPGE
G
L
P
P
Y136
Y92
P
A
W118
E
D99
Critical prolines
D
EGPALPP
P
P
G
E
Y136
Y92
L
A
W118
P
D99

Figure 4.10 Testing the interaction between PtdIns*P*K IIα and a panel of SH3-domains

SH3 domains were expressed as GST fusion proteins in *E. coli* and purified as described previously (Gout *et al.*, 1993). Approximately 1.5 μg of each fusion protein or GST only bound to GSH-Sepharose beads was incubated with 1.5 μg of thrombin-cleaved, baculovirus-expressed PtdIns*P*K IIα (Section 3.2.8) for 2 h at 4°C. GSH-Sepharose beads were washed 4 times in buffer containing 100 mM Tris.HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, followed by twice in PtdIns*P*K assay buffer (Section 2.5.2.b). Immobilised proteins were analysed by SDS-PAGE and silver stained. Beads were also assayed for PtdIns*P*K activity (not shown).

Lanes: p85α, N-terminal SH3 from p85α; GAP, SH3 from Ras GTPase-activating protein; PLCγ, SH3 from PLCγ; Src, SH3 from c-Src; Crk and Crk-FL, SH3 from c-Crk and the full-length protein respectively; spectrin, SH3 from spectrin; N-, C-, and FL-Grb2, the N-terminal SH3, C-teminal SH3 and full-length Grb2 proteins respectively; Fgr, SH3 from Fgr; Csk, SH3 from Csk; Nck-N1, -N2, -N3, and -FL, the first, second and third SH3 domains from Nck and the full-length protein respectively.


GST
p85α
GAP
PLCγ
Src
Crk
Fgr
spectrin
N
C
FL
Grb2
Fyn
Csk
N1
N2
N3
FL
Nck
Crk-FL
PtdInsPK IIα
21
30
46
53 kDa
66
kDa

catalytic domain (Yamamoto *et al.*, 1995), no discrete kinase domain could be identified by analysis of PtdIns*P*K homologues. Consequently, studies were initiated with the aim of delimiting a minimal kinase domain within the PtdIns*P*K IIα molecule. The initial approach was to construct a series of truncation mutants by PCR and assay the recombinant proteins for PtdIns*P*K activity (Section 4.3). However, it was found that all mutants lacked kinase activity even when as few as 48 amino acids were deleted from the N-terminus. This was unexpected since sequence alignment of these 48 amino acids shows that the N-terminal region contains residues that are conserved in only some members of the PtdIns*P*K family and are therefore unlikely to be involved in catalysis. The observation that the boundaries of the kinase domain could not be determined by simple linear truncations is similar to findings reported from the studies of PI 3-kinase p110α (Dhand *et al.*, 1994) and PI 3-kinase p110γ (Stoyanova *et al.*, 1997). This may be due to the presence of essential structures in the N-terminus which interact with the kinase core or are required for correct folding of the intact polypeptide. It is also possible that the N-terminus has some regulatory function such as interaction with substrates.

With the aim of identifying nucleotide-binding residues, recombinant PtdIns*P*K IIα was labelled with the reactive ATP analogue FSBA. [^{14}C]-FSBA has been used extensively to probe the active sites of protein kinases because of the ability of this compound to covalently modify the nucleotide binding site of these enzymes (Kamps *et al.*, 1984; Russo *et al.*, 1985; Zoller *et al.*, 1981; Zoller and Taylor, 1979). However, [^{14}C]-FSBA is an exceptionally costly reagent so a novel approach was employed in which trypsin-digested products of the FSBA reaction were analysed by MALDI-TOF mass spectrometry to identify peptides containing the nucleotide-binding lysine residue. Comparison of FSBA-labelled and unlabelled peptide masses revealed the loss of a peptide due to modification of, and therefore protection of a trypsin cleavage site. The mass of this peptide corresponded to that of a peptide in a highly conserved region of PtdIns*P*K IIα containing the conserved lysine residue K145. Accordingly, it was hypothesised that K145 was a FSBA-binding residue. In order to test this K145 was mutated to an alanine residue, which led to a complete loss of activity, thereby demonstrating that K145 is required for catalysis and adding weight to the hypothesis that K145 is a nucleotide binding residue. In PKA the corresponding ATP-binding residue has been determined to be an invariant lysine residue in subdomain II (Zoller *et al.*, 1981). Closer scruitiny of the PtdIns*P*K IIα sequence surrounding K145 indicated similarity to subdomain II of PKA: subdomain II of PtdIns*P*K IIα has the sequence IIK, while that of PKA has the sequence AMK (Figure 4.5). Using subdomain II as a point of reference, it was possible to construct a model of the PtdIns*P*K IIα kinase domain using the subdomain organisation of PKA (Figure 4.5). In order to test this model two further mutations were constructed, at K91 and K213. As expected, mutation of the conserved residue K213 which is invariant in PtdIns*P*Ks and maps to subdomain VIb, led to a complete loss of activity. The K91A mutation which is predicted to lie outside the

catalytic core, was found to have approximately 80% the activity of the wild type enzyme (Figure 4.6).

The catalytic subunit of PKA has been extensively studied using site-directed mutagenesis and the crystal structure solved (Knighton *et al.*, 1991). Several structural features of PKA which are conserved in other phosphotransferase enzymes, are also conserved in the PtdIns*P*K family. Most significantly, crucial residues implicated in nucleotide binding and catalysis are amongst the most highly conserved. These are summarised in Figure 4.1 and Table 4.1.

No P-loop consensus was found in the PtdIns*P*Ks but when the sequence just upstream of K145 was analysed less stringently, a potential loop region with the PtdIns*P*K consensus sequence GXSGSXF can be identified. The fact that this was not detected in the initial analysis of PtdIns*P*K IIα is reminiscent of the PI 3-kinases where P-loops are not recognisable (Zvelebil *et al.*, 1996). Although the majority of nucleotide binding proteins and protein kinases conform to the GXXXXG consensus sequence (Saraste *et al.*, 1990), several do not, indicating that variation in this structure is allowed. Whether or not the PtdIns*P*K consensus sequence GXSGSXF represents a phosphate binding motif needs to be determined experimentally but it is reasonable to assume that this represents the P-loop because of its position close to K145 and the fact that this is well conserved in the PtdIns*P*Ks (see Figures 4.2 and 4.7).

One of the most highly conserved regions of homology in the PtdIns*P*K family resembles the protein kinase subdomain II which, on the basis of FSBA-labelling experiments, contains the nucleotide-binding site in protein kinases (Zoller *et al.*, 1981; Zoller and Taylor, 1979), PI 3-kinases (Wymann *et al.*, 1996) and PtdIns*P*K (this study) families. Importantly, this residue is absolutely conserved and even conservative mutations at this position lead to a loss of activity (Wymann *et al.*, 1996). The high degree of conservation reflects the proposed role of this residue in anchoring the α and β-phosphates of the ATP molecule (Knighton *et al.*, 1991; Zheng *et al.*, 1993).

Subdomain VIb in protein-, PI 3- and PtdIns 4Ks represents the catalytic loop containing the catalytic aspartic and asparagine residues in the consensus DXXXXN. Modification or mutation of either of these residues leads to an inactive protein (Buechler and Taylor, 1988). The corresponding sequence in PtdIns*P*K IIα (DLKGST) contains K213 which also gives an inactive protein when mutated to an alanine residue. Most protein serine/threonine kinases also contain a lysine residue at this position which is responsible for binding the γ-phosphate, but protein tyrosine kinases contain either an arginine or an alanine residue and in the PI 3-kinase family this position is conservatively substituted to a histidine residue. It is noteworthy that no residue corresponding to N171 in the highly conserved subdomain VIb can be identified in the PtdIns*P*Ks. In PI3/4- and protein kinases this asparagine residue lies in the DXXXXN consensus sequence of the catalytic loop and although not directly implicated in catalysis, this residue stabilises the loop by hydrogen bonding to the backbone carbonyl groups of D166 (Knighton *et al.*,

1991). The corresponding sequence in PtdIns*P*K IIα is DLKGST and it is not known if the threonine residue can hydrogen bond in a similar manner.

Subdomain VII containing the DFG sequence is present in PtdIns*P*Ks as the invariant DYS motif. The critical amino acid in this sequence is the aspartic acid residue as it is invariant in protein and PI kinases and as its mutation in PKA abolishes activity. It is thought that the invariant aspartic acid residue is involved in chelating the primary magnesium ion (Knighton *et al.*, 1991; Zheng *et al.*, 1993). The changes from phenylalanine to tyrosine and glycine to serine residues represent conservative changes which are also found in protein kinases such as PVPK1 from *Phaseolus vulgaris*, which has the sequence DFD (Lawton *et al.*, 1989), and the β-andrenergic receptor kinase, which has the sequence DLG (Benovic *et al.*, 1989).

Determining the position of the nucleotide binding residue has allowed a more detailed analysis of the subdomain organisation of the PtdIns*P*K family and although the importance of other critical residues such as D211 and D273 has not been addressed in this study, these residues can now be aligned with the protein and PI-3 and PtdIns 4Ks with greater confidence. The data presented here clarify the structural relationship between the PtdIns*P*Ks and the PI/protein kinase superfamily and support the hypothesis that the PtdIns*P*K family is related to other phosphotransferases (Yamamoto *et al.*, 1995). Furthermore, as virtually all the conserved residues implicated in catalytic function in protein kinases also appear to be present in PtdIns*P*Ks, a distinct phosphotransferase mechanism, as previously suggested (Boronenkov and Anderson, 1995; Loijens *et al.*, 1996) is unlikely. Therefore, despite considerable diversity the PtdIns*P*Ks and members of the protein/PI kinase family appear to have evolved similar structures and do not constitute a completely unrelated family as previously stated (Hunter, 1995). The ongoing effort of our lab to solve the crystal structure of PtdIns*P*K IIα should determine to what extent the PtdIns*P*K family is related to other kinases by a common tertiary structure.

In addition to providing basic information on structure, the construction of kinase-dead mutants provides important reagents for the future study of the PtdIns*P*K function. It is anticipated that subtlely mutated, kinase-defficient PtdIns*P*Ks may inhibit signalling processes by acting as dominant negative proteins. These may prove to be useful in determining the mechanism of regulation of PtdIns*P*Ks, in particular the type II enzymes which have been proposed to lie in a novel PtdIns(4,5)P_2 biosynthetic pathway (Rameh *et al.*, 1997).

C-terminal proline-rich domain

None of the PtdIns*P*Ks contains any identifiable molecular interaction motif (Section 3.2.1). Limited homology to the chaperonin TCP-1 has been noted in the PtdIns(3)*P* 5-kinase Fab1p and the putative PtdIns*P*K *C.e* ORF1 (Hsuan *et al.*, 1998; Yamamoto *et al.*, 1995, also see Figure 1.6b), although the function of this region is unknown. We and others have noted the presence of two short proline-rich sequences between amino acids

313 and 314 in the insert region of PtdIns*P*K IIα which have been suggested to act as ligands for SH3 domain-containing molecules (Boronenkov and Anderson, 1995).

SH3 domain interactions may be important in the assembly of multimeric signalling complexes and also, as in the case of dynamin, in enzymatic activation (Gout *et al.*, 1993). Preferred SH3 ligands have been identified by screening phage display and combinatorial peptide libraries (Chen *et al.*, 1993; Chen *et al.*, 1995) and all have been found to contain the sequence PXXP. NMR and mutagenesis studies of Src SH3 domain-ligand complexes suggest that these conserved proline residues contact the binding pocket (Feng *et al.*, 1994). Modelling an interaction with the Src SH3 domain predicted that the PtdIns*P*K IIα peptides PPDSPGN and PPLAPGE could be accomodated by the Feng model (Figure 4.9). However, it is very unlikely that the Src SH3 domain could form a stable interaction with these two ligands because both contain a negatively charged residue that would prevent formation of the salt bridge with Src D99, which normally interacts with an arginine residue of the cognate intermolecular ligand PLPPLAR (Feng *et al.*, 1994). Recombinant PtdIns*P*K IIα failed to bind a panel of SH3 domains *in vitro* including those from p85α and PLCγ (Figure 4.10). Nevertheless, it remains possible that PtdIns*P*K IIα interacts with an unknown SH3 domain or that the interaction is in some way regulated. While it is not possible to decide whether or not PPDSPGN and PPLAPGE act as ligands, it is likely that if a binding partner for these proline-rich motifs exists, it will require a positively charged residue at the corresponding position to D99 in Src in order to form a stable interaction.

5.0 Monoclonal Antibodies to PtdIns*P* kinase IIα

5.1 Introduction

Initial biochemical characterisation studies of mammalian PtdIns*P*Ks indicated that there were only two immunologically distinct types termed type I and type II (Bazenet *et al.*, 1990; Jenkins *et al.*, 1994). However, in recent years, the rapid expansion of nucleotide databases and the use of degenerate PCR based on conserved PtdIns*P*K sequences has led to the isolation of a variety of novel phosphoinositide kinase homologues. As a consequence of these new approaches to cloning, the mammalian PtdIns*P*K family has expanded to include 6 distinct genes. The existence of multiple isoforms with closely related sequences greatly complicates the study of PtdIns*P*K function and few highly specific reagents exist. This problem is exemplified by the type IIα, -β, and -γ PtdIns*P*Ks which display a very high degree of sequence identity and cannot be easily separated by SDS-PAGE (see Table 8.3 for identity scores). Further heterogeneity can arise through the generation of a large number of splice variants (Carvajal *et al.*, 1995; Carvajal *et al.*, 1996; Loijens and Anderson 1996) and possibly by proteolysis (Loijens *et al.*, 1996).

No specific PtdIns*P*K inhibitors exist and although the type I and type II PtdIns*P*Ks can be distinguished *in vitro* on the basis of phosphatidic acid activation, this is often unreliable and not possible in intact cell systems. Distinguishing type I and type II subgroups by their *in vitro* substrate specificity is currently very difficult, for example requiring specific phosphatase treatment followed by HPLC (Rameh *et al.*, 1997). Isotype-specific polyclonal antibodies (Jenkins *et al.*, 1994) and type II-specific monoclonal antibodies have been described (Brooksbank *et al.*, 1993) but in both cases these were raised to proteins purified from animal tissues and were therefore likely to contain a mixture of isozymes. The specificity of these reagents for different isozymes has yet to be addressed.

Consequently, with the aim of generating immunological reagents for the *in vitro* and *in vivo* analysis of type IIα PtdIns*P*K function, murine monoclonal antibodies have been generated using highly pure, baculovirus-expressed, recombinant human PtdIns*P*K IIα. Such antibodies will prove valuable for the study PtdIns*P*K IIα expression in animal cells and tissues. Immunochemical experiments will reveal subcellular localisation and perhaps resolve outstanding questions such as whether cytosolic PtdIns*P*K IIα translocates to a membrane compartment on agonist stimulation. The ability to immunoprecipitate endogenous protein should help determine whether PtdIns*P*K IIα exists in complex with other signalling molecules such as membrane receptors. An immunoprecipitating antibody will also be of use in discovering which stimuli, if any, activate PtdIns*P*K IIα activity and whether or not this involves a postranslational modification such as phosphorylation.

5.2 Results

5.2.1 PtdIns*P* kinase IIα monoclonal antibodies

All animal handling, dissection, splenocyte fusion, and hybridoma cloning was performed by the UCL/Middlesex Hospital Monoclonal Antibody Unit. Briefly, female BALB/c mice were immunised with baculovirus-expressed PtdIns*P*K IIα from which the GST affinity tag had previously been removed by thrombin digestion in PBS (Section 3.2.8). All immunisations were performed by intravenous injection of 50 μg of native protein without adjuvants. After 30 days the mice were injected again and ten days later test bleeds were taken from the tails of both mice for analysis by ELISA, western blotting and immunoprecipitation. Antiserum from both mice tested positive in all assays (Figure 5.1), indicating that the immune response was sufficient for the generation of monoclonal antibodies.

On day 50, mouse 2 was further immunised and 48 h later given a final boost 48 hours prior to euthanasia and splenectomy. Fusion and selection were performed according to standard methods (Harlow and Lane, 1988).

Approximately 20 ELISA-positive hybridomas were obtained by fusion of splenocytes with X63Ag8.653 and selection in hypoxanthine-aminopterin-thymidine medium. Five of these were lost due to mortality. The remaining 15 were expanded in 12-well plates and the supernatants again screened by ELISA, western blotting, and immunoprecipitation (Figure 5.2). At least one antibody (Number 1) was unable to immunoprecipitate activity yet was strongly positive by ELISA and western blotting suggesting that this antibody bound an epitope resulting in the inhibition of activity. When specific, such inhibitory antibodies can be very useful for characterising mixtures of activities but cannot be used to immunoprecipitate activity. The isotype of supernatants testing positive by the above criteria was determined by antibody capture on PtdIns*P*K IIα-coated plates followed by ELISA with anti-isotype secondary antibodies (Harlow and Lane, 1988). Western blotting antibodies recognise low mass bands in addition to the major 53 kDa PtdIns*P*K IIα band (Figure 5.2a). This is due to the presence of minor proteolytic products and indicates specificity for different epitopes. The results of the screening experiments are summarised in Table 1.

Two hybridomas, 11 and 19 (subsequently renamed 4H1 and 7H8, respectively), were cloned by dilution and the supernatants screened by ELISA and immunoprecipitation. Hybridoma 7H8 was found to stably secrete an IgG1 antibody which was strongly positive in ELISA, western blotting and immunoprecipitation. Consequently, 7H8 was chosen for further characterisation and was grown in RPMI containing 10% serum (as it failed to grow in serum-free medium) and supernatants used directly for western blotting, immunoprecipitation and immunofluorescence.

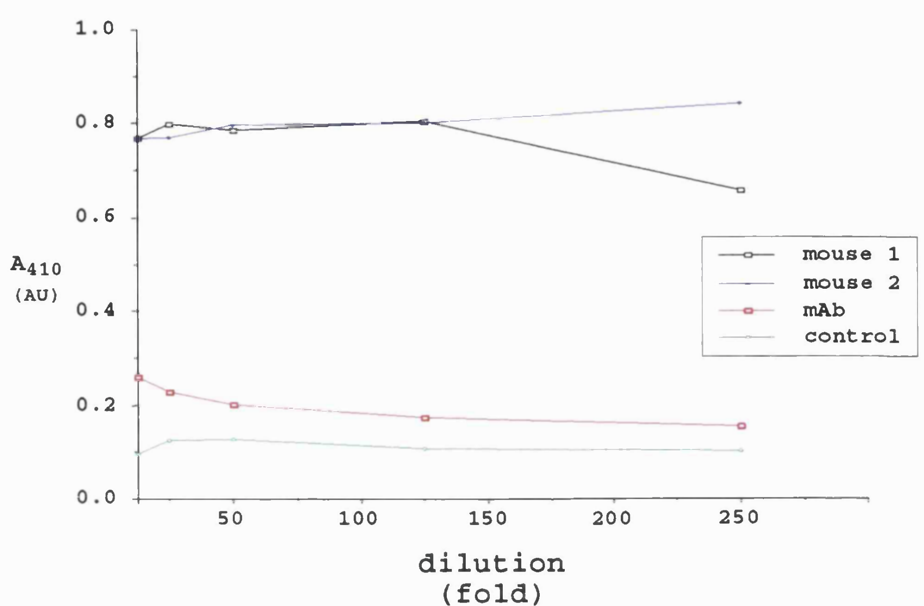
Figure 5.1 Analysis of polyclonal serum from PtdIns*P*K IIα immunised mice

A. ELISA of test bleeds from mouse 1 and mouse 2, or serum from a control mouse with a similar genetic background (dilutions shown were of neat serum). mAb, a commercial αPtdIns*P*K IIα monoclonal antibody (Affinity, dilutions shown were from a 0.1 mg/ml stock).

B. Immunoprecipitation of PtdIns*P*K activity by test serum. Serum was adsorbed to protein-G Sepharose and incubated for 40 min with 0.1 μg PtdIns*P*K IIα in PBS containing 0.1 % Triton X-100 and 1 mg/ml BSA (PBS-TB). After three washes in PBS-TB the immunoprecipitates were assayed for PtdIns*P*K activity and the lipid products of the reaction analysed by TLC followed by autoradiography.

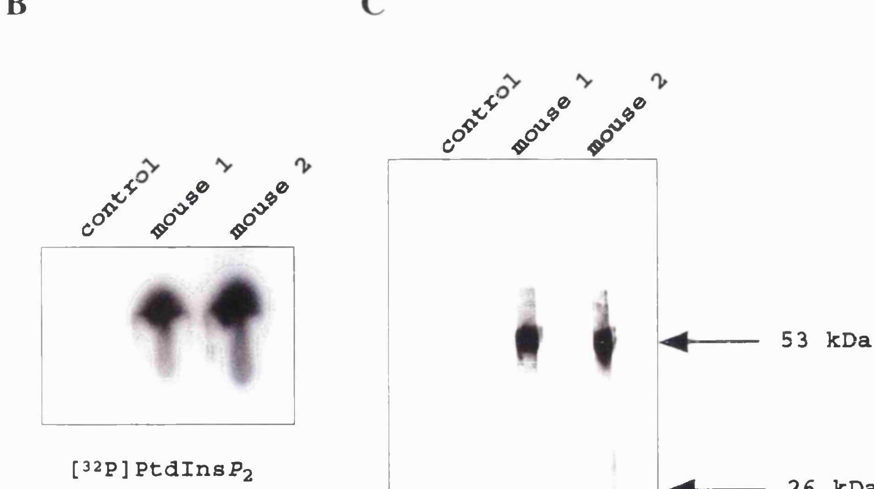
C. Western blotting of test serum. Equal amounts of PtdIns*P*K IIα (53 kDa) and GST (26 kDa) were separated by SDS-PAGE, transferred to PVDF and the membrane cut into narrow strips before probing with test serum or control serum (1:500 dilution) as indicated.

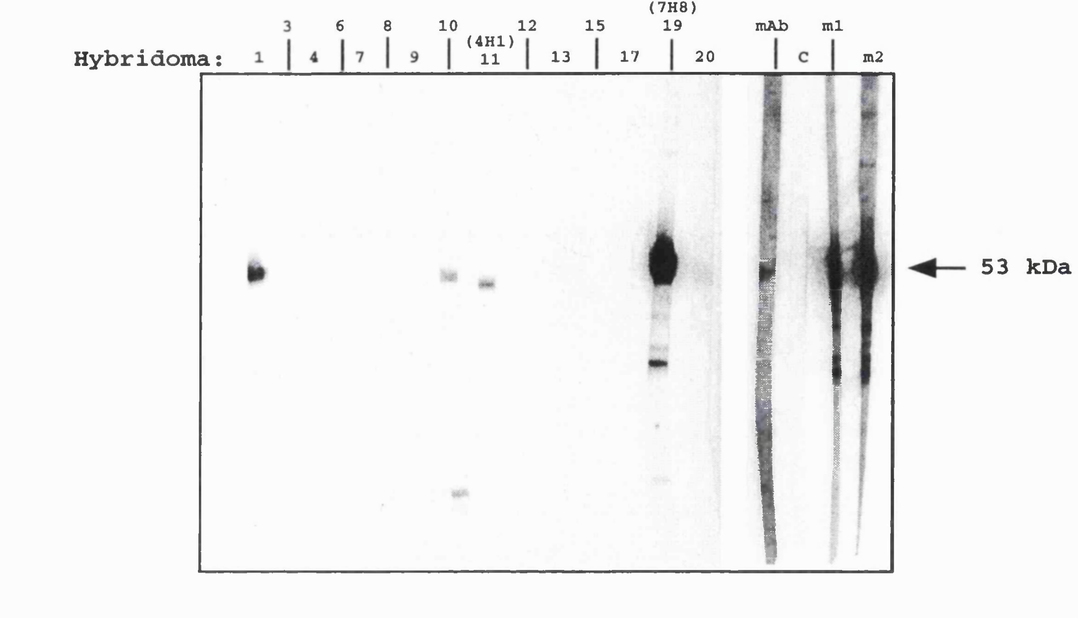
A



B

C





B

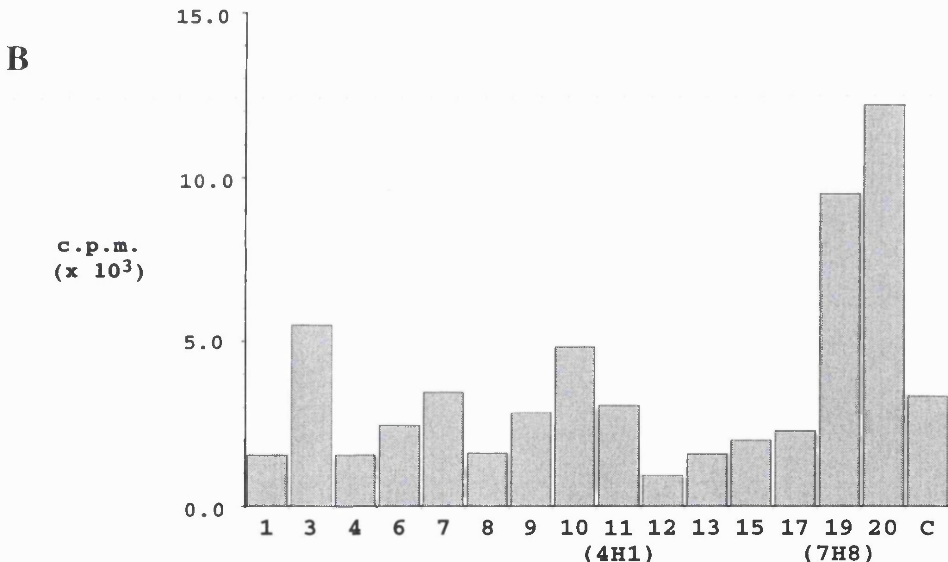


Figure 5.2 Screening of ELISA positive hybridomas by western blotting and immunoprecipitation

A. Equal amounts of PtdIns*P*K IIα and GST was transferred to PVDF membrane. This was cut into 4 mm strips which were probed with hybridoma supernatants (1:500 dilution). mAb, commercial anti-PtdIns*P*K IIα monoclonal antibody (Affiniti, Transduction Labs); C, control serum (as Figure 5.1); m1 and m2, mouse 1 and mouse 2 polyclonal serum as positive controls.

B. Hybridoma supernatants (1ml) were adsorbed to protein-G Sepharose and after washing, these were incubated with PtdIns*P*K IIα in 0.1 % Triton X-100 and 1 mg/ml BSA. Immune complexes were extensively washed in PBS containing 0.1% Triton X-100 and assayed for PtdIns*P*K activity as described previously (Section 2.5.2.b). After extraction, the organic phase was subjected to Cerenkov counting.

Hybridoma	ELISA	IP-activity	WB	Isotype
1	+++	no	+++	IgM
3	++	yes	-	nd
4	+	no	-	nd
6	+	no	-	nd
7	++	no	-/+	nd
8	++	no	-/+	nd
9	+	no	-	nd
10	+++	yes	+	IgG
(4H1) 11	+++	no	+	IgG1
12	+	no	-	nd
13	+	no	-	nd
15	+	no	-	nd
17	+	no	-	nd
(7H8) 19	+++	yes	+++	IgG1
20	+++	yes	+	IgM
control	no	no	-	na

Table 5.1 Summarised results of hybridoma screening

Relative signal strength is shown as follows: -, negative; +, weak positive; ++, positive; +++, strong positive. (WB, western blotting; nd, not determined; na, not applicable).

5.2.2 Western blotting: species and cell specificity

Total cell lysates and membrane fractions were probed with 7H8 by western blotting with the aim of identifying (i) the isoform specificity of 7H8, (ii) the specificity of this reagent against a complex background of cellular proteins and, (iii) assessing the relative levels of expression of PtdIns*P*K IIα in a range of commonly used mammalian cell lines (Figure 5.4).

7H8 was found to specifically recognise a 53 kDa protein which was particularly well expressed in U937, Jurkat and Bac1 cells, all of which are of heamatopoietic origin. The 53 kDa band was absent from salt-washed human erythrocyte ghosts (HEG). The antibody also recognised a similar sized protein in MDCK, HEK-293 and A431 epithelial cells but only very small amounts were present in human and rat fibroblasts (HFF and REF). The apparent absence of a 53 kDa protein in the REF cell line is likely to be the result of a low level of PtdIns*P*K IIα expression in this cell line. PtdIns*P*K IIα is virtually absent from human fibroblasts (HFF) and the fact that 7H8 cross-reacts with murine and canine proteins suggests that the alternative possibility, that the 7H8 epitope is absent in rat PtdIns*P*K IIα is unlikely. HEK-293 cells and A431 plasma membranes contained a cross-reacting band of approximately 47 kDa which was also visible in A431 and MDCK cell lysates, but not in other cell lines, on longer exposures (not shown). 7H8 did not cross-react with human recombinant STM7, a type I PtdIns(4)*P* 5-kinase.

5.2.3 Immunofluorescence

Unpurified 7H8 was tested for its ability to stain endogenous PtdIns*P*K IIα in A431 and MDCK cells. Only weak immunofluorescence was observed in these cells compared to cells stained with hybridoma medium alone (results not shown). The observation that 7H8 hybridoma culture supernatants did not produce a sufficient immunofluorescent signal from the endogenous PtdIns*P*K IIα may be due to several factors: firstly, levels of antibody are typically low in these supernatants (0.1-1.0 μg/ml) and secondly, undiluted supernatant contains relatively large amounts of serum proteins, including bovine immunoglobulins, which can cause non-specific immunofluorescence and intefere with recognition of PtdIns*P*K IIα. In an attempt to overcome these problems, Swiss 3T3 fibroblasts, which do not contain significant amounts of endogenous PtdIns*P*K IIα, (Figure 5.3) were microinjected with a mammalian expression construct containing epitope-tagged PtdIns*P*K IIα (Section 3.2.6). Cells were fixed and incubated with hybridoma supernatant from cultures of 7H8 diluted 1/10 in PBS and processed for immunofluorescence microscopy (Section 2.8.3). PtdIns*P*K IIα displayed a predominantly cytosolic localisation in Swiss 3T3 fibroblasts stained with 7H8 (Figure 5.4a). A similar pattern of immunofluorescence was observed when the same cells were stained with the anti-myc antibody 9E10 (Figure 5.4d). In addition to the cytosolic localisation, local concentrations of PtdIns*P*K IIα were also observed in actin-rich

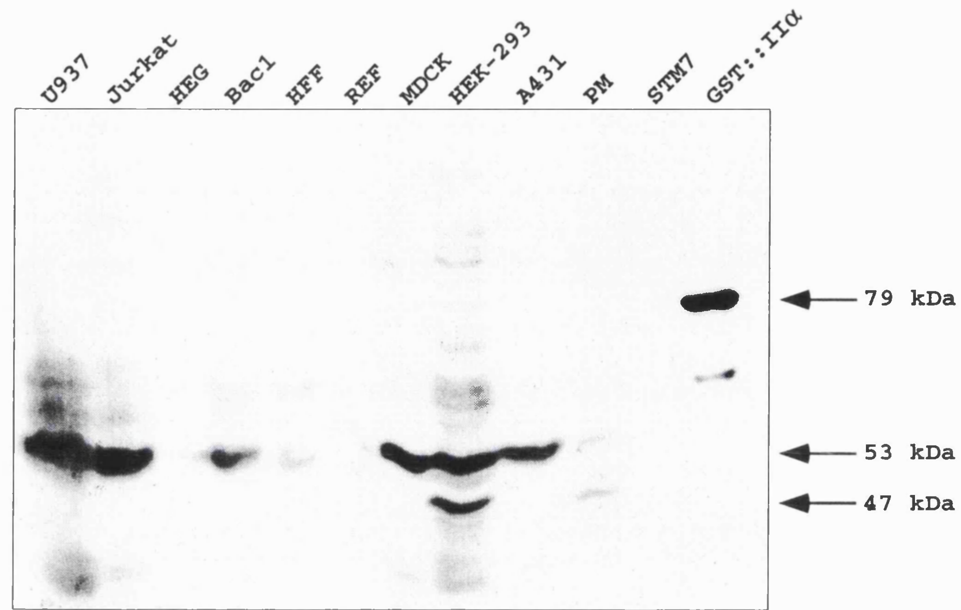


Figure 5.3 Species and cell specificity of mAb 7H8

Approximately 75 μg of total protein were separated by SDS-PAGE and transferred to PVDF membrane for western immunoblotting as described in Material and Methods. Samples are as follows: U937, human histiocytic lymphoma; Jurkat, human jurkat T-cell lymphoma; HEG, human erythrocyte ghost (salt washed); Bac1, murine macrophage; HFF, human foreskin fibroblast; REF, rat embryo fibroblast; MDCK, Madin-Derby canine kidney cells; HEK-293, human embryonic kidney cells; A431, human epidermoid carcinoma (total lysate); PM, A431 plasma membrane preparation; STM7, bacterially expressed 68 kDa human type I PtdIns(4)*P* 5-kinase; GST::IIα, recombinant PtdIns*P*K IIα-GST fusion protein.

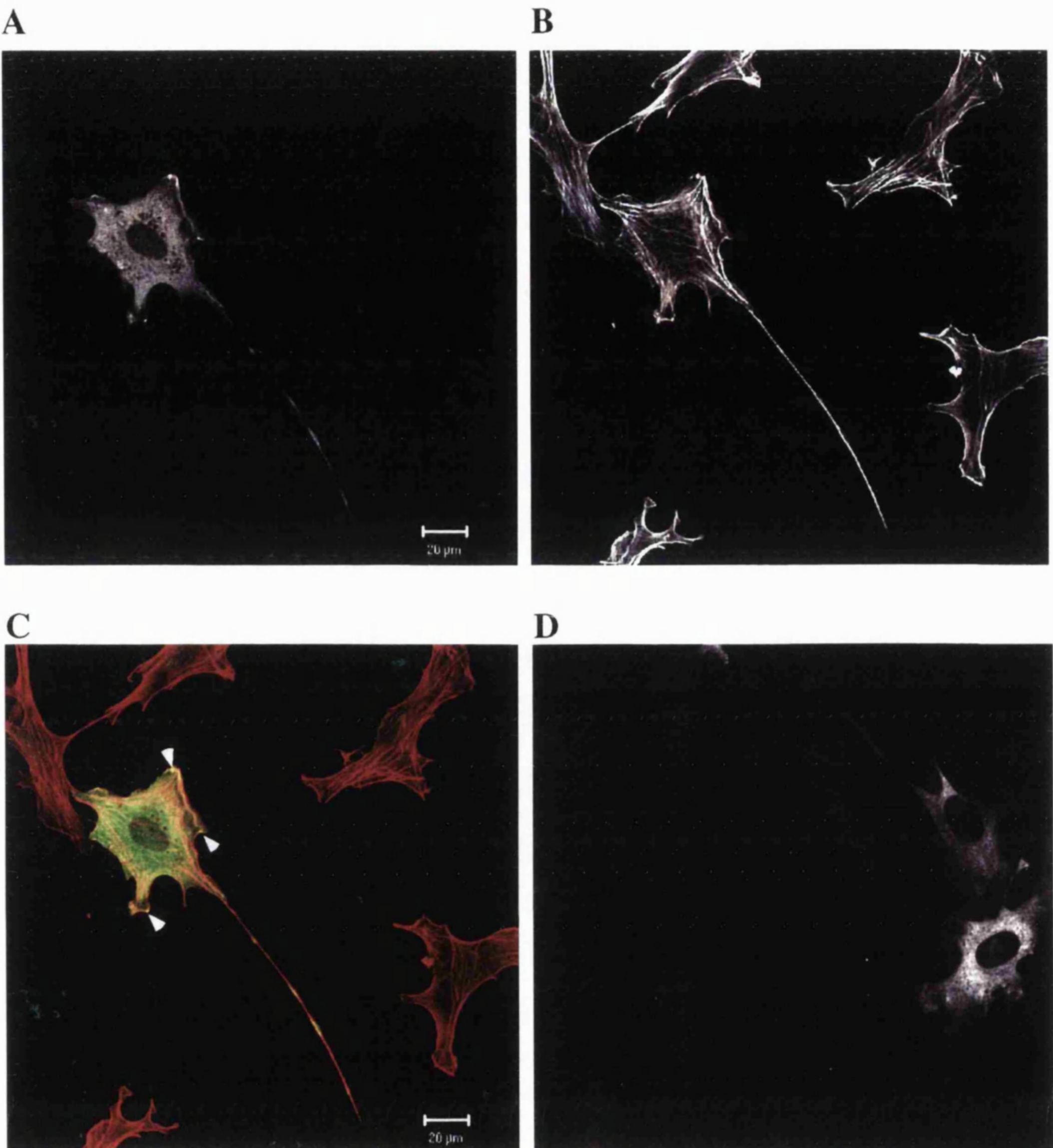


Figure 5.4 Immunofluorescence of Swiss 3T3 cells overexpressing PtdIns*P*K IIα

Swiss 3T3 cells were microinjected with a plasmid expressing myc epitope-tagged PtdIns*P*K IIα (Section 3.2.7).

A. A typical microinjected cell stained with mAb 7H8. B. The same cell stained with rhodamine-labelled phalloidin. C. Images A and B superimposed to show co-localisation of PtdIns*P*K IIα with actin; green indicates PtdIns*P*K IIα, red indicates the actin cytoskeleton, and yellow indicates co-localisation of PtdIns*P*K IIα and actin. Arrows indicate local concentrations of PtdIns*P*K IIα. (D) A microinjected Swiss 3T3 cell stained with mAb 9E10.

A B

Figure 5.5 Subcellular localisation of endogenous PtdIns*P*K IIα in HFF cells

A. HFF cells were stained with a mAb raised to a bacterially expressed polypetide corresponding to residues 42-206 of PtdIns*P*K IIα (Transduction Laboratories, Lexington, KY, USA).

B Cells were co-stained for actin using rhodamine-labelled phalloidin and analysed by confocal microscopy as described in Section 2.8.3. Arrowheads indicate actin fibres (1) and putative membrane ruffles/lamellopodia (2) where PtdIns*P*K IIα appears to be concentrated in (A).

peripheral regions of the 7H8 stained cells which were similar to membrane ruffles (5.4c). The latter observation was unexpected so I sought to confirm this by immuno-staining HFF cells (which express low levels of PtdIns*P*K IIα, Section 5.2.2) with a commercially available anti-PtdIns*P*K IIα mAb. As shown in Figure 5.5, endogenous PtdIns*P*K IIα localises to membrane ruffles and stress fibres in HFF cells.

5.3 Discussion

PtdIns*P*K IIα is one of three known mammalian type II PtdIns*P*Ks which are highly related. Recombinant PtdIns*P*K IIα was used to raise murine monoclonal antibodies with the aim of generating effective tools for the study of PtdIns*P*K IIα function. Of the hybridomas obtained in this study, 7H8 was found to secrete an IgG_1 antibody which could recognise PtdIns*P*K IIα by ELISA, western blotting, immunopreciptation and immunofluorescence. Western blotting showed that PtdIns*P*K IIα was most highly expressed in cells of haematopoietic origin. Relatively large amounts of PtdIns*P*K IIα were also observed in epithelial cells derived from human and canine kidney and human cervical carcinoma (A431) but PtdIns*P*K IIα appears to be expressed at relatively low levels in HFF and was not detected in REF fibroblasts. Salt washed human erythrocyte ghosts did not contain detectable amounts of PtdIns*P*K IIα whereas an unwashed A431 plasma membrane preparation did contain appreciable amounts of PtdIns*P*K IIα. This is consistent with earlier reports where a type II activity could be removed from erythrocyte membranes with a high salt wash (Bazenet *et al.*, 1990; Jenkins *et al.*, 1994; Ling *et al.*, 1989).

As expected from the high degree of conservation between PtdIns*P*K IIα homologues (Table 8.3), 7H8 recognised an epitope common to the human, murine and canine forms of PtdIns*P*K IIα.

The appearence of a 47 kDa band in some lysates suggests that 7H8 may cross react with PtdIns*P*K IIβ, an isoform with 76% sequence identity with PtdIns*P*K IIα, although the possibility that the lower band is a proteolytic product of PtdIns*P*K IIα cannot be ruled out without further experimentation. Recently a third type II isoform, the 47 kDa PtdIns*P*K IIγ has been cloned from rat brain which is 61.1% identical to the human PtdIns*P*K IIα (Itoh *et al.*, 1998). The mobility of this isoform on SDS-PAGE is similar to that of PtdIns*P*K IIβ which is 76% identical to PtdIns*P*K IIα (Castellino *et al.*, 1997) but because these two isoforms migrate faster than PtdIns*P*K IIα on SDS-PAGE it will be possible to distinguish them in future western blotting experiments. The fact that 7H8 does not recognise a type I PtdIns*P*K probably indicates that any possible cross reactivity is limited to the type II subgroup of PtdIns*P*Ks.

Immunostaining with 7H8 showed the antibody to be an effective immunofluoresecnce reagent. In Swiss 3T3 fibroblasts ectopically-expressing PtdIns*P*K IIα , 7H8 indicated a predominantly cytosolic localisation. This is consistent with the results obtained on microinjection of MDCK and Swiss 3T3 cells with epitope-tagged PtdIns*P*K IIα stained with mAb 9E10 (Section 3.2.7 and Section 5.2.3). The weak immunofluorescence signal

observed in A431 and MDCK cells (not shown) is expected to be a consequence of the relatively low levels of endogenous PtdIns*P*K IIα in these cells compared with microinjected cells. It is likely that 7H8 hybridoma supernatants are too impure to be used directly, however, the fact that 7H8 can recognise ectopically-expressed PtdIns*P*K IIα suggests that affinity-purified 7H8 may be capable of detecting endogenous PtdIns*P*K IIα by immunofluorescence.

Interestingly, PtdIns*P*K IIα ectopically expressed in Swiss 3T3 cells also localised to actin-rich regions of the cell periphery which were morphologically similar to membrane ruffles. Furthermore, PtdIns*P*K IIα appeared to be concentrated at discrete points in these structures (Figure 5.4). This phenomenon was not observed when cells were stained for the myc epitope tag, nevertheless, endogenous PtdIns*P*K IIα showed a similar pattern of immunofluorescence in HFF cells stained with a commercially available PtdIns*P*K IIα mAb (Figure 5.5) suggesting that the 7H8 immunofluorescence was unlikely to be the result of overexpression. The observation that 9E10 does not give the same pattern of staining may be due to the fact that the 9E10 epitope is somehow masked in the actin-localised PtdIns*P*K IIα. Immunoflourescence of HFF cells also revealed several potentially important observations: firstly, that in HFF cells at least, PtdIns*P*K IIα is not detected in the cytosol; secondly, that PtdIns*P*K IIα co-localises with actin fibres, and finally, that PtdIns*P*K IIα is concentrated at the leading edge of the cells (Figure 5.5). The observation that endogenous HFF PtdIns*P*K IIα is not cytosolic suggests that overexpression of PtdIns*P*K IIα in mammalian cells may lead to a delocalised pool of cytosolic PtdIns*P*K IIα, and furthermore, it is possible that the strong cytoplasmic immunofluorescence obscures the actin fibre localisation.

6.0 Cloning and Characterisation of a PtdIns 4-kinase

6.1 Introduction

PtdIns 4K activities have been purified from many mammalian sources including brain (Endemann *et al.*, 1987; Scholz *et al.*, 1991; Gehrmann *et al.*, 1996), liver (Hou *et al.*, 1988) human epidermoid carcinoma A431 cells (Walker *et al.*, 1988), erythrocytes (Jenkins *et al.*, 1991; Wetzker *et al.*, 1991; Graziani *et al.*, 1992), adrenal medulla (Husebye and Flatmark, 1988), and bovine uterus (Porter *et al.*, 1988; Li *et al.*, 1989). PtdIns 4K activity has been found associated with numerous membrane fractions such as plasma membrane, Golgi, lysosome, coated vesicle and nuclear membrane (Pike 1992, for review). PtdIns 4K activity has also been purified from *S. cerevisiae* (Flanagan and Thorner, 1992). However, the reported 55 kDa and 45 kDa membrane-associated yeast PtdIns 4K activities (Belunis *et al.*, 1988; Nickels *et al.*, 1992) have not been characterised at the molecular level and no unreported genes with PtdIns-kinase homology can be found in the complete genome of *S. cerevisiae* (Mewes *et al.*, 1997). It is possible that the 45 and 55 kDa activities represent proteolytic products of the tightly membrane-associated *STT4* gene product or that they arise from a novel family of PI kinases.

The prevalent activity in brain and A431 cells appears to be the membrane bound 55 kDa type II enzyme which accounts for up to 43-95% of total PI kinase activity (Endemann *et al.*, 1987; Pike, 1992). The type II isoform has been implicated in many important signalling processes (Section 1.5.1.a) and associates with integrin $\alpha_3\beta_1$ (Berditchevski *et al.*, 1997; Yauch et al., 1998), the CD4-p56^{lck} complex (Prasad *et al.*, 1993; Pertile and Cantley, 1995), and the EGF receptor in an EGF-dependent manner (Cochet *et al.*, 1991; Kauffmann Zeh *et al.*, 1995). In spite of much effort this isoform remains uncloned, largely because the type II enzyme has a strong tendency to aggregate with other proteins (Wetzker *et al.*, 1991). Consequently, it has proven difficult to obtain preparations of sufficient quality for peptide sequencing. Despite the apparent abundance of this protein, homology-based strategies such as degenerate PCR and cDNA database searching have yet to yield any appropriate PtdIns 4K clones, and it seems increasingly likely that the type II activity may belong to an as yet uncharacterised family of phosphoinositide kinases. This idea stems from the following reasoning: (i) the abundance of protein in diverse tissues implies a relatively abundant message, which suggests that an EST should exist; (ii) the biochemical properties of the type II PtdIns 4K in regard to detergent activation, K_m for ATP, the ability to renature following SDS-PAGE, and inhibition by adenosine, Ca^{2+} and wortmannin, are significantly different to the known PtdIns 4Ks.

	p55 type II¶	p92 (PtdIns4K β)	p97† (PtdIns 4Kα)	p230 (type III/PtdIns4α)	References
Activators	Triton X-100, phosphorylat-ion, spermine and spermidine	Triton X-100	Triton X-100	Triton X-100	Pike, 1992; Kaufmann-Zeh *et .al.*, 1994; Wong & Cantley, 1994; Nakagawa *et.al.*, 1996b
Inhibitors	mAb 4C5G, adenosine (IC_{50} = 20-100 μM)	wortmannin (IC_{50} = 100 nM), adenosine (IC_{50} = 200 μM)	mAb 4C5G, adenosine (IC_{50} = 20-100 μM)	wortmannin (IC_{50} =0.5 mM), adenosine (IC_{50} >1mM)	Pike, 1992; Nakagawa *et al.*, 1996b; Wong & Cantley, 1994;
Subcellular localisation	plasma membrane, vesicles	Golgi, vesicles*, cytosol	cytosol	Golgi, vesicles*	Pike, 1992; Wong *et al.*, 1997;
Effect of Ca^{2+}	inhibits (K_i = 0.3-0.4 mM)	nd	nd	does not inhibit	Pike, 1992
Effect PtdIns(4,5)P_2	inhibits	nd	nd	nd	
Km ATP	20-100 μM	nd	nd	150-750 μM	Pike, 1992
Km PtdIns	30-60 μM	nd	nd	15 μM	Pike, 1992

Table 6.1 The mammalian PtdIns 4Ks.

nd, not determined. *, Subcellular localisation is very similar for p230 and p92 may be artefactual due to use of strong overexpression and/or incomplete posttranslational modification. †, Does not correspond to any previously activity (see Section 6.1). ¶, Has yet to be cloned.

The fact that the type II enzyme is such an abundant and 'sticky' enzyme can lead to false conclusions in experimental data. Significant amounts of PtdIns 4K activity can be found associated with many partially purified proteins and following immuno-precipitation procedures. This has lead to the publication of a PtdIns 4K which, despite demonstrable activity, later turned out to be a fatty acyl CoA synthetase (Yamakawa *et al.*, 1991; Yamakawa *et al.*, 1992). In other studies where the significance of PtdIns 4K is not well understood, the possibility of contamination should be seriously considered in the absence of proper controls to demonstrate that activity is intrinsic rather than associated.

In common with other PI kinases, the study of PtdIns 4Ks is greatly complicated by the existence of multiple isoforms (Table 6.1) and a lack of specific immunological and biochemical tools. Acceptable evidence of the identification of the type II isoform is usually provided by activation by detergent, inhibition by relatively low concentrations of adenosine, a 10-20-fold lower K_m for ATP than type III isoforms and the fact that only the 55 kDa type II isoform will renature from SDS-PAGE gel slices. However the 4C5G mAb raised against the type II PtdIns 4K (Endemann *et al.*, 1991) can also inhibit p97, a probable splice variant of the 230 kDa type III enzyme (Wong and Cantley, 1994), which brings into question the specificity of this widely used reagent.

Recently a soluble adrenal PtdIns 4K activity has been described which is sensitive to the PI 3-kinase inhibitors wortmannin and LY294002 (Downing *et al.*, 1996; Nakanishi *et al.*, 1995). Wortmannin-sensitivity now appears to be a property of several PtdIns 4Ks including the mammalian type III/PtdIns 4Kα (Table 6.1) and the yeast *STT4* proteins. The yeast *Pik1* protein is insensitive to wortmannin even at high concentrations (J. Thorner, personal communication) as is the type II activity present in Jurkat and A431 cell membranes (Nakanishi *et al.*, 1995 and M. Waugh, unpublished observations).

The first mammalian PtdIns 4K to be characterised at the molecular level was a human 97 kDa PtdIns 4Kα isoform which appears to be a soluble splice variant of the 230 kDa membrane-associated type III enzyme. It is notable that a 97 kDa activity had not been described prior to its cloning, despite extensive purification studies. Also, since it's cloning, no further data has been presented to confirm the existence of the p97 isoform. Therefore, the possibility that this isoform is a very minor splice variant should be seriously considered.

The surprising discovery that the type II PtdIns*P* kinases can phosphorylate the D-4 position of phosphoinositides (Rameh *et al.*, 1997) raises the possibility that as yet uncloned PtdIns 4Ks may be encoded by a PtdIns*P* kinase homologue or a third family of PI kinases. The type II PtdIns*P* kinase described in Chapter 3 has a similar mobility to the type II PtdIns 4K but does not use PtdIns as substrate or renature following SDS-PAGE. A human erythrocyte and platelet PtdIns(3)*P* 4-kinase has been described which has an apparent molecular mass of 110 kDa by gel filtration (Graziani *et al.*, 1992; Yamamoto *et al.*, 1990). This is consistent with the properties of the 53 kDa PtdIns*P*K IIα which is able to dimerise in two-hybrid assays (M. dos Santos and S. Minogue, unpublished data). PtdIns(3,4)P_2 has an important signalling role in the activation of the proto-oncogene Akt (Section 1.5.4.a) and the novel substrate specificty of PtdIns*P*K IIα (and other type II PtdIns*P*Ks; see Section 1.5.2.c) indicates they have the potential to act in such a pathway.

This Chapter describes the cloning of a novel member of the PtdIns 4K family with striking homology to the yeast *Pik1* gene product. Biochemical characterisation of the protein indicates properties inconsistent with the 55 kDa type II PtdIns 4K.

6.2 Results

6.2.1 Cloning and sequence analysis

Given the problems associated with purifying the type II PtdIns 4K (discussed in Section 1.5.1.a), alternative routes to cloning this crucial signalling isoform were sought which did not rely on sequencing a purified protein. To this end, degenerate PCR primers were designed with homology to the conserved PtdIns 4K catalytic domain (Figure 6.1), with the expectation that the relatively abundant type II isoform would be well represented at the mRNA level. Degenerate PCR using random-primed first-strand cDNA derived from

A431 and Jurkat cells as template produced only one sequence with homology to the PtdIns 4Ks and which upon closer analysis was found to be identical to the sequence of PtdIns 4Kα (Wong and Cantley, 1994).

Searching of databases containing transcribed human sequences for motifs conserved between the known PtdIns 4Ks identified a partial cDNA clone (Genbank Z42324). The translated sequence of this short cDNA predicted a polypeptide with marked similarity to mammalian and yeast PtdIns 4Ks (Figure 6.1). The sequence of this EST was used to design oligonucleotide primers Z4fwd (5'-ATATGGATCCCAGTTCTCTGTGGACAGC) and Z4rev (5'-ATATGCGGCCGCTCTTGCCGAAGGTCATCC) for use in PCR. A single 271 bp PCR product, corresponding to the expected size, was isolated using human A431 first strand cDNA template and cloned into pBluescript. After sequencing, the 271 bp cDNA was used to probe human placenta and foetal brain cDNA libraries from which five overlapping clones with homology to PtdIns 4Ks were obtained. In consideration of this and of the experimental data obtained subsequently, this cDNA was named PtdIns 4K β (PtdIns 4Kβ). The clones extending furthest in the 5'- direction all contained a Kozak consensus sequence preceded by an in-frame stop codon. The aligned consensus sequence predicted an open reading frame of 801 codons (Figure 1.2). A minor splice variant (PtdIns 4KβI) was also isolated in which a serine-rich insert of 15 amino acid residues in length was present. A MOTIFS (GCG, University of Wisconsin) search of this sequence identified serine residues 316 and 325 as potential substrates for the protein serine/threonine kinase, casein kinase II.

Sequence comparisons with known PI kinases confirmed PtdIns 4Kβ to be a member of the PI 3/4-kinase superfamily (Zvelebil *et al.*, 1996, and Figure 1.3). Comparison with known PI kinases showed that the C-terminus of PtdIns 4Kβ, contains the catalytic core, and is most closely related to the PtdIns 4K family which currently includes mammalian PtdIns 4K α/type III, yeast STT4p, the archetypal PtdIns 4K from *S. cerevisiae*, Pik1p and open reading frames from *C. elegans*, *D. discoideum* and *S. pombe* all of which are predicted to be PtdIns 4Ks. PtdIns 4Kβ showed the closest similarity to Pik1p with 33.8% overall identity and 44% identity in the kinase domain. Homology with other PI kinases was also noted outside the kinase domain between residues 140-201, which corresponds to the lipid kinase unique or lipid kinase homology 2 (LKH2) domain (Figures 1.4 and 6.3). This is a feature of PI 3- and PtdIns 4Ks that has been described previously (Hsuan *et al.*, 1998; Zvelebil *et al.*, 1996), but its function remains unknown. An interesting feature of the LKH2 domain in Pik1p and PtdIns 4Kβ is its position close to the N-terminus in contrast to all other PI 3/4-kinases (see Figure 1.4). PtdIns 4β and Pik1p can also be distinguished from the other PtdIns 4Ks by the presence of a third lipid kinase homology domain (LKH3) which is discussed in detail below (Section 6.2.7).

H.s. Z42324	LSVI**V**KCGDD**L**RQE(End)
D.D 4K	VSVIVKTGDDCRQEQMAVQLISKFDEIWKETRL
S.c. PIK 1	CSVIAKTGDDLRQEAFAYQMIQAMANIWVKEKV
H.s. 4Kα	QAAIFKVGDDCRQDMLALQIIDLFKNIFQLVGL
4K consensus:	XXO**I**O**KXGDD**O**RQ**ZXO**A**O**Q**O**I**XXOXX**I**OXXXXO
H.s. Vps34	YPVIFKHGDDLRQDQLILQIISLMDKLLRKENL
H.s. p110α	NEIIFKNGDDLRQDMLTLQIIRIMENIWQNQGL
H.s. p110γ	IGIIFKHGDDLRQDMLILQILRIMESIYETESL
3K CONSENSUS:	XXO**IFKXGDDLRQD**X**L**X**LQI**OXO**M**ZXOXXXXX**L**
PIK CONSENSUS:	XXO**I**O**KXGDD**O**RQ**ZXOXO**Q**OOXXOXXOXXXXXO

Figure 6.1 Strategy used for database searching and design of primers for degenerate PCR

The high degree of sequence homology between the catalytic subunits of the PI kinases (PIKs) makes the specific identification of novel PtdIns 4-kinase clones difficult. In an attempt to identify features unique to the PtdIns 4-kinase catalytic core all available PtdIns 4-kinase and PI 3-kinase sequences were compiled and aligned as shown above. Several features were used to identify database clone Z42324: (i) PtdIns 4-kinases contain a cysteine or leucine residue at the conserved hydrophobic position in the sequence GDDO, whereas in PI 3-kinases this is an invariant leucine. (ii) The PtdIns 4-kinases prefer a glutamic acid residue at the conserved acidic position in the sequence GDDORQZ which is an invariant aspartic acid in the PI 3-kinases.

Note that only a limited number of PIK homologues were available when this comparison was made. Definitions are given in Figures 1.4 and 1.8. Symbols used: O, hydrophobic residue; Z, acidic residue; X, any residue.

```
  1  CCGCGGCCGCTGTCAGGGAAGCGCAGGCGGCCAATGGAACCCGGGAGCGGTCGCTGCTGC   60
     GGCGCCGGCGACAGTCCCTTCGCGTCCGCCGGTTACCTTGGGCCCTCGCCAGCGACGACG
              .         .         .         .         .         .
 61  TGAGGCGGCAGTGTCGGCAGTCCAACCGCGACTGCCCGCACCCCCTCCGCGGGGGTCCCC  120
     ACTCCGCCGTCACAGCCGTCAGGTTGGCGCTGACGGGCGTGGGGGAGGCGCCCCCAGGGG
     *        .         .         .    >>>  .         .         .
121  CAGAGCTTGGAAGCTCGAAGTCTGGCTGTGGCCATGGGAGATACAGTAGTGGAGCCTGCC  180
     GTCTCGAACCTTCGAGCTTCAGACCGACACCGGTACCCTCTATGTCATCACCTCGGACGG
                                      M  G  D  T  V  V  E  P  A      9
              .         .         .         .         .         .
181  CCCTTGAAGCCAACTTCTGAGCCCACTTCTGGCCCACCAGGGAATAATGGGGGGTCCCTG  240
     GGGAACTTCGGTTGAAGACTCGGGTGAAGACCGGGTGGTCCCTTATTACCCCCCAGGGAC
     P  L  K  P  T  S  E  P  T  S  G  P  P  G  N  N  G  G  S  L     29
              .         .         .         .         .         .
241  CTAAGTGTCATCACGGAGGGGGTCGGGGAACTATCAGTGATTGACCCTGAGGTGGCCCAG  300
     GATTCACAGTAGTGCCTCCCCCAGCCCCTTGATAGTCACTAACTGGGACTCCACCGGGTC
     L  S  V  I  T  E  G  V  G  E  L  S  V  I  D  P  E  V  A  Q     49
              .         .         .         .         .         .
301  AAGGCCTGCCAGGAGGTGTTGGAGAAAGTCAAGCTTTTGCATGGAGGCGTGGCAGTCTCT  360
     TTCCGGACGGTCCTCCACAACCTCTTTCAGTTCGAAAACGTACCTCCGCACCGTCAGAGA
     K  A  C  Q  E  V  L  E  K  V  K  L  L  H  G  G  V  A  V  S     69
              .         .         .         .         .         .
361  ATCAGAGGCACCCCACTGGAGTTGGTCAATGGGGATGGTGTGGACAGTGAGATCCGTTGC  420
     TAGTCTCCGTGGGGTGACCTCAACCAGTTACCCCTACCACACCTGTCACTCTAGGCAACG
     I  R  G  T  P  L  E  L  V  N  G  D  G  V  D  S  E  I  R  C     89
              .         .         .         .         .         .
421  CTAGATGATCCACCTGCCCAGATCAGGGAGGAGGAAGATGAGATGGGGGCCGCTGTGGCC  480
     GATCTACTAGGTGGACGGGTCTAGTCCCTCCTCCTTCTACTCTACCCCCGGCGACACCGG
     L  D  D  P  P  A  Q  I  R  E  E  E  D  E  M  G  A  A  V  A    119
              .         .         .         .         .         .
481  TCAGGCACAGCCAAAGGAGCAAGAAGACGGCGGCAGAACAACTCAGCTAAACAGTCTTGG  540
     AGTCCGTGTCGGTTTCCTCGTTCTTCTGCCGCCGTCTTGTTGAGTCGATTTGTCAGAACC
     S  G  T  A  K  G  A  R  R  R  R  Q  N  N  S  A  K  Q  S  W    139
              .         .         .         .         .         .
541  CTGCTGAGGCTGTTTGAGTCAAAACTGTTTGACATCTCCATGGCCATTTCATACCTGTAT  600
     GACGACTCCGACAAACTCAGTTTTGACAAACTGTAGAGGTACCGGTAAAGTATGGACATA
     L  L  R  L  F  E  S  K  L  F  D  I  S  M  A  I  S  Y  L  Y    159
              .         .         .         .         .         .
601  AACTCCAAGGAGCCTGGAGTACAAGCCTACATTGGCAACCGGCTCTTCTGCTTTCGCAAC  660
     TTGAGGTTCCTCGGACCTCATGTTCGGATGTAACCGTTGGCCGAGAAGACGAAAGCGTTG
     N  S  K  E  P  G  V  Q  A  Y  I  G  N  R  L  F  C  F  R  N    179
              .         .         .         .         .         .
661  GAGGACGTGGACTTCTATCTGCCCCAGTTGCTTAACATGTACATCCACATGGATGAGGAC  720
     CTCCTGCACCTGAAGATAGACGGGGTCAACGAATTGTACATGTAGGTGTACCTACTCCTG
     E  D  V  D  F  Y  L  P  Q  L  L  N  M  Y  I  H  M  D  E  D    199
              .         .         .         .         .         .
721  GTGGGTGATGCCATTAAGCCCTACATAGTCCACCGTTGCCGCCAGAGCATTAACTTTTCC  780
     CACCCACTACGGTAATTCGGGATGTATCAGGTGGCAACGGCGGTCTCGTAATTGAAAAGG
     V  G  D  A  I  K  P  Y  I  V  H  R  C  R  Q  S  I  N  F  S`   219
              .         .         .         .         .         .
781  CTCCAGTGTGCCCTGTTGCTTGGGGCCTATTCTTCAGACATGCACATTTCCACTCAACGA  840
     GAGGTCACACGGGACAACGAACCCCGGATAAGAAGTCTGTACGTGTAAAGGTGAGTTGCT
     L  Q  C  A  L  L  L  G  A  Y  S  S  D  M  H  I  S  T  Q  R    239
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 841  CACTCCCGTGGGACCAAGCTACGGAAGCTGATCCTCTCAGATGAGCTAAAGCCAGCTCAC   900
      GTGAGGGCACCCTGGTTCGATGCCTTCGACTAGGAGAGTCTACTCGATTTCGGTCGAGTG
      H  S  R  G  T  K  L  R  K  L  I  L  S  D  E  L  K  P  A  H    259

 901  AGGAAGAGGGAGCTGCCCTCCTTGAGCCCGGCCCCTGACACAGGGCTGTCTCCCTCCAAA   960
      TCCTTCTCCCTCGACGGGAGGAACTCGGGCCGGGGACTGTGTCCCGACAGAGGGAGGTTT
      R  K  R  E  L  P  S  L  S  P  A  P  D  T  G  L  S  P  S  K    279

 961  AGGACTCACCAGCGCTCTAAGTCAGATGCCACTGCCAGCATAAGTCTCAGCAGCAACCTG   1020
      TCCTGAGTGGTCGCGAGATTCAGTCTACGGTGACGGTCGTATTCAGAGTCGTCGTTGGAC
      R  T  H  Q  R  S  K  S  D  A  T  A  S  I  S  L  S  S  N  L    299

1021  AAACGAACAGCCAGCAACCCTAAAGTGGAGAATGAGGATGAGGAGCTCTCCTCCAGCACC   1080
      TTTGCTTGTCGGTCGTTGGGATTTCACCTCTTACTCCTACTCCTCGAGAGGAGGTCGTGG
      K  R  T  A  S  N  P  K  V  E  N  E  D  E  E  L  S  S  S  T    319

1081  GAGAGTATTGATAATTCATTCAGTTCCCCTGTTCGACTGGCTCCTGAGAGAGAATTCATC   1140 Eco RI
      CTCTCATAACTATTAAGTAAGTCAAGGGGACAAGCTGACCGAGGACTCTCTCTTAAGTAG
      E  S  I  D  N  S  F  S  S  P  V  R  L  A  P  E  R  E  F  I    339

1141  AAGTCCCTGATGGCGATCGGCAAGCGGCTGGCCACGCTCCCCACCAAAGAGCAGAAAACA   1200
      TTCAGGGACTACCGCTAGCCGTTCGCCGACCGGTGCGAGGGGTGGTTTCTCGTCTTTTGT
      K  S  L  M  A  I  G  K  R  L  A  T  L  P  T  K  E  Q  K  T    359

1201  CAGAGGCTGATCTCAGAGCTCTCCCTGCTCAACCATAAGCTCCCTGCCCGAGTCTGGCTG   1260
      GTCTCCGACTAGAGTCTCGAGAGGGACGAGTTGGTATTCGAGGGACGGGCTCAGACCGAC
      Q  R  L  I  S  E  L  S  L  L  N  H  K  L  P  A  R  V  W  L    379

1261  CCCACTGCTGGCTTTGACCACCACGTGGTCCGTGTACCCCACACACAGGCTGTTGTCCTC   1320
      GGGTGACGACCGAAACTGGTGGTGCACCAGGCACATGGGGTGTGTGTCCGACAACAGGAG
      P  T  A  G  F  D  H  H  V  V  R  V  P  H  T  Q  A  V  V  L    399

1321  AACTCCAAGGACAAGGCTCCCTACCTGATTTATGTGGAAGTCCTTGAATGTGAAAACTTT   1380
      TTGAGGTTCCTGTTCCGAGGGATGGACTAAATACACCTTCAGGAACTTACACTTTTGAAA
      N  S  K  D  K  A  P  Y  L  I  Y  V  E  V  L  E  C  E  N  F    419

1381  GACACCACCAGTGTCCCTGCCCGGATCCCCGAGAACCGAATTCGGAGTACGAGGTCCGTA   1440 Bam HI
      CTGTGGTGGTCACAGGGACGGGCCTAGGGGCTCTTGGCTTAAGCCTCATGCTCCAGGCAT
      D  T  T  S  V  P  A  R  I  P  E  N  R  I  R  S  T  R  S  V    439

1441  GAAAACTTGCCCGAATGTGGTATTACCCATGAGCAGCGAGCTGGCAGCTTCAGCACTGTG   1500
      CTTTTGAACGGGCTTACACCATAATGGGTACTCGTCGCTCGACCGTCGAAGTCGTGACAC
      E  N  L  P  E  C  G  I  T  H  E  Q  R  A  G  S  F  S  T  V    459

1501  CCCAACTATGACAACGATGATGAGGCCTGGTCGGTGGATGACATAGGCGAGCTGCAAGTG   1560
      GGGTTGATACTGTTGCTACTACTCCGGACCAGCCACCTACTGTATCCGCTCGACGTTCAC
      P  N  Y  D  N  D  D  E  A  W  S  V  D  D  I  G  E  L  Q  V    479

1561  GAGCTCCCCGAAGTGCATACCAACAGCTGTGACAACATCTCCCAGTTCTCTGTGGACAGC   1620
      CTCGAGGGGCTTCACGTATGGTTGTCGACACTGTTGTAGAGGGTCAAGAGACACCTGTCG
      E  L  P  E  V  H  T  N  S  C  D  N  I  S  Q  F  S  V  D  S    499

1621  ATCACCAGCCAGGAGAGCAAGGAGCCTGTGTTCATTGCAGCAGGGGACATCCGCCGGCGC   1680
      TAGTGGTCGGTCCTCTCGTTCCTCGGACACAAGTAACGTCGTCCCCTGTAGGCGGCCGCG
      I  T  S  Q  E  S  K  E  P  V  F  I  A  A  G  D  I  R  R  R    519
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          .         .         .         .         .         .
1681 CTTTCGGAACAGCTGGCTCATACCCCGACAGCCTTCAAACGAGACCCAGAAGATCCTTCT  1740
     GAAAGCCTTGTCGACCGAGTATGGGGCTGTCGGAAGTTTGCTCTGGGTCTTCTAGGAAGA
     L  S  E  Q  L  A  H  T  P  T  A  F  K  R  D  P  E  D  P  S   539
          .         .         .         .         .         .
1741 GCAGTTGCTCTCAAAGAGCCCTGGCAGGAGAAAGTACGGCGGATCAGAGAGGGCTCCCCC  1800
     CGTCAACGAGAGTTTCTCGGGACCGTCCTCTTTCATGCCGCCTAGTCTCTCCCGAGGGGG
     A  V  A  L  K  E  P  W  Q  E  K  V  R  R  I  R  E  G  S  P   559
          .         .         .         .         .         .
1801 TACGGCCATCTCCCCAATTGGCGGCTCCTGTCAGTCATTGTCAAGTGTGGGGATGACCTT  1860
     ATGCCGGTAGAGGGGTTAACCGCCGAGGACAGTCAGTAACAGTTCACACCCCTACTGGAA
     Y  G  H  L  P  N  W  R  L  L  S  V  I  V  K  C  G  D  D  L   579
          .         .         .         .         .         .
1861 CGGCAAGAGCTTCTGGCCTTTCAGGTGTTGAAGCAACTGCAGTCCATTTGGGAACAGGAG  1920
     GCCGTTCTCGAAGACCGGAAAGTCCACAACTTCGTTGACGTCAGGTAAACCCTTGTCCTC
     R  Q  E  L  L  A  F  Q  V  L  K  Q  L  Q  S  I  W  E  Q  E   599
          .         .         .         .         .         .
1921 CGAGTGCCCCTTTGGATCAAGCCATACAAGATTCTTGTGATTTCGGCTGATAGTGGCATG  1980
     GCTCACGGGGAAACCTAGTTCGGTATGTTCTAAGAACACTAAAGCCGACTATCACCGTAC
     R  V  P  L  W  I  K  P  Y  K  I  L  V  I  S  A  D  S  G  M   619
          .         .         .         .         .         .
1981 ATTGAACCAGTGGTCAATGCTGTGTCCATCCATCAGGTGAAGAAACAGTCACAGCTCTCC  2040
     TAACTTGGTCACCAGTTACGACACAGGTAGGTAGTCCACTTCTTTGTCAGTGTCGAGAGG
     I  E  P  V  V  N  A  V  S  I  H  Q  V  K  K  Q  S  Q  L  S   639
          .         .         .         .         .         .
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     L  L  D  Y  F  L  Q  E  H  G  S  Y  T  T  E  A  F  L  S  A   659
          .         .         .         .         .         .
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     GTCGCGTTAAAACACGTTTCAACACGACCCATGACGAACCAGACGATGGACGACGTTCAG
     Q  R  N  F  V  Q  S  C  A  G  Y  C  L  V  C  Y  L  L  Q  V   679
          .         .         .         .         .         .
2161 AAGGACAGACACAATGGGAATATCCTTTTGGACGCAGAAGGCCACATCATCCACATCGAC  2220
     TTCCTGTCTGTGTTACCCTTATAGGAAAACCTGCGTCTTCCGGTGTAGTAGGTGTAGCTG
     K  D  R  H  N  G  N  I  L  L  D  A  E  G  H  I  I  H  I  D   699
          .         .         .         .         .         .
2221 TTTGGCTTCATCCTCTCCAGCTCACCCCGAAATCTGGGCTTTGAGACGTCAGCCTTTAAG  2280
     AAACCGAAGTAGGAGAGGTCGAGTGGGGCTTTAGACCCGAAACTCTGCAGTCGGAAATTC
     F  G  F  I  L  S  S  S  P  R  N  L  G  F  E  T  S  A  F  K   719
          .         .         .         .         .         .
2281 CTGACCACAGAGTTTGTGGATGTGATGGGCGGCCTGGATGGCGACATGTTCAACTACTAT  2340
     GACTGGTGTCTCAAACACCTACACTACCCGCCGGACCTACCGCTGTACAAGTTGATGATA
     L  T  T  E  F  V  D  V  M  G  G  L  D  G  D  M  F  N  Y  Y   739
          .         .         .         .         .         .
2341 AAGATGCTGATGCTGCAAGGGCTGATTGCGCTCGGAAACCTCAAAGAGAGGTTCCACATG  2400
     TTCTACGACTACGACGTTCCCGACTAACGCGAGCCTTTGGAGTTTCTCTCCAAGGTGTAC
     K  M  L  M  L  Q  G  L  I  A  L  G  N  L  K  E  R  F  H  M   759
          .         .         .         .         .         .
2401 AGCATGACTGAGGAGCAGCTGCAGCTGCTGGTGGAGCAGATGGTGGATGGCAGTATGCGG  2460
     TCGTACTGACTCCTCGTCGACGTCGACGACCACCTCGTCTACCACCTACCGTCATACGCC
     S  M  T  E  E  Q  L  Q  L  L  V  E  Q  M  V  D  G  S  M  R   779
          .         .         .         .         .         .
2461 TCTATCACCACCAAACTCTATGACGGCTTCCAGTACCTCACCAACGGCATCATGTGACAC  2520
     AGATAGTGGTGGTTTGAGATACTGCCGAAGGTCATGGAGTGGTTGCCGTAGTACACTGTG
     S  I  T  T  K  L  Y  D  G  F  Q  Y  L  T  N  G  I  M  *
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                      .         .         .         .         .         .
2521  GCTCCTCAGCCCAGGAGTGGTGGGGGGTCCAGGGCACCCTCCCTAGAGGGCCCTTGTCTG  2580
      CGAGGAGTCGGGTCCTCACCACCCCCCAGGTCCCGTGGGAGGGATCTCCCGGGAACAGAC
                      .         .         .         .         .         .
2581  AGAAACCCCAAACCAGGAAACCCGACCTACCCAACCATCCACCCAAGGAAATGGAAGGC  2640
      TCTTTGGGGTTTGGTCCTTTGGGCTGGATGGGTTGGTAGGTGGGTTCCCTTTACCTTCCG
                      .         .         .         .         .         .  2641
      AAGAAACACGAAGGATCATGTGGTAACTGCGAGAGCTTGCTGAGGGGTGGAGAGCCAGC  2700
      TTCTTTGTGCTTCCTAGTACACCATTGACGCTCTCGAACGACTCCCCACCCTCTCGGTCG
                      .         .         .         .         .         .
2701  TGTGGGGTCCAGACTTGTTGGGGCTTCCCTGCCCCTCCTGGTCTGTGTCAGTATTACCAC  2760
      ACACCCCAGGTCTGAACAACCCCGAAGGGACGGGGAGGACCAGACACAGTCATAATGGTG
                      .         .         .         .         .         .
2761  CAGACTGACTCCAGGACTCACTGCCCTCCAGAAAACAGAGGTGACAAATGTGAGGGACAC  2820
      GTCTGACTGAGGTCCTGAGTGACGGGAGGTCTTTTGTCTCCACTGTTTACACTCCCTGTG
                      .         .         .         .         .         .
2821  TGGGGCCTTTCTTCTCCTTGTAGGGGTCTCTCAGAGGTTCTTTCCACAGGCCATCCTCTT  2880
      ACCCCGGAAAGAAGAGGAACATCCCCAGAGAGTCTCCAAGAAAGGTGTCCGGTAGGAGAA
                      .         .         .         .         .         .
2881  ATTCCGTTCTGGGGCCCAGGAAGTGGGGAAGAGTAGGTTCTCGGTAATTAGGAACTGATC  2940
      TAAGGCAAGACCCCGGGTCCTTCACCCCTTCTCATCCAAGAGCCATTAATCCTTGACTAG
                      .         .         .         .         .         .
2941  CTGTGGTTGGCCAATGGCCATGGTGTGGCCCAGTTATACCCCTCCCAGGGACCTACCCTT  3000
      GACACCAACCGGTTACCGGTACCACACCGGGTCAATATGGGGAGGGTCCCTGGATGGGAA
                      .         .         .         .         .         .
3001  CCCAGGGACGACCCTGGCCCAAGCTCCCCTTGTTGGCGGGCGCTGCGTGGGCCCTGCACT  3060
      GGGTCCCTGCTGGGACCGGGTTCGAGGGGAACAACCGCCCGCGACGCACCCGGGACGTGA
                      .         .         .         .         .         .
3061  TGCTGAGGTTCCCCATCATGGGCAAGGAAGGGAATTCCCACAGCCCTCCAGTGTACTGAG  3120
      ACGACTCCAAGGGGTAGTACCCGTTCCTTCCCTTAAGGGTGTCGGGAGGTCACATGACTC
                      .         .         .         .         .         .
3121  GGTACTGGCCTAGCCATGTGGAATTCCCTACCCTGACTCCTTCCCCAAACCCAGGGAAAA  3180
      CCATGACCGGATCGGTACACCTTAAGGGATGGGACTGAGGAAGGGGTTTGGGTCCCTTTT
                      .         .         .         .         .         .
3181  GAGCTCTCAATTTTTTATTTTTAATTTTTGTTTGAAATAAAGTCCTTAGTTAGCCAAAAA  3240
      CTCGAGAGTTAAAAAATAAAAATTAAAAACAAACTTTATTTCAGGAATCAATCGGTTTTT
                      .         .
3241  AAAAAAAAAAAAAAAAAAAAAAAAAA  3266
      TTTTTTTTTTTTTTTTTTTTTTTTTT
```
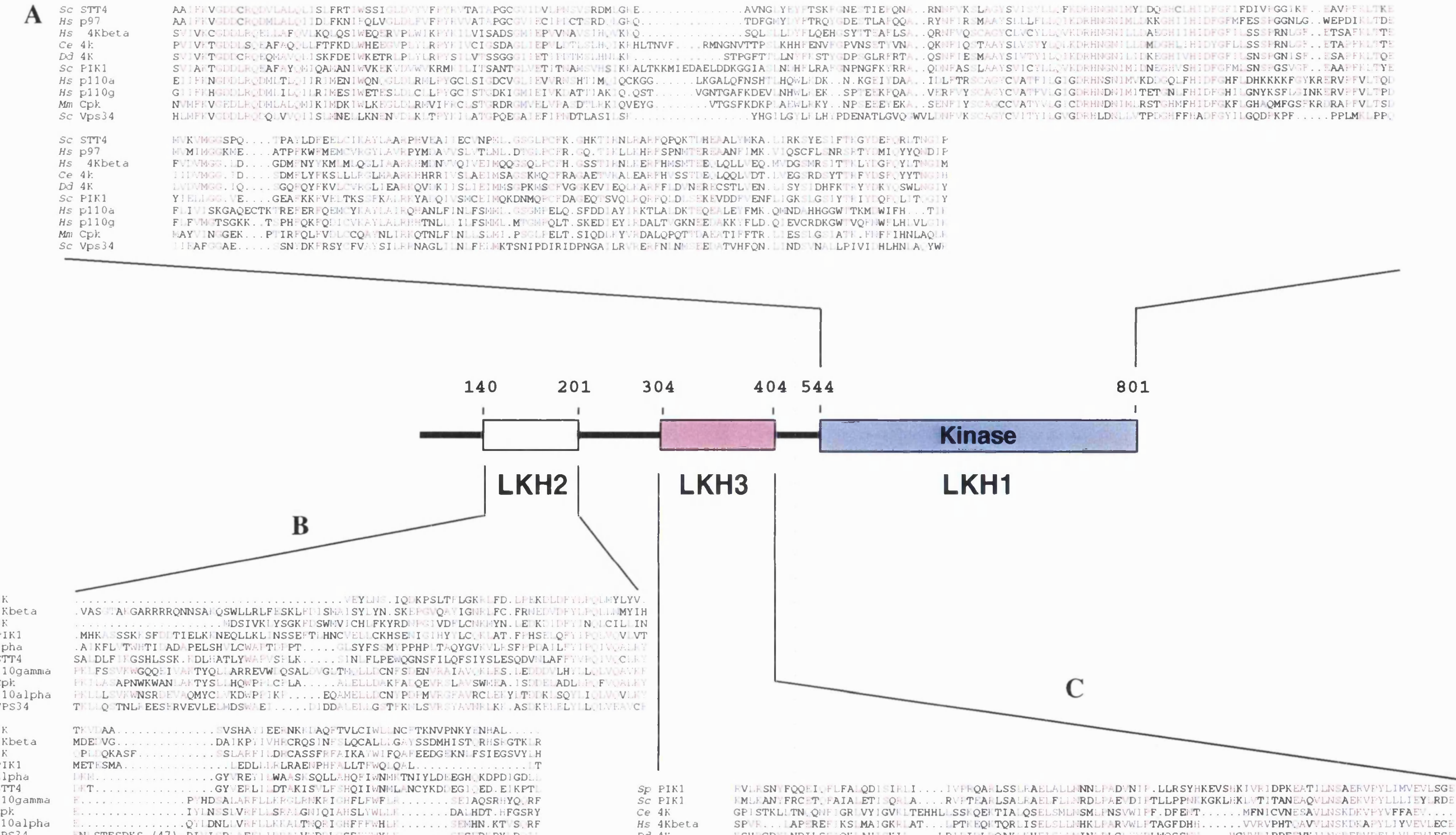

Figure 6.2 Nucleotide sequence of PtdIns 4-kinase β

The full nucleotide sequence and predicted amino acid sequence is shown in single letter code. >>> indicates the initiation codon and * indicates a stop codon. The sequence of the PCR-generated probe is underlined and the region predicted to contain a casein kinase II site is shown in red.

Figure 6.3 The domain structure of PtdIns 4Kβ

Sequence analysis of PtdIns 4Kβ indicated the presence of three distinct regions of homology with known and putative PI kinases. LKH1 (A) represents the highly conserved catalytic core of the enzyme. LKH2 (B) is found in all known PI 3-kinases whereas LKH3 (C) is found only within the Pik1p-related PtdIns 4Ks (Section 1.5.3.b). Alignments were generated using the PILEUP program (GCG, Madison, WI). Identity is shown in red and conservation in blue. Gaps have been introduced to optimise alignments (dots). Definitions and references relating to PtdIns 4Ks are given in Figure 1.4, those relating to PI 3-kinases are given in Figure 1.8



A
B
C
140
201
304
404
544
801
Kinase
LKH2
LKH3
LKH1

6.2.2 Northern analysis

The distribution of PtdIns 4Kβ in human tissues was determined by high stringency northern hybridisation to an 800 bp probe representing nucleotides 150-950. A single 4.1 kb, medium to low abundance transcript was detected in all tissues examined and was most abundant in skeletal muscle, heart, brain and pancreas (Figure 6.4). The discrepancy between the size of the clones isolated and the 4.1 kb transcript suggests that approximately 900 nucleotides of extra non-coding sequence are present in the mature mRNA.

6.2.3 Expression of recombinant PtdIns 4Kβ

The PtdIns 4Kβ cDNA was cloned into the *NcoI* and *XbaI* sites of pEFBOS (Mizushima and Nagata, 1990) and expressed under the control of the human EF-1α promoter with an N-terminal myc epitope tag following lipid-mediated transfection of COS M1 cells. Western blotting with the 9E10 mAb detected a band of approximately 106 kDa in total cell lysates from transfected, but not mock transfected cells (Figure 6.5).

6.2.4 Subcellular localisation of PtdIns 4Kβ isoforms

The majority of the total cellular PtdIns 4K activity is tightly membrane bound. However, hydropathy analysis of the predicted amino acid sequence of PtdIns 4Kβ using PEPPLOT (GCG) predicted no hydrophobic segments which might indicate a transmembrane domain. Subcellular fractionation and western blotting of transfected COS cells showed that PtdIns 4Kβ was indeed a soluble enzyme although a fraction remained in high speed pellets which could be extracted with 1M NaCl (Figure 6.5).

Immunofluorescence of MDCK and A431 cells expressing epitope-tagged PtdIns 4Kβ and PtdIns 4Kβ I indicated a predominantly cytosolic localisation (Figure 6.6).

6.2.5 Activity

PtdIns 4Kβ was also cloned and expressed in *E. coli* as a $(H)_6$-tagged fusion protein. After purification by Ni^{2+}-affinity chromatography, PtdIns 4Kβ was found to be active towards micellar PtdIns and the product of this reaction was confirmed as PtdIns(4)*P* using TLC in the presence of borate ions (Figure 6.7).

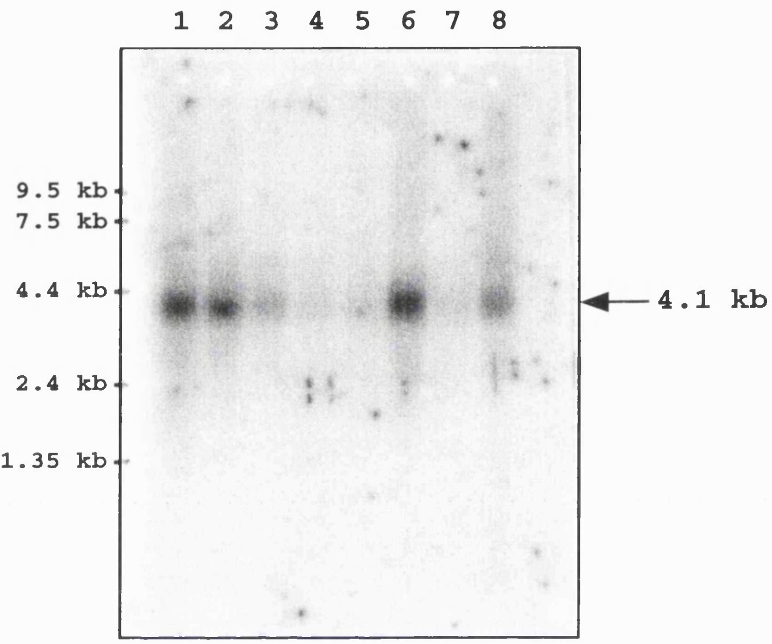


Figure 6.4 Expression of PtdIns 4-kinase β mRNA in human tissues

A human multiple tissue northern blot (Clontech) containing 2 μg of poly(A)+ mRNA per lane, was probed at high stringency with a [^{32}P]-labelled, PCR generated probe spanning nucleotides 150-950. Lanes: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas. The positions of size markers are indicated.

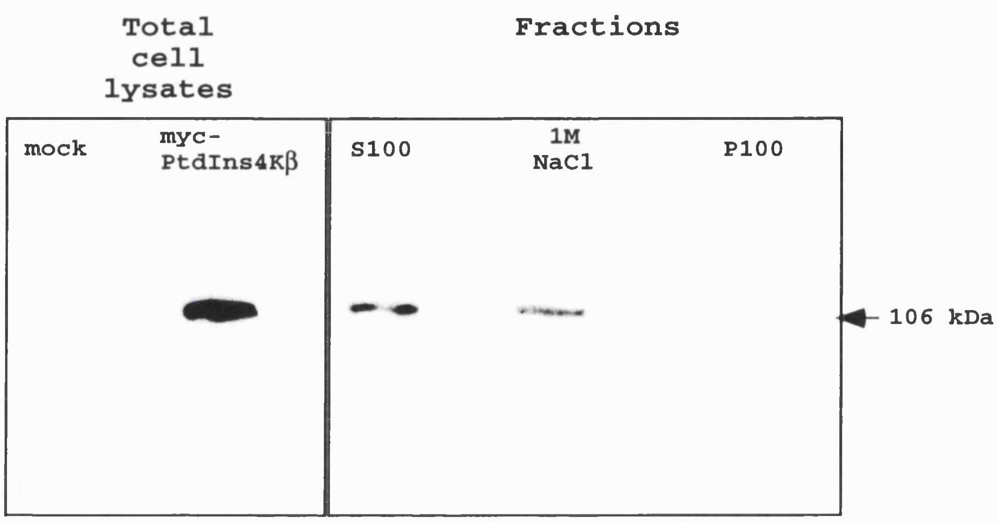


Figure 6.5 Transient expression of myc-PtdIns 4Kβ in COS cells and subcellular fractionation

Approximately 2 x 10^7 cells in mid-log phase were electroporated with 20 μg of plasmid DNA, plated on 150 mm dishes and grown for 48 h. Monolayers of transfected or mock-transfected cells were washed twice in cold PBS and then either lysed in SDS-PAGE sample buffer (total lysate) or scraped into homogenisation buffer containing 100 mM Tris.HCl, pH 8.0, 100 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.5 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin A. This was then homogenised and centrifuged at 100,000 xg for 1 h after which the soluble fraction (S100) was removed and the insoluble pellet carefully washed with homogenisation buffer. The pellet was then resuspended in homogenisation buffer containing 1M NaCl and stirred on ice for 20 minutes before centrifugation at 100,000 *xg* for a further 1 h. This supernatant (1 M NaCl) and the pellet (P100) was collected. These fractions were analysed by western blotting with mAb 9E10 followed by ECL detection.

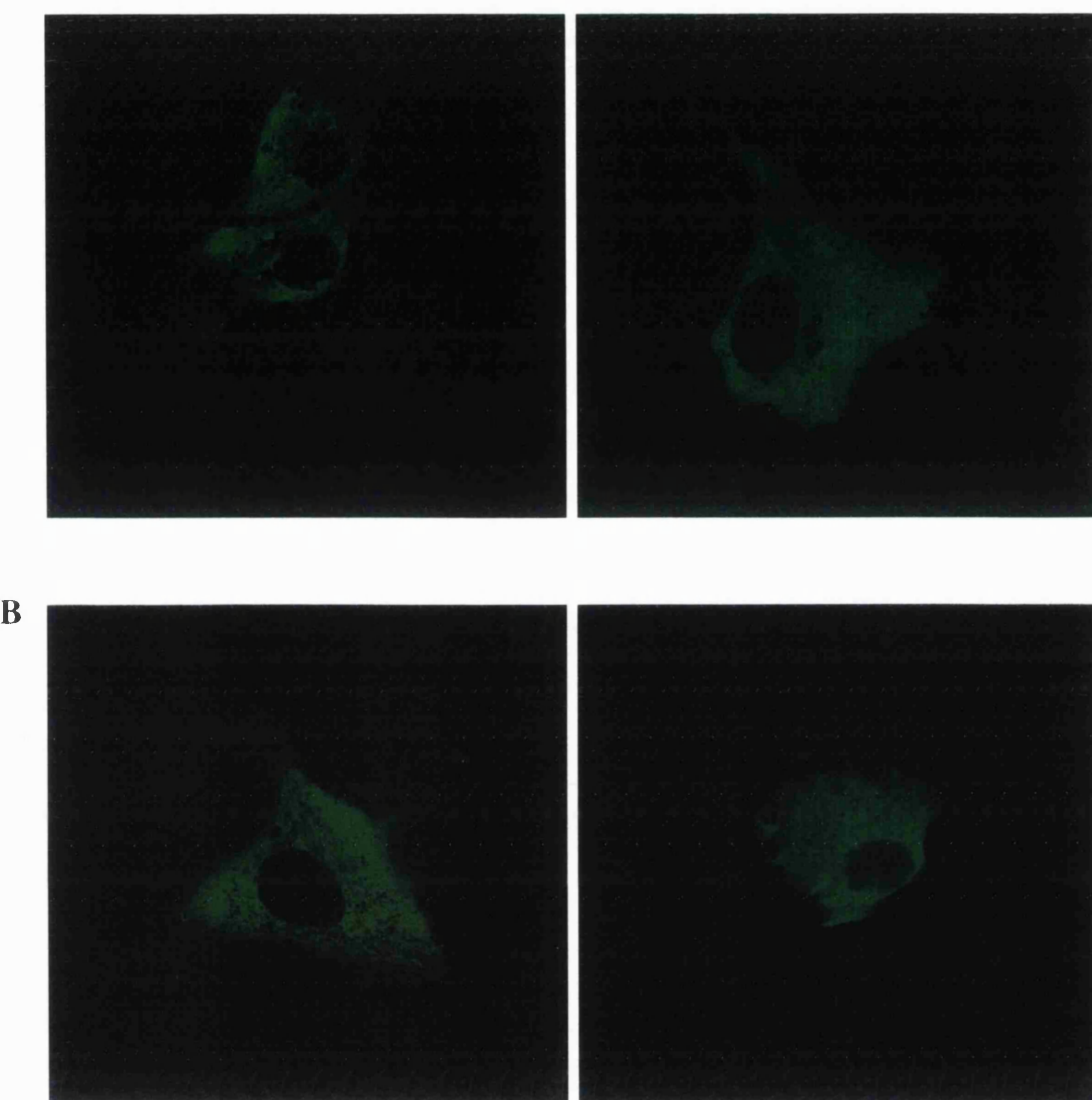


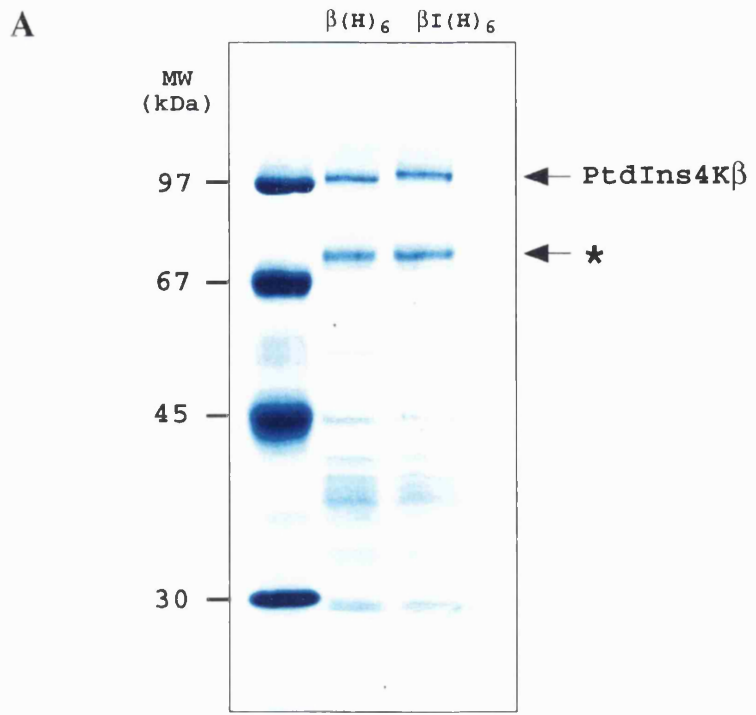
Figure 6.6 Subcellular localisation of PtdIns 4Kβ and PtdIns 4KβI

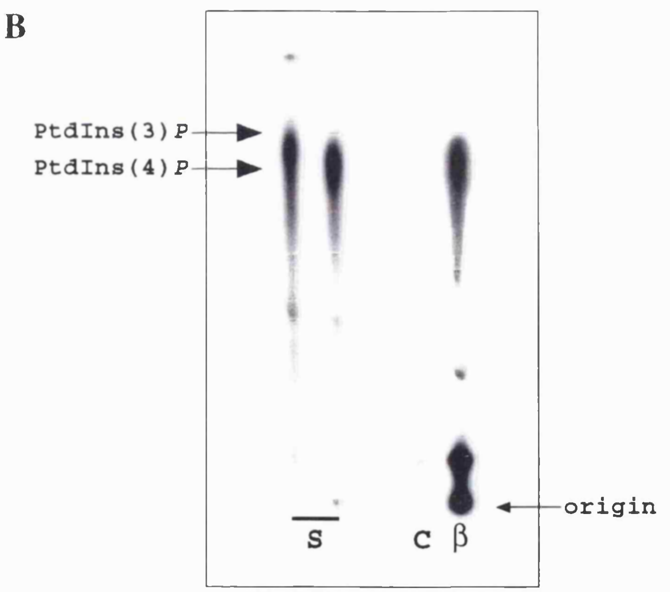
A431 cells were grown on coverslips and transfected with pEF-BOS containing myc epitope-tagged PtdIns 4Kβ (A) and PtdIns 4KβI (B) with Lipofectin as described in Chapter 2.0. After growth for 48 h cells were fixed and stained for immunofluorescence as described in Section 2.8.3 and visualised by confocal microscopy.

Figure 6.7 Expression and activity

PtdIns 4Kβ and PtdIns4KβI cDNAs were cloned into the bacterial expression vector pET 21d (Invitrogen) for expression as C-teminal $(H)_6$-fusions. Lysates were prepared from *E. coli* BL21 carrying PtdIns 4Kβ$(H)_6$ or PtdIns 4KβI$(H)_6$ and purified on Ni-NTA-agarose as detailed in Chapter 2.0. Recombinant proteins were analysed by SDS-PAGE (A). The asterix indicates a co-purifying protein which is likely to be the *E. coli DNAK* gene product, a commonly observed 70 kDa contaminant.

PtdIns 4Kβ$(H)_6$ was assayed for PtdIns 4K activity (B) and the PtdIns(4)*P* product confirmed using TLC in borate buffer (Walsh *et al.,* 1991). TLC lanes: S, PtdIns(3)*P* and PtdIns(4)*P* standards generated using recombinant human Vps34p and salt-washed A431 membranes, respectively; C, control assay performed on Ni-NTA-agarose purified lysates from BL21 cells lacking pET 21d-PtdIns 4Kβ$(H)_6$; β, assay performed with purified PtdIns 4Kβ$(H)_6$. Similar results were obtained with PtdIns 4KβI (data not shown).


A
β(H)6 βI(H)6
MW
(kDa)
97
67
45
30
PtdIns4Kβ
*


B
PtdIns(3)P
PtdIns(4)P
origin
s
c β

6.2.6 Inhibition of PtdIns 4-kinase β by wortmannin

An approximately 100 kDa bovine adrenal PtdIns 4K, sensitive to inhibition by wortmannin has recently been partially characterised (Downing *et al.*, 1996; Nakanishi *et al.*, 1995). The observation that this activity is soluble in high salt is consistent with the properties of PtdIns 4Kβ. To determine the IC_{50} for wortmannin, bacterially expressed PtdIns 4Kβ was assayed with increasing concentrations of wortmannin using conditions
described previously (Nakanishi *et al.*, 1995). Both PtdIns4Kβ-$(H)_6$ and PtdIns4KβI-$(H)_6$ were inhibited by wortmannin with an IC_{50} of approximately 100 nM (Figure 6.8).

6.2.7 Identification of a novel conserved domain

In addition to the C-terminal kinase domain, a region of homology was identified in a subset of PtdIns 4Ks and termed LKH3. Extensive database searching indicates that this region, which lies between amino acids 329 and 349 in PtdIns 4Kβ, is probably unique to PtdIns 4Ks and more specifically, to the Pik1-like PtdIns 4Ks (Figure 6.3). The expression of this region as a GST-fusion protein in *E. coli* produced poor quality protein (results not shown) and was not pursued further.

6.2.8 PtdIns 4Kβ is unable to rescue a *Pik1* null mutation

Sequence alignments show that Pik1p and PtdIns 4Kβ are 53 % similar overall and 67 % similar within the kinase core. This suggested that PtdIns 4Kβ could perform similar cellular functions to Pik1p. In order to test this hypothesis, a *S. cerevisiae* strain heterozygous for the *Pik1* allele was transformed with the high copy number yeast expression vector pRS14/Gal10 (Sikorski and Hieter, 1989) containing either PtdIns 4Kβ or PtdIns 4KβI. Expression was confirmed by western blotting of total cell lysates with an anti-PtdIns 4Kβ polyclonal antibody (Meyers and Cantley, 1997) and is shown in Figure 6.9. When *pik1/PIK1* diploids are subjected to sporulation and ascus dissection, spore viability segregates 2:2 in every tetrad (Flanagan *et al.*, 1993). This ratio was not altered in *Pik1* heterozygotes overexpressing PtdIns 4Kβ or PtdIns 4KβI, indicating that neither protein could complement the *Pik1* null allele (data not shown).

6.2.9 PtdIns 4Kβ is a substrate for casein kinase II *in vitro*

Figure 6.10 shows a schematic representation of PtdIns 4Kβ and PtdIns 4KβI, showing how the short insert sequence is positioned directly before the LKH3 region. The acidic, serine-rich nature of this sequence suggested that it could act as a substrate for serine/threonine protein kinases, in particular casein kinase II (CKII) whose preferred substrates all contain a serine or threonine acceptor residue in acidic sequences (Pearson

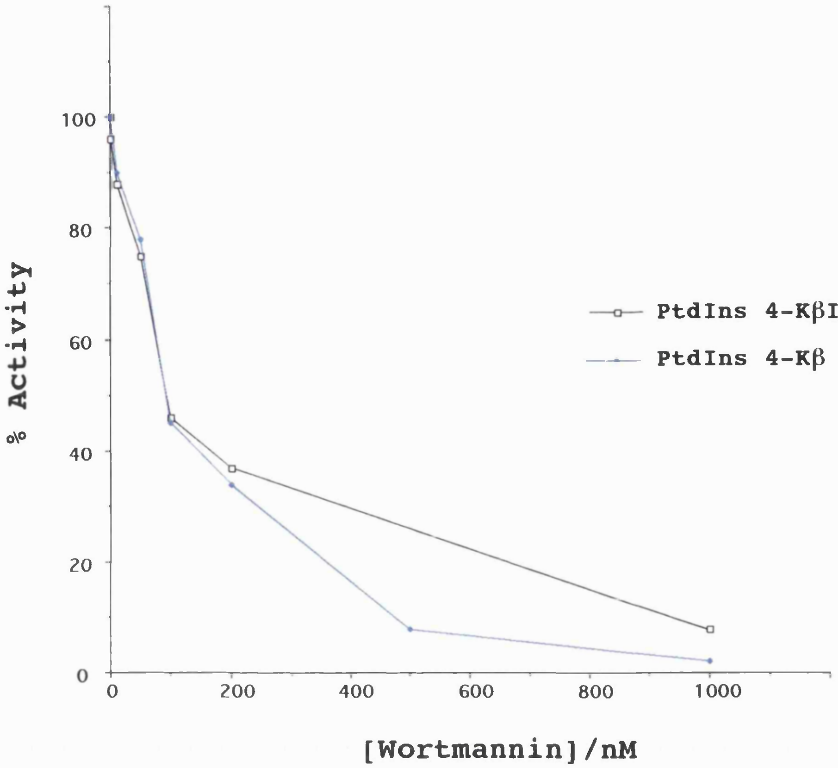


Figure 6.8 Inhibition of PtdIns 4Kβ by wortmannin

Approximately 100 ng of PtdIns 4Kβ$(H)_6$ or PtdIns 4KβI$(H)_6$ proteins were assayed for PtdIns 4K activity using assay conditions described in Section 2.5.2.a except that the reaction mixtures were pre-incubated with increasing concentrations of wortmannin as indicated for 15 min prior to the addition of ATP. The concentration of vehicle (DMSO) was kept constant (1%) and had no effect on activity alone (not shown). Note that this experiment was performed only once, however, the sensitivity of PtdIns 4Kβ to wortmannin has been confirmed independently (see text).

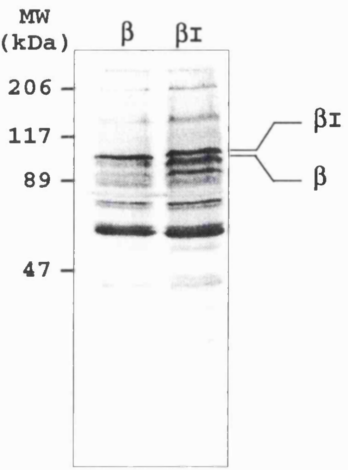


Figure 6.9 Expression of PtdIns 4Kβ and PtdIns 4KβI in *Pik1* homozygotes

A diploid strain (*pik1D1::LEU2/PIK1,* Flanagan *et al.*, 1993) was transformed with the yeast expression vector pRS14/Gal10 (Sikorski and Hieter, 1989) containing the PtdIns 4Kβ or PtdIns 4KβI ORFs as follows: single colonies of freshly grown cells were picked and dispersed in 300 μl of buffer containing 0.1 M lithium acetate, 45% PEG 4000, 10 mM Tris.HCl, pH 8.0, 1 mM EDTA. 3 μg of plasmid DNA was added to the cell suspension and mixed thoroughly. After over-night incubation at 30°C, cells were plated on selective medium. PtdIns 4Kβ and PtdIns 4KβI transformants were grown in 3 ml liqid cultures for 48 hrs at 30°C, induced with 5 % galactose and incubated for a further 3 hours. Cell lysates were prepared vortexing cells with glass beads for 5 min. Expression of proteins was confirmed by western blotting with an anti-PtdIns 4Kβ polyclonal antibody (Meyers and Cantley, 1997). Tetrad analysis of *pik1D1::LEU2/PIK1* yeast transfected with either PtdIns 4Kβ construct showed that PtdIns 4Kβ could not rescue the Pik1 mutant.

and Kemp, 1991). *In vitro* kinase assays showed that while human recombinant CKII could incorporate [^{32}P]-phosphate into both PtdIns 4Kβ and PtdIns 4KβI, PtdIns 4KβI was clearly the preferred substrate (Figure 6.10). Minimal autokinase activity was observed with $(H)_6$-fusion proteins in the absence of CKII (see Figure 6.11). Phosphoamino acid analysis of PtdIns 4KβI indicated that only serine residues were phosphorylated. Mass spectrometric analysis of the trypsin-digested PtdIns 4KβI phosphoprotein confirmed that a single residue was phosphorylated in a tryptic peptide with a mass corresponding to residues 308-331 spanning the insert region (data not shown).

6.2.10 PtdIns 4Kβ is phosphorylated by cell lysates

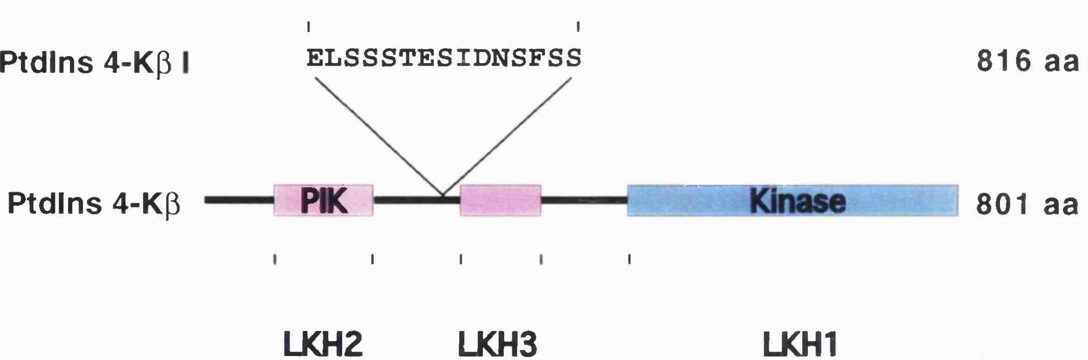
The ability of cellular protein kinases to phosphorylate PtdIns 4Kβ and -βI was examined by incubating $(H)_6$-tagged fusion proteins with Jurkat cell lysates and [γ-^{32}P]-ATP. Surprisingly, it was found that PtdIns 4Kβ-$(H)_6$ was a better substrate for protein kinases in this lysate than PtdIns 4KβI-$(H)_6$ (Figure 6.11). This observation suggested that PtdIns 4Kβ may be a substrate for kinases other than CKII.

Evidence exists for regulation of CKII activity by EGF in A431 epidermoid cells (Ackerman and Osheroff, 1989) and A431 cells are known to contain PtdIns 4Kβ mRNA (Section 6.1, above). To see if EGF-activated CKII could phosphorylate PtdIns 4Kβ isoforms, soluble fractions were prepared from A431 cells treated with 200 nM EGF for 50 min and these were used to phosphorylate $(H)_6$-fusion proteins as before. Also, in order to assess the contribution of other protein kinases, the assays were performed in the presence of excess unlabelled GTP. Since CKII has the unusual property of being able to use GTP as a phosphate donor, it is possible to use unlabelled GTP to selectively eliminate the CKII signal in *in vitro* kinase assays (Ackerman and Osheroff, 1989; Charriaut Marlangue *et al.*, 1991). Figure 6.11 shows that there is a high basal level of kinase activity towards PtdIns 4Kβ and -βI and that there is an increase in PtdIns 4Kβ phosphorylation on EGF treatment. When the same experiment was repeated with 10 mM GTP, only low basal levels of phosphorylation were observed but when stimulated with EGF a large increase in PtdIns 4Kβ phosphorylation was observed suggesting that a distinct, EGF-dependent protein kinase phosphorylates PtdIns 4Kβ.

6.3 Discussion

The identification of a PI kinase homologue in the EST database has led to the molecular characterisation of PtdIns 4Kβ and PtdIns 4KβI, both novel PtdIns 4Ks, whose biochemical properties most closely resemble the soluble wortmannin-sensitive PtdIns 4K described previously (Downing *et al.*, 1996; Nakanishi *et al.*, 1995). Northern analysis indicated that PtdIns 4Kβ is widely expressed at medium to low levels in the 8

A



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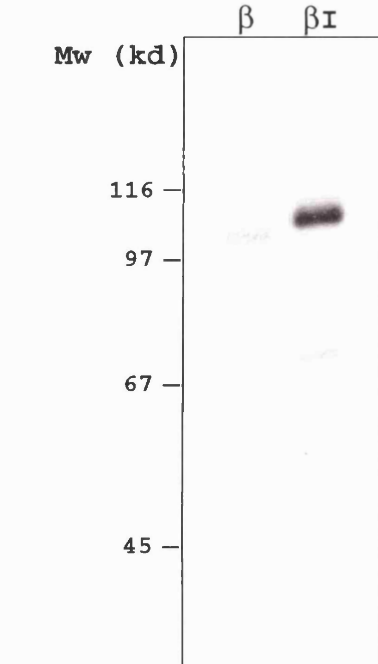


Figure 6.10 Phosphorylation of PtdIns 4Kβ by casein kinase II

A. Schematic representation of PtdIns 4Kβ showing the position of the insert region which contains two putative casein kinase II phosphorylation sites as indicated.

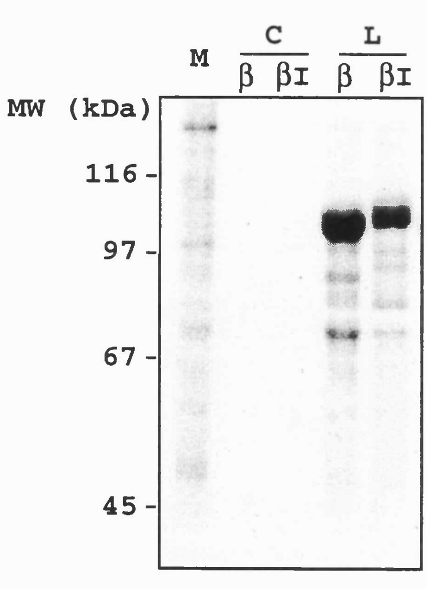
B. Equivalent amounts of bacterially expressed PtdIns 4K β-(H)6-fusion protein were incubated with recombinant human casein kinase II (Calbiochem) in a reaction containing 50 mM Tris.HCl, pH 7.4, 50 mM NaCl, 30 mM imidazole, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and 1 mM $MnCl_2$ and 20 mM (10 μCi) $\gamma[^{32}P]$ATP for 20 min at room temperature, after which the reaction was terminated by the addition of SDS-PAGE sample buffer. After electrophoresis the gel was exposed to X-ray film.

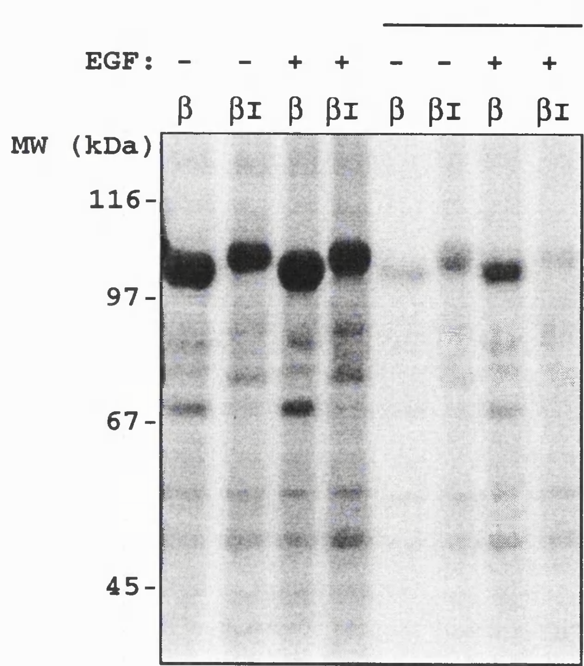
Figure 6.11 Phosphorylation of PtdIns 4Kβ by cell lysates

A. Equal amounts of PtdIns 4Kβ-$(H)_6$ and PtdIns 4KβI-$(H)_6$ protein bound to Talon resin were incubated for 20 min at room temperature with 50 μg of a Triton X-100 Jurkat cell lysate in a buffer containing 50 mM Tris.HCl, pH 7.4, 50 mM NaCl, 50 mM β-glycerophosphate, 25 mM KCl, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 μg/ml okadaic acid and 20 μM (10 μCi) [γ^{32}P]ATP. Lanes: M; an excess of Talon affinity matrix. C; PtdIns 4Kβ-$(H)_6$ and PtdIns 4KβI-$(H)_6$ incubated with γ[^{32}P]ATP in the absence of cell lysate (autokinase controls). L; PtdIns 4Kβ-$(H)_6$ and PtdIns 4KβI-$(H)_6$ incubated with cell lysate.

B. Phosphorylation of PtdIns 4Kβ isoforms by cell lysates from EGF-treated A431 cells. Soluble fractions were prepared from serum starved A431 cells as described in Figure 6.5 above except that monolayers were treated with or without 200 nM EGF for 50 min. Protein kinase assays were performed as in A, above with and without 10 mM GTP.

A





human tissues examined. RT-PCR also confirmed the presence of PtdIns 4Kβ mRNA in human Jurkat T-lymphocyte, human A431 epidermoid carcinoma and human HL60 granulocyte cells (data not shown). Whilst the significance of the observed higher levels of message in skeletal muscle, heart, brain and pancreas is unclear, this wide pattern of expression is consistent with PtdIns 4Kβ having a general cellular role. The relative level of expression of the transcript corresponding to PtdIns 4KβI could not be determined by northern analysis because of the small difference (45 bp) in size of the predicted mRNAs, however, only one of a total of five library clones contained this insert and it is therefore possible that this is a minor splice variant in brain and placenta.

Sequence alignments show that the known PtdIns 4Ks can be subdivided into two groups (Figure 1.4) and the domain structure of PtdIns 4Kβ is most similar to the yeast *Pikl* group. The *S. cerevisiae* STT4 gene product along with the mammalian type III enzymes (including p97 PtdIns 4Kα, and p230) all comprise one group and contain a PH domain. PtdIns 4Kβ and Pik1p appear to define a new group which lacks a PH domain but contains the short region of homology illustrated in Figure 6.3. This motif is unlike any described previously and nothing similar can be found in any proteins other than PtdIns 4Ks. The fact that this is present in even the most rudimentary of eukaryotes suggests that the domain has some functional significance.

Pik1p is essential for yeast viability: gene disruption is lethal and temperature-sensitive mutants undergo a rapid growth arrest on shift to the non-permissive temperature (Flanagan *et al.*, 1993; Garcia-Bustos *et al.*, 1994). Pik1p has been found associated with nuclear fractions (Garcia-Bustos *et al.*, 1994) although in other experiments it has been found only in the cytosolic fraction (Flanagan *et al.*, 1993; Flanagan and Thorner, 1992). More recently it has been found exclusively in the cytosol by direct immunofluorescence (Thorner, 1996). The function of Pik1p remains mysterious but it was initially suggested that it plays a role in pheromone signalling (Flanagan *et al.*, 1993) and in cytokinesis (Garcia-Bustos *et al.*, 1994). The similarity between PtdIns 4Kβ and Pik1p suggested the possibility that these two enzymes are functionally conserved and this hypothesis was tested using *Pikl* null mutants. However, overexpression of PtdIns 4Kβ and PtdIns 4KβI in *S. cerevisiae* strains lacking a functional *Pikl* gene was not sufficient to restore *Pikl* function. The inability of even high levels of PtdIns 4Kβ to complement the *Pikl* phenotype may be due to the inability of yeast regulatory proteins to recognise PtdIns 4Kβ.

The subcellular localisation of PtdIns 4Kα and PtdIns 4Kβ isoforms has recently been studied using immunofluorescence (Nakagawa *et al.*, 1996; Wong *et al.*, 1997). PtdIns 4Kα was found to be membrane bound and associated with the endoplasmic reticulum, whereas PtdIns 4Kβ was found to be mainly cytosolic with a fraction being associated with the Golgi. The association with the endoplasmic reticulum and Golgi apparatus is suggestive of a role in vesicle trafficking, a process in which PtdIns 4Ks have been implicated (Sections 1.5.1.a and 1.11.2). These results are consistent the cell

fractionation data shown in Figure 6.5 using myc-tagged PtdIns 4Kβ expressed in COS cells to study their subcellular localisation. Both isoforms were also expressed in A431 and MDCK cells using plasmid transfection and microinjection (Figure 6.6). The fact that both PtdIns 4Kβ isoforms lack membrane targeting sequences and yet are not exclusively cytosolic, raises the question of the mechanism for membrane localisation. PtdIns 4Kβ and PtdIns 4KβI showed identical immunofluorescence staining, suggesting that the insert region was unlikely to direct subcellular localisation under the conditions used in this study. It is possible that interaction with membranes, whether direct or indirect, is mediated by the LKH3 region or by posttranslational modification, although this remains to be determined. It is also not known whether a fraction is constitutively associated with membranes or if PtdIns 4Kβ membrane-association is regulated. Furthermore, it must be considered that the use of overexpression may cause the accumulation of a delocalised pool of PtdIns 4Kβ in the cytosol, whereas under normal conditions a smaller amount of PtdIns 4Kβ might be exclusively membrane localised.

The finding that PtdIns 4Kβ is sensitive to PI 3-kinase inhibitors is important because it calls the specificity of such reagents into question. Wortmannin has been a valuable tool in dissecting PI 3-kinase function because of the potency of this fungal metabolite and its apparent specificity (Arcaro and Wymann, 1993; Ui *et al.*, 1995). Recently enzymes other that PI 3-kinases, some with important roles in signalling, have been found to be inhibited at concentrations in the nanomolar range, for example the phospholipase PLA_2 (Cross *et al.*, 1995), and protein kinases such as myosin light chain kinase (Nakanishi *et al.*, 1992). The mammalian type III PtdIns 4K has also been found to be sensitive to wortmannin (Balla *et al.*, 1997) and a structural analogue of wortmannin, demethoxyviridin, has been found to inhibit a *S. pombe* membrane PtdIns 4K activity (Woscholski *et al.*, 1994). This is likely to be an STT4-like activity since STT4p is inhibited by wortmannin (Cutler *et al.*, 1997). Given the structural relationship between the catalytic domains of PI 3-kinases and PtdIns 4Ks and the invariance of the wortmannin-binding lysine residue (Wymann *et al.*, 1996) within the catalytic domains of this family, it is hardly surprising that wortmannin should have multiple targets amongst the PI3/4-kinases. Such findings advocate caution when interpreting inhibition data, in particular with whole cell experiments which frequently use elevated inhibitor concentrations in order to overcome the problem of instability. Also, because wortmannin inhibits by irreversible covalent binding to its target, the pre-incubation time used in different experiments is a critical parameter. The existence of multiple wortmannin targets which may lie in the same pathways, and the fact that some PI 3-kinases such as the class II and class III proteins display reduced sensitivity to wortmannin (Domin *et al.*, 1997), may explain the wide range of effects of PI 3-kinase inhibitors on whole cells and complex preparations.

There is mounting evidence for the existence of a wortmannin-sensitive cellular pool of PtdIns(4)*P* and PtdIns(4,5)P_2. However, it was initially reported that wortmannin

inhibited angiotensin II-stimulated inositol phosphate and calcium signalling in adrenal glomerulosa cells (Nakanishi *et al.*, 1995). It was subsequently shown that incubation of cultured adrenal glomerulosa cells or human Jurkat cells with wortmannin lead to a decrease in total PtdIns(4)P and PtdIns(4,5)P_2 levels in unstimulated cells. Furthermore, upon stimulation with angiotensin II, PDGF, or the TCR agonist OKT-3, the initial increase in PtdIns(4,5)P_2, InsP_3 and calcium mobilisation were not substantially affected. However, production of these key messengers during the sustained phase from 2-10 min post stimulation was significantly impaired (Nakanishi *et al.*, 1995). In these studies wortmannin was used at a concentration of 10 μM, a concentration known to completely inhibit PtdIns 4Kβ *in vitro*, however this concentration of wortmannin would also inhibit the type III enzyme whose yeast homologue may lie upstream of PKC (Yoshida *et al.*, 1994). Alternatively, wortmannin sensitivity may arise if a PtdIns 4K activity is regulated by the product of a PI 3-kinase as proposed for PLCγ regulation (Bae *et al.*, 1998; Gratacap *et al.*, 1998; Rameh *et al.*, 1998). It is interesting to note that a previous study of PI turnover in T-cells postulated that the sustained phase of PtdIns(4,5)P_2 synthesis may involve the activation of a distinct PtdIns(4)P-generating pathway (Inokuchi and Imboden, 1990); this pathway may correspond to the wortmannin-sensitive PI pool. It will be crucial to test whether overexpression of, or reconstitution with exogenous PtdIns 4Kβ can overcome inhibition of PtdIns(4,5)P_2 signalling by PI kinase inhibitors.

During the cloning of PtdIns 4Kβ a splice variant was isolated which contained a short sequence with a large proportion of acidic and serine residues. Analysis of the sequence suggested that it contained a casein kinase II phosphorylation site. Also, because of its close juxtaposition to LKH3, a novel PtdIns 4K-specific sequence of unknown function, it was hypothesised that CKII phosphorylation may have role in regulating the function of LKH3. *In vitro* kinase assays with recombinant human CKII showed that PtdIns 4Kβ could act as a substrate for CKII and that PtdIns 4KβI was phosphorylated to a much greater degree. Using mass spectrometry the phosphorylation site was mapped to the insert region of PtdIns 4KβI indicating that alternative splicing generates a protein with potential for regulation by CKII. The effect of CKII phosphorylation on PtdIns 4K activity was measured and it was found that CKII phosphorylation of PtdIns 4Kβ and -βI led to only modest increases in activity (25 ±5% and 46 ±11%, respectively, data not shown). Estimates of the stoichiometry of CKII phosphorylation, assuming a single phosphorylation site, were in the order of 1.5×10^{-3} mol phosphate/mol for PtdIns 4KβI and two orders of magnitude lower for PtdIns 4Kβ. Consequently, the figures for activation could be underestimates. It is also possible that other factors are required to activate CKII towards PtdIns 4KβI *in vivo*.

I next looked at the ability of cell lysates to phosphorylate PtdIns 4Kβ isoforms with the aim of characterising cellular protein kinases. Incubation of both PtdIns 4K isoforms with Triton X-100 extracts from Jurkat cells led to a massive phosphorylation of PtdIns

4Kβ and PtdIns 4KβI suggesting that more than one protein kinase was involved. Further experiments were designed in order to assess the contribution by CKII in the cell lysates. CKII is known to be regulated by extracellular signals and stimulation with EGF has been shown to activate CKII towards physiological substrates in EGF-treated A431 cells (Ackerman and Osheroff, 1989). Also, because CKII can use GTP as a phosphate donor (a property unique amongst the protein kinases), an excess of unlabelled GTP was used to quench the CKII signal. The results were surprising: PtdIns 4Kβ and PtdIns 4KβI were basally phosphorylated by soluble protein preparations of serum-starved A431 cells (β > βI). Upon EGF stimulation an increase in PtdIns 4Kβ and PtdIns 4KβI phosphorylation is observed (increase in β >> increase in βI). Quenching of the CKII signal with GTP revealed that most of the phosphorylation of PtdIns 4Kβ and PtdIns 4KβI was due to CKII and that PtdIns 4Kβ was also phosphorylated by an unknown kinase in an EGF-dependent manner. These preliminary results suggest that PtdIns 4Kβ and PtdIns 4KβI are differentially phosphorylated in response to growth factors. As with phosphorylation by purified CKII *in vitro*, no significant activation of PtdIns 4K activity was observed upon EGF-stimulation (results not shown). The GTP used to quench the CKII signal also has the potential to activate G-protein pathways with unknown effects on PtdIns 4Kβ phosphorylation; specific CKII inhibitors should be employed in future studies and it may be necessary to identifiy the phosphorylation sites. The ability of cell lysates to phosphorylate recombinant PtdIns 4Kβ and the fact that PtdIns 4Kβ and PtdIns 4KβI both bound large amounts of kinase activities from cell lysates (data not shown) suggests that it may be possible to affinity purify the cognate protein kinases.

The cloning of the human PtdIns 4Kβ defines a novel group of PtdIns 4Ks with substantial homology to the yeast *Pikl* gene. Database searches have confirmed the existence of other metazoan Pik1p homologues in the genomes of *C. elegans* and *D. discoideum*. A particularly striking feature of this subbranch of the PtdIns 4Ks is a conserved domain organisation which distinguishes the Pik1- from STT4-related PtdIns 4Ks, specifically by the presence LKH3 region positioned between the LKH2 and the kinase domain, LKH1. Preliminary experiments suggest that PtdIns 4Kβ may be phosphorylated by CKII and other protein kinases. Further experiments will be nesessary to establish the regulatory importance *in vivo*.

7.0 Discussion

7.1 Synopsis

This thesis has presented the cloning of two novel enzymes, PtdIns*P* K IIα and PtdIns 4Kβ. PtdIns*P*K IIα belongs to a subgroup of the PtdIns*P*K family whose members may generate PtdIns(4,5)P_2 *in vivo* by phosphorylation of PtdIns(5)*P* (Itoh *et al.*, 1998; Rameh *et al.*, 1997). Like PtdIns*P*K IIα, PtdIns 4Kβ phosphorylates the D-4 position of the inositol headgroup but the preferred substrate is PtdIns. A combination of affinity labelling and site-directed mutatgenesis was used to gain insight into the structure and function of the PtdIns*P*K IIα catalytic core, and the data presented in Chapter 4 concluded that the PtdIns*P*K family is structurally related to both protein and PI kinases. PtdIns*P*K IIα was expressed in insect cells and used to generate monoclonal antibodies; one of these, 7H8, was characterised and found to be effective in immunoprecipitation, ELISA, immunofluorescence and western blotting. The availability of a specific immunological probe such as this should be useful in contributing to our future understanding of the cellular function of the type II PtdIns*P*Ks and the significance of the putative PtdIns(4,5)P_2 biosynthetic pathway via PtdIns(5)*P*.

PtdIns 4Kβ was cloned with the aim of further defining the mammalian PtdIns 4Ks which catalyse an important first step in the synthesis of phosphoinositides. PtdIns 4Kβ was found to have several features in common with the yeast PtdIns 4K Pik1p but failed to rescue the essential function of Pik1p when overexpressed in *pik1* mutants. A putative casein kinase II site was identified in a splice variant of PtdIns 4Kβ (named PtdIns 4KβI) and recombinant casein kinase II was found to preferntially phosphorylate PtdIns 4KβI *in vitro*. *In vitro* kinase assays using lysates from EGF-treated A431 cells indicate that PtdIns 4Kβ and -βI proteins are differentially phosphorylated by more than one protein kinase. These phosphorylation events caused only small increases in activity and their significance is unclear.

7.2 The Function of PtdIns 4-kinases and PtdIns*P* kinases

At the time of writing some 7 PI 3-kinase, 3 PtdIns 4K, and 6 PtdIns*P*K isoforms are known in mammals (Chapter 1). The rapid expansion of cDNA databases and the use of homology-based PCR strategies allowed the majority of these to be identified on the basis of similarity with known proteins. Consequently, it has proven far easier to clone homologues than to assign physiological function.

The study of PI-kinase function is hampered by several basic problems: firstly, the large number of PI 3-K, PtdIns 4K, and PtdIns*P*K isoforms makes placing specific gene products in linear pathways extremely difficult. This is largely a consequence of the combinatorial complexity of the problem, but difficulties also arise from the absence of a comprehensive panel of isoform-specific reagents at present. It is also extremely difficult

to infer biological function from *in vitro* experiments involving substrates presented in artificial form, for example using micelles instead of membranes. Investigators have sought ways to overcome this fundamental problem by using whole cells assays with a "biological readout". For example, constitutively targetting PI 3-kinase to the plasma membrane has been used extensively to study PI 3-kinase signalling because this leads to the activation of several downstream signalling pathways (Section 1.5.4). However, no biological readout has yet been described for a cloned PtdIns 4K or a type II PtdIns*P*K. It is likely that the function of the type I PtdIns*P*Ks will be elucidated first since these are known to cause changes in the actin cytoskeleton when overexpressed (Ishihara *et al.*, 1998; Shibasaki *et al.*, 1997) and are effective in reconstituting secretion from PC12 cells (Hay *et al.*, 1995).

In Chapter 3 I described the cloning of the type IIα PtdIns*P*K. This isoform was chosen not only because it was available in a highly purified form but because, unlike the type I isoforms, it was reported to be inactive towards membrane substrate (Bazenet *et al.*, 1990). The latter property could be explained by the hypothesis that the type II enzyme was subject to stringent regulation *in vivo* and thus was considered a candidate for the receptor-associated and agonist-regulated PtdIns*P*K activity (Cochet *et al.*, 1991). A number of approaches were used to investigate the cellular function of PtdIns*P*K IIα. Overexpression in mammalian cells did not lead to any apparent morphological changes or increases in phospholipids or inositol phosphates, nor was PtdIns*P*K IIα found to associate with activated EGF receptors (Section 3.2.9). The kinase-defficient mutant proteins described in Chapter 4 may be useful in elucidating the function of PtdIns*P*K IIα since these have the potential to inhibit PI signalling by acting as dominant negatives. Immunofluorescent staining of ectopically expressed protein showed PtdIns*P*K IIα to be cytosolic in epithelial cells (Section 3.2.7), however similar experiments using Swiss 3T3 fibroblasts and monoclonal antibody 7H8 suggested that PtdIns*P*K IIα was concentrated at focal contacts and stress fibres (Figure 5.4) and a similar result was obtained when human fibroblasts were stained with a comercially available monoclonal antibody (Figure 5.5). This observation is suggestive of an involvement in cytoskeletal organisation for which some evidence exists (Section 1.11). It is anticipated that the use of the monoclonal antibody 7H8, whose generation and characterisation is described in Chapter 5, will be an effective tool in determining the significance of this observation.

PtdIns 4Kβ cDNA was isolated on the basis of homology with yeast, *D. discoideum*, and mammalian PtdIns 4Ks as a candidate for the agonist-regulated type II PtdIns 4K (Section 1.5.1.a). However, biochemical characterisation of recombinant PtdIns 4Kβ revealed it to be a wortmannin-sensitive activity with properties inconsistent with the type II PtdIns 4K (Section 6.2.6). The primary structure of PtdIns 4Kβ revealed a close similarity to the yeast PtdIns 4K Pik1p and a topological similarity to several eukaryotic PtdIns 4K homologues, which define a subbranch of the PtdIns 4Ks characterised by the

presence of LKH3, a region of unknown function (Section 1.5.1). PtdIns 4Kβ is a candidate for the wortmannin-sensitive PtdIns 4K activity which regulates a latent, hormone responsive pool of phosphoinositides (Downing *et al.*, 1996; Nakanishi *et al.*, 1995). However, the recent finding that other PtdIns 4Ks are wortmannin-sensitive means that this activity will have to be more extensively characterised before the isoform responsible can be identified.

Genetic studies have the potential to contribute greatly to our understanding of PI kinase function and the identification of PI-kinase homologues in model metazoan systems such as *C. elegans*, *D. melanogaster*, mouse, and rat opens the possibility that the question of PI-kinase function can be addressed by using gene knockouts. Such approaches have had mixed successes: for example, the finding that *D. discoideum* PLC null mutants are viable and that embryonic fibroblasts from PLCγ1 knockout mice respond normally to RTK activation (Drayer *et al.*, 1994; Ji *et al.*, 1998) suggests that at least in some cell types, PLCs may lie in redundant pathways. However, if more mutants in parallel and downstream pathways can be generated, then we may be able to study how different gene products interact and perhaps overcome the problem of compensation by alternative pathways or enhanced downstream signalling. A paradigm among these approaches is the genetic studies of *D. melanogaster* phototransduction which have been use to describe a PLCβ-linked pathway in extraordinary detail (Section 1.7.2). Rat and mouse gene knockouts are the preferred model systems for investigating the role of PI signalling in complex mammalian CNS functions. PKCγ, PtdInsTPα, PLCβ isoforms, and InsP_3 receptor mutants all have neurological phenotypes (Abeliovich *et al.*, 1993a; Abeliovich *et al.*, 1993b; Hamilton *et al.*, 1997; Kim *et al.*, 1997; Matsumoto *et al.*, 1996), suggesting important roles for PI signalling in the brain.

7.3 The substrate specificity of PtdIns*P* kinases: new pathways?

A surprising development in phosphoinositide signalling has been the finding that the type I PtdIns*P*Ks, which were previously thought to phosphorylate only PtdIns(4)*P*, can in fact phosphorylate a range of substrates at different positions on this inositol headgroup. It is now known that the type I PtdIns*P*Ks can phosphorylate at the D-5 position of PtdIns, PtdIns(4)*P*, PtdIns(3)*P*, and PtdIns(3,4)P_2 to generate the corresponding D-5 phosphate (Zhang *et al.*, 1997). Intriguingly, the type I enzyme alone can also catalyse the sequential phosphorylation of PtdIns(3)*P* at the D-4 and D-5 positions to form PtdIns(3,4,5)P_3 (Zhang *et al.*, 1997). Although these reactions have only been demonstrated *in vitro*, they indicate the potential ability of these enzymes to generate a complex array of biologically active species *in vivo*, and suggest that the regulation of these isoforms is likely to be equally complex. The newly recognised substrate specificities of these enzymes also raises the possibility that PtdIns(3,5)P_2,

PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3 are responsible for the effects of the type I enzymes on the actin cytoskeleton (Shibasaki *et al.*, 1997; Ishihara *et al.*, 1998). When the biological significance of the *in vitro* activities is understood, it may be necessary to re-evaluate the roles of type I PtdIns*P*Ks in cytoskeletal reorganisation and exocytosis.

The availablility of synthetic PtdIns(5)*P* substrate and the use of sophisticated chromatographic techniques to analyse reaction products, has led to the finding that the type IIα PtdIns*P*K (and therefore, presumably, the other closely-related type II isoforms) generate PtdIns(4,5)P_2 by phosphorylation of PtdIns(5)*P* at the D-4 position (Rameh *et al.*, 1997). This has raised the possibility of a novel PtdIns(4,5)P_2 biosynthetic pathway, previously unrecognised because of the difficulty of distinguishing PtdIns(4)*P* and PtdIns(5)*P*. Although PtdIns(5)*P* has been detected in resting fibroblasts, there is as yet, no direct evidence that this pathway exists *in vivo*. Nevertheless, the generation of PtdIns(4,5)P_2 by this alternative pathway remains conceptually attractive. Cellular levels of PtdIns(5)*P* are low in resting fibroblasts (approximately 2% of PtdIns(4)*P*) (Rameh *et al.*, 1997). PtdIns(5)*P* synthesis may be a rate-limiting step and therefore a crucial point of regulation of this pathway. This is consistent with the finding in this thesis that overexpression of PtdIns*P*K IIα in various cell types led to no detectable increases in phosphoinositides or inositol phosphates (Chapter 3). Such a pathway may also satisfy the requirements for an agonist-sensitive pool (Section 1.2.1) by metabolically compartmentalising the synthesis of PtdIns(4,5)P_2. The identity of the PtdIns 5-kinase involved in this putative pathway is unknown. At present only the type I PtdIns*P*Ks are known to catalyse the *in vitro* phosphorylation of PtdIns at the D-5 position, but other candidates include the various PI-kinase homologues with no established lipid kinase activity or as yet unidentified enzymes.

An important implication arising from the discovery of a PtdIns(5)*P* —> PtdIns(4,5)P_2 pathway is that for many years PtdIns(5)*P* had been misidentified as PtdIns(4)*P*. Cellular PtdIns monophosphate had always been assumed to be PtdIns(4)*P* (apart from a small fraction of PtdIns(3)*P* which does not change significantly on stimulation). This assumption is fundamental to many studies of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 synthesis in response to agonist where non-equilibrium labelling has been used to determine the order of phosphate transfer to the inositol ring (for example Stephens *et al.*, 1993). Since routine HPLC cannot distinguish PtdIns(4)*P* from PtdIns(5)*P*, it had been assumed that PtdIns(4,5)P_2 could only be generated by the conventional PtdIns(4)*P* —> PtdIns(4,5)P_2 route or by the D-3 dephosphorylation of PtdIns(3,4,5)P_3. Since most of these studies analysed total cellular PIs, the contribution of the PtdIns(5)*P* —> PtdIns(4,5)P_2 pathway has been overlooked.

Alternatively, the type II PtdIns*P*Ks may catalyse the biosynthesis of PtdIns(3,4)P_2 which is an important regulator of Akt (Section 1.5.4.a). Although PtdIns(3,4,5)P_3 is thought to be the primary lipid generated by class I PI 3-kinase activity, PtdIns(3,4)P_2 activates Akt *in vitro* (Franke *et al.*, 1997; Stokoe *et al.*, 1997). Agonist induced

PtdIns(3,4)P_2 may be formed by the action of a 5-phosphatase such as SHIP (Section 1.6.2); there is also evidence for the synthesis of PtdIns(3,4)P_2 by a type II PtdIns 3-kinase and a PtdIns(3)P 4-kinase upon activation of integrin $\alpha_{2b}\beta_3$ in platelets (Banfic *et al.*, 1998). Interestingly, the type II PtdIns$P$K has been found associated with the cytoskeleton of thrombin-stimulated platelets (Hinchliffe *et al.*, 1996), however, immunodepletion of type II PtdIns$P$K activity had no effect on PtdIns(3,4)P_2 synthesis in platelets, suggesting that a novel PtdIns(3)P 4-kinase is responsible (Banfic *et al.*, 1998).

7.4 The structure of PtdIns*P*K II

In Chapter 4 of this thesis, a nucleotide-binding residue of PtdIns$P$K IIα was identified. The position of this residue, K145, was used to optimise alignments of PtdIns$P$Ks with the serine/threonine protein kinase PKA such that it was possible to identify four regions of local homology corresponding to subdomains I, II, VIb and VII of the protein kinases. This study established that although the PtdIns$P$Ks are the most divergent branch of the protein-PI 3/PtdIns 4K superfamily, homologies can be identified which correspond to structures involved in catalysis. Chapter 4 provided evidence for this relationship, and allowed the construction of rationally designed, catalytically inactive mutants for functional studies. However, proof of the relationship could only be provided by the three-dimensional structure of a PtdIns$P$K.

The crystal structure of bacterially expressed recombinant PtdIns$P$K IIβ (an isoform which displays 77 % identity to the kinase domain of PtdIns$P$K IIα) has recently been solved at 3.0 Å resolution (Rao *et al.*, 1998) and is the first lipid kinase structure reported. As predicted in Chapter 4, an invariant lysine residue in PtdIns$P$K IIα (K145) corresponds to the ATP binding lysine residue (K72) of PKA and lies below the predicted PtdIns$P$K P-loop in a central cleft which represents the ATP-binding pocket (Figure 4.8; Rao *et al.,* 1998). Rao *et al.* used similarities between the crystal structures of PtdIns$P$K IIβ and PKA to produce a model of the PtdIns$P$K catalytic core in much the same way as the identification of the nucleotide binding residue of PtsIns$P$K IIα was used to predict the positions of the catalytic and Mg^{2+}-binding loops in Chapter 4. However, contrary to this thesis, Rao *et al.* predicted that the PtdIns$P$K catalytic loop (PKA subdomain VIb) was the invariant sequence MDYSL in the PtdIns$P$Ks (Figure 4.2), and the Mg^{2+}-binding loop (PKA subdomain VII) was IID which is also invariant in the PtdIns$P$Ks. Aligning the primary structure of PtdIns$P$Ks with PKA so that the MDYSL and IID sequences map onto the catalytic and Mg^{2+}-binding loops respectively, made it necessary to introduce three major gaps in the kinase core (Rao *et al.*, 1998), an alignment that would not have been statistically optimal in the absence of structural data. However, both the MDYSL and IID motifs are well conserved and fold into a central cleft which also contains the ATP-binding site (Rao *et al.*, 1998). PtdIns$P$K IIβ was not crystallised in the presence of ATP, divalent ions, PtdIns(5)P substrate or a competitive

inhibitor such as PtdIns(4,5)P_2. Thus it is difficult to determine unequivocally whether the side chains of the critical residues are positioned appropriately for catalysis, especially since binding substrates and cofactor may alter the conformation of the catalytic cleft such that crucial residues are positioned differently.

An important question still remains: how are the substrate-binding sites of the PtdIns*P*Ks, in particular the type I isoforms, able to accomodate such an unusually wide range of lipid headgroups? This question is only likely to be answered by a high resolution structure of a PtdIns*P*K enzyme bound to different substrates. A more detailed understanding of the structural differences between PI kinases that are specific for different lipid substrates may allow the design of novel specific inhibitors for research and clinical applications.

An interesting feature of the PtdIns*P*K IIβ structure is that it is predicted to be a disc-shaped homodimer that interacts with lipid bilayers via a flattened, positively charged face. Like the β isoform, PtdIns*P*K IIα was predicted to be dimeric using two-hybrid analysis and gel filtration (M. dos Santos, S. Minogue, and J. Hsuan unpublished data) raising the possibility that this is a general property of type II PtdIns*P*Ks. The relatively large membrane interface of the PtdIns*P*K IIβ suggests the potential to form a stable interaction with membranes that could be further stabilised by interaction with a membrane protein, phosphorylation or some other posttranslational modification. This putative property of the type II PtdIns*P*K dimer may explain the observation that type II PtdIns*P*K activity is distributed between the cytosolic and membrane fractions (Bazenet *et al.*, 1990; Jenkins *et al.*, 1994).

The PtdIns*P*K IIβ sequence that binds the TNFα receptor is conserved in other type II PtdIns*P*Ks (Castellino *et al.*, 1997) and maps to a cleft on the opposite face from the membrane interface (Rao *et al.*, 1998). This raises the possibility that all type II isoforms can interact with cell surface receptors. PtdIns 4- and PtdIns*P*K activities are reported to associate with a basic juxtamembrane region of the EGF receptor (Cochet *et al.*, 1991), however the TNFα receptor does not contain such a sequence and neither do members of the trans-membrane 4 superfamily of proteins which were recently found to co-purify with the type II PtdIns 4K (Yauch *et al.*, 1998).

7.5 Conclusion

There are many unresolved questions related to the physiological roles of PtdIns 4- and PtdIns*P*Ks and significant challenges remain. Not least is the question of how extracellular signals regulate the biosynthesis and flux of PtdIns(4,5)P_2 through PLC and PI 3-kinase pathways. To address this question it will be necessary to identify the isoforms involved and dissect the mechanisms that regulate the activities of the relevant PtdIns- and PtdIns*P*Ks. Of particular interest is the identity of possibly novel enzymes including the type II PtdIns 4K which has been implicated in a large number of PI

signalling systems (Section 1.5.1.a) and the EGFR-associated PtdIns*P*K (Cochet *et al.*, 1991), which remains completely uncharacterised.

The metabolism of PtdIns(4,5)P_2 which underlies exocytosis represents another important cellular model for PtdIns 4- and PtdIns*P*K function. However, it is not known how the supply of PtdIns(4,5)P_2 is regulated or what the precise function of this lipid is. While the ability to reconstitute exocytotsis from permeablised cells using cytosolic components provides a basis for assaying recombinant proteins and inhibitors, complimentary experiments are required to ensure that the correct isozymes are identified.

The putative role of phosphoinositides in regulating the cytoskeleton has recieved much attention since the finding that Rho regulates a PtdIns*P*K activity (Chong *et al.*, 1994) and that expression of type I PtdIns*P*Ks in various cell types leads to dramatic changes in the actin cytoskeleton (Ishihara *et al.*, 1998; Shibasaki *et al.*, 1997, and Section 1.10.1). Despite a growing body of data supporting the involvement of PtdIns*P*Ks (and phosphoinositides in general) in the regulation of the actin cytoskeleton, much remains to be determined regarding the signalling pathways and molecular events which lead to this phenomenom.

Finally, perhaps the greatest challenge to investigators is to understand the bewildering complexity of phosphoinositide signalling which results from the combined complexities of multiple isoforms, compartmentation phenomena, the heterogeneity of signalling pathways, and crosstalk between signalling pathways. Before we can achieve a better understanding of these aspects of cellular PI function we will need to characterise the proteins implicated in the regulation of these pathways in different cell types and generate corresponding isoform-specific reagents.

8.0 Appendix

8.1 Abbreviations

The abbreviations used in this thesis follow the guidelines of the IUAPC-IUBMB Joint commission on Biochemical Nomenclature (summarised in *Biochemical Journal*, **321**, 1-16, 1997). In addition, the following abbreviations are used:

Abbreviation	Meaning
AMV RT	(Avian myoblastosis virus reverse transcriptase)
APMSF	(p-amidino)phenylmethylsulphonylfluoride
BLAST	basic local alignment search tool
CDTA	*trans*-1,2-diaminocyclohexane *N,N,N',N'*, tetraacetic acid
DAG	diacylglycerol
ddH_2O	double distilled water
DEPC	diethyl pyrocarbonate
ECL	Enhanced Chemiluminescence
EGF(R)	epidermal growth factor (receptor)
EPB	electroporation buffer
EST	expressed sequence tag
EtBr	ethidium bromide
FSBA	5'-[*p*-(fluorosulphonyl)benzoyl]adenosine
GSH	reduced glutathione
GSH-Sepharose	glutathione coupled to sepharose beads
GST	glutathione-S-transferase (GST::[protein] denotes an N-terminal GST fusion protein)
GTE	glucose-Tris-EDTA buffer
GTP[γ]S	guanosine 5'-*o*-(3'-thiotrisphosphate)
$(H)_6$	hexahistidine affinity purification tag
HRP	horse radish peroxidase
MMTLV RT	murine Moloney T-cell lymphotrophic virus reverse transcriptase
Ni-NTA -(agarose)	nickle nitrilotriacetic acid
OD	optical density
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PH	pleckstrin homology (domain)
PI	phosphoinositide
PLC	phospholipase (or phosphoinositidase) C
PNK	polynucleotide kinase
PtdCho	phosphatidylcholine
PtdIns	phosphatidylinositol
PtdIns-TP	phosphatidylinositol transfer protein
Ptd*InsP*	phosphatidylinositol monophosphate
$PtdInsP_2$	phosphatidylinositol bisphosphate
$PtdInsP_3$	phosphatidylinositol trisphosphate
PtdOH	phosphatidic acid
RACE	rapid amplification of cDNA ends
RIPA	receptor immunoprecipitation buffer
SH2	Src homology 2 (domain)
SH3	Src homology 3 (domain)
SSC	standard sodium citrate buffer
SSPE	standard sodium phosphate-EDTA buffer
TB	teriffic broth

8.2 Supplimentary Information

8.2.1 p53 peptide sequence data

p 53 digest 1: lys -C	digest 2: asp-N	digest 3: lys-C
f 31 kIYIDDNNK	f 30 DGTHPVG DAKKKAAHAAKTVKHGAG	f 55 KHVQDPVM
f 34 kENMPSFK	f 31 DLKGSTVAREAS	f 58 kAIIDILTHYDAK kLMDYSXXgi
f 36 kVFLEK kDNDFINEGQK	f 36 DSQARSGARFHTSY	
f 37 KVDNHL	f 40 DKEKAKELPTLK	
f 38 KVDNHLFNK	f 43 DILTHYy	**N-terminal**
f 41 kELPTLK	f 45 DGQKIYi	1. XWSLPGXRVPPPADpEG
f 43 kHGAGAEISTV	f 46 DrRYVIKTITSE ddnnkkvflkl	2. EWSLPGGRVPRPAD
f 44 kHGAGAEISTVNPEQYSK	f 50 DDFKAYSKIKv DFQNSLTrSAplp dvef	
f 48 kTITSEDVAEMHNILL	f 51 DFQNSLTRSAPLPN	
f 60 kDVEFLAOLK kTITSEDVAEMNHILK	f 57 DSPGNTLNSSPPLAPGEF	
f 77 kS/GFLDFI	f 58 DAKKKAAHAAKTVKHGAG- -AEISTVNPEQY	
	f 59 DYSLHVGIA DFIGLILHT dakkkaat	
	f 60 DFINEGQKIYI EISTVNPEQYSKRF	
	f 62 ELSHVOIPVMl	
	f 66 EVYFMAI	
	f 68 DVEFLAQLKLM	

Table 8.1 p53 PtdIns*P*K peptide sequence data

The 53 kDa PtdIns*P*K II, purified from porcine platelets (Divecha et al, 1992), was gel-purified and digested with endoproteinases lys-C and asp-N. Peptides were HPLC purified and fractions subjected to Edman microsequenceing (Totty et al, 1992). The sequences of peptides in each fraction are indicated in single letter amino acid code. Lower case indicates a relatively low signal.

All of the peptides in Table 8.1 could be aligned with the predicted amino acid sequnece of human PtdIns*P*K IIα with the exception of the N-terminal sequences which are derived from contaminating porcine platelet factor 4 (Genbank 585702). The porcine PtdIns*P*K IIα cDNA sequence has been determined (GenBank U96135) and displays 98% homology overall with the human sequence.

8.2.2 Pairwise homology scores for selected PtdIns*P* kinase catalytic domains

	S.c. Fab1	*H.s.* IIγ	*H.s.* IIβ	*H.s.* IIα	*M.m.* Iγ	*H.s.* Iβ	*H.s.* Iα
S.c. Mss4	31.1 (55.4)	35.4 (58.5)	37.2 (61.5)	36.4 (59)	35.5 (54.3)	39 (56.4)	36 (57)
S.c. Fab1	-	20.4 (47)	26.7 (52)	28.9 (52.1)	27.9 (51.2)	27.6 (54.2)	25.8 (50)
H.s. IIγ	-	-	65.2 (80)	64.4 (81.7)	34.1 (55.9)	34.2 (55.3)	33.6 (53.4)
H.s. IIβ	-	-	-	77.4 (89.4)	32.6 (52)	33.9 (57.1)	34.4 (53.6)
H.s. IIα	-	-	-	-	29.4 (51.43)	29.9 (54.5)	31 (52.9)
M.m. Iγ	-	-	-	-	-	68.8 (80.1)	66 (80.4)
H.s. Iβ	-	-	-	-	-	-	65.2 (78.5)

Table 8.3 Homology between members of the PtdIns*P*K gene family

Pairwise identity and similarity scores were determined using the GAP alignment program (GCG, University of Wisconsin, Madison WI). Similarity scores are given in brackets. Definitions and acession numbers are given in Figure 1.6.

8.2.3 Pairwise homology scores for selected PtdIns 4-kinase catalytic domains

	S.c. STT4	*H.s.* p92	*F35H -12.4*	*S.c.* Pik1	*S.p.* Pik1	*D.d* PIK4
p97 /230	53.4 (74.8)	40.4 (66.2)	37.8 (62.2)	36.7 (62.9)	34.3 (60.6)	42.8 (64.2)
S.c. STT4	-	40.9 (61.9)	38.7 (58.9)	39.5 (60.1)	37.9 (57.7)	42.2 (61.1)
H.s. p92	-	-	56.8 (76.6)	45.3 (67.8)	42.9 (69.3)	47.3 (59.9)
F35H -12.4	-	-	-	42.4 (64.7)	46.8 (65.4)	50.0 (69.2)
S.c. Pik1	-	-	-	-	58.8 (75.5)	46.0 (68.0)
S.p. Pik1	-	-	-	-	-	46.9 (67.0)

Table 8.4 Homology between members of the PtdIns 4K gene family

Pairwise identity and similarity scores were determined using the GAP alignment program (GCG, University of Wisconsin, Madison WI). Similary scores are given in brackets. Definitions and accession numbers are given in Figure 1.4.

8.3 References

Abeliovich, A., Chen, C., Goda, Y., Silva, A.J., Stevens, C.F. and Tonegawa, S. (1993a) Modified hippocampal long-term potentiation in PKC gamma-mutant mice. *Cell*, **75**, 1253-1262.

Abeliovich, A., Paylor, R., Chen, C., Kim, J.J., Wehner, J.M. and Tonegawa, S. (1993b) PKC gamma mutant mice exhibit mild deficits in spatial and contextual learning. *Cell*, **75**, 1263-1271.Ackerman, P. and Osheroff, N. (1989) Regulation of casein kinase II activity by epidermal growth factor in human A-431 carcinoma cells. *J. Biol. Chem.*, **264**, 11958-11965.

Anderson, D., Koch, C.A., Grey, L., Ellis, C., Moran, M.F. and Pawson, T. (1990) Binding of SH2 domains of phospholipase C gamma 1, GAP, and Src to activated growth factor receptors. *Science*, **250**, 979-982.

Brown, R.A., Ho, L.K., Weber Hall, S.J., Shipley, J.M. and Fry, M.J. (1997) Identification and cDNA cloning of a novel mammalian C2 domain-containing phosphoinositide 3-kinase, HsC2-PI3K. *Biochem. Biophys. Res. Commun.*, **233**, 537-544.

Ahmed, S., Lee, J., Wen, L.P., Zhao, Z., Ho, J., Best, A., Kozma, R. and Lim, L. (1994) Breakpoint cluster region gene product-related domain of n-chimaerin. Discrimination between Rac-binding and GTPase-activating residues by mutational analysis. *J. Biol. Chem.*, 269, 17642-17648.

Akimoto, K., Takahashi, R., Moriya, S., Nishioka, N., Takayanagi, J., Kimura, K., Fukui, Y., Osada, S., Mizuno, K., Hirai, S., Kazlauskas, A. and Ohno, S. (1996) EGF or PDGF receptors activate atypical PKClambda through phosphatidylinositol 3-kinase. *EMBO J..*, **15**, 788-798.

Alessi, D.R. and Cohen, P. (1998) Mechanism of activation and function of protein kinase B. *Curr. Opin. Genet. Dev.*, **8**, 55-62.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B.A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J..*, **15**, 6541-6551.

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B. and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr. Biol.*, **7**, 261-269.

Antonetti, D.A., Algenstaedt, P. and Kahn, C.R. (1996) Insulin receptor substrate 1 binds two novel splice variants of the regulatory subunit of phosphatidylinositol 3-kinase in muscle and brain. *Mol Cell Biol*, **16**, 2195-2203.

Arcaro, A. and Wymann, M.P. (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol(3,4,5)-trisphosphate in neutrophil responses. *Biochem. J.*, **296**, 297-301.

Attree, O., Olivos, I.M., Okabe, I., Bailey, L.C., Nelson, D.L., Lewis, R.A., McInnes, R.R. and Nussbaum, R.L. (1992) The Lowe's oculocerebrorenal syndrome gene encodes a protein highly homologous to inositol polyphosphate-5-phosphatase. *Nature*, **358**, 239-242.

August, A., Sadra, A., Dupont, B. and Hanafusa, H. (1997) Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase. *Proc. Natl. Acad. Sci. USA.*, **94**, 11227-11232.

Backer, J.M., Myers, M.G., Jr., Shoelson, S.E., Chin, D.J., Sun, X.J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E.Y., Schlessinger, J. and et al. (1992) Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J..*, **11**, 3469-3479.

Bae, Y.S., Cantley, L.G., Chen, C.S., Kim, S.R., Kwon, K.-S. and Rhee, S.G. (1998) Activation of phospholipase C-gamma by phosphatidylinositol(3,4,5)trisphosphate. *J. Biol. Chem.*, **273**, 4465-4469.

Bairoch, A. and Cox, J.A. (1990) EF-hand motifs in inositol phospholipid-specific phospholipase C. *FEBS-Lett.*, **269**, 454-456.

Balla, T., Downing, G.J., Jaffe, H., Kim, S., Zolyomi, A. and Catt, K.J. (1997) Isolation and molecular cloning of wortmannin-sensitive bovine type III phosphatidylinositol 4-kinases. *J. Biol. Chem.*, **272**, 18358-18366.

Ballou, L.R., Barker, S.C., Postlethwaite, A.E. and Kang, A.H. (1991) Interleukin 1 stimulates phosphatidylinositol kinase activity in human fibroblasts. *J. Clin. Invest.*, **87**, 299-304.

Baltensperger, K., Kozma, L.M., Jaspers, S.R. and Czech, M.P. (1994) Regulation by insulin of phosphatidylinositol 3'-kinase bound to alpha- and beta-isoforms of p85 regulatory subunit. *J. Biol. Chem.*, **269**, 28937-28946.

Banerjee, A., Barry, V.A., DasGupta, B.R. and Martin, T.F.J. (1996) N-Ethylmaleimide-sensitive factor acts at a prefusion ATP-dependent step in Ca^{2+}-activated exocytosis. *J. Biol. Chem.*, **271**, 20223-20226.

Banfic, H., Downes, C.P. and Rittenhouse, S.E. (1998a) Biphasic activation of PKBalpha/Akt in platelets. Evidence for stimulation both by phosphatidylinositol 3,4-bisphosphate, produced via a novel pathway, and by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.*, **273**, 11630-11637.

Banfic, H., Tang, X., Batty, I.H., Downes, C.P., Chen, C. and Rittenhouse, S.E. (1998b) A novel integrin-activated pathway forms PKB/Akt-stimulatory phosphatidylinositol 3,4-bisphosphate via phosphatidylinositol 3-phosphate in platelets. *J. Biol. Chem.*, **273**, 13-16.

Bankaitis, V.A., Aitken, J.R., Cleves, A.E. and Dowhan, W. (1990) An essential role for a phospholipid transfer protein in yeast Golgi function. *Nature*, **347**, 561-562.

Bankaitis, V.A., Malehorn, D.E., Emr, S.D. and Greene, R. (1989) The Saccharomyces cerevisiae SEC14 gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex. *J. Cell. Biol.*, **108**, 1271-1281.

Bansal, V.S., Caldwell, K.K. and Majerus, P.W. (1990) The isolation and characterization of inositol polyphosphate 4-phosphatase. *J. Biol. Chem.*, **265**, 1806-1811.

Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V. and Schlessinger, J. (1993) SH3 domains direct cellular localization of signaling molecules. *Cell*, **74**, 83-91.

Barrett, T., Xiao, B., Dodson, E.J., Dodson, G., Ludbrook, S.B., Nurmahomed, K., Gamblin, S.J., Musacchio, A., Smerdon, S.J. and Eccleston, J.F. (1997) The structure of the GTPase-activating domain from p50rhoGAP. *Nature*, **385**, 458-461.

Batty, I.H., Currie, R.A. and Downes, C.P. (1998) Evidence for a model of integrated inositol phospholipid pools implies an essential role for lipid transport in the maintenance of receptor-mediated phospholipase C activity in 1321N1 cells. *Biochem. J.*, **330**, 1069-1077.

Bauerfeind, R., Takei, K. and De Camilli, P. (1997) Amphiphysin I is associated with coated endocytic intermediates and undergoes stimulation-dependent dephosphorylation in nerve terminals. *J. Biol. Chem.*, **272**, 30984-30992.

Bazenet, C.E., Ruano, A.R., Brockman, J.L. and Anderson, R.A. (1990) The human erythrocyte contains two forms of phosphatidylinositol 4-phosphate 5-kinase which are differentially active towards membranes. *J. Biol. Chem.*, **265**, 18012-18022.

Bellacosa, A., Testa, J.R., Staal, S.P. and Tsichlis, P.N. (1991) A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science*, **254**, 274-277.

Belunis, C.J., Bae Lee, M., Kelley, M.J. and Carman, G.M. (1988) Purification and characterization of phosphatidylinositol kinase from Saccharomyces cerevisiae. *J. Biol. Chem.*, **263**, 18897-18903

Benovic, J.L., DeBlasi, A., Stone, W.C., Caron, M.G. and Lefkowitz, R.J. (1989) Beta-adrenergic receptor kinase: primary structure delineates a multigene family. *Science*, **246**, 235-240.

Berditchevski, F., Tolias, K.F., Wong, K., Carpenter, C.L. and Hemler, M.E. (1997) A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J. Biol. Chem.*, **272**, 2595-2598.

Berridge, M.J. (1983) Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. *Biochem. J.*, **212**, 849-858.

Berridge, M.J. (1993) Inositol trisphosphate and calcium signalling. *Nature*, **361**, 315-325.

Berstein, G., Blank, J.L., Jhon, D.Y., Exton, J.H., Rhee, S.G. and Ross, E.M. (1992) Phospholipase C-beta 1 is a GTPase-activating protein for Gq/11, its physiologic regulator. *Cell*, **70**, 411-418.

Bondeva, T., Pirola, L., Bulgarelli-Leva, G., Rubio, I., Weztker, R. and Wymann, M.P. (1998) Bifurcation of lipid and protein kinase signals od PI 3K gamma to the protein kinases PKB and MAPK. *Science*, **282**, 293-296.

Bork, P. and Margolis, B. (1995) A phosphotyrosine interaction domain [letter]. *Cell*, **80**, 693-694.

Boronenkov, I.V. and Anderson, R.A. (1995) The sequence of phosphatidylinositol-4-phosphate 5-kinase defines a novel family of lipid kinases. *J. Biol. Chem.*, **270**, 2881-2884.

Brooksbank, C.E.L., Hutchings, A., Butcher, G.W., Irvine, R.F. and Divecha, N. (1993) Monoclonal antibodies to phosphatidylinositol 4-phosphate 5-kinase: Distribution and intracellular location of the C isoform. *Biochem. J.*, **291**, 77-82.

Brown, E.J., Beal, P.A., Keith, C.T., Chen, J., Shin, T.B. and Schreiber, S.L. (1995) Control of p70 s6 kinase by kinase activity of FRAP in vivo [published erratum appears in Nature 1995 Dec 7;378(6557):644]. *Nature*, **377**, 441-446.

Buechler, J.A. and Taylor, S.S. (1988) Identification of aspartate-184 as an essential residue in the catalytic subunit of cAMP-dependent protein kinase. *Biochemistry*, **27**, 7356-7361.

Bullough, D.A. and Allison, W.S. (1986) Inactivation of the bovine heart mitochondrial F1-ATPase by 5'-p-fluorosulfonylbenzoyl[^{3}H]inosine is accompanied by modification of tyrosine 345 in a single beta subunit. *J. Biol. Chem.*, **261**, 14171-14177.

Burgering, B.M. and Coffer, P.J. (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature*, **376**, 599-602.

Burgoyne, R.D. and Morgan, A. (1993) Regulated exocytosis. *Biochem. J.*, **293**, 305-316.

Carpenter, C.L., Auger, K.R., Duckworth, B.C., Hou, W.M., Schaffhausen, B. and Cantley, L.C. (1993) A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. *Mol Cell Biol*, **13**, 1657-1665.

Carpenter, C.L., Duckworth, B.C., Auger, K.R., Cohen, B., Schaffhausen, B.S. and Cantley, L.C. (1990) Purification and characterization of phosphoinositide 3-kinase from rat liver. *J. Biol. Chem.*, **265**, 19704-19711.

Carrera, A.C., Alexandrov, K. and Roberts, T.M. (1993) The conserved lysine of the catalytic domain of protein kinases is actively involved in the phosphotransfer reaction and not required for anchoring ATP. *Proc. Natl. Acad. Sci. USA.*, **90**, 442-446.

Carter, R.H., Park, D.J., Rhee, S.G. and Fearon, D.T. (1991) Tyrosine phosphorylation of phospholipase C induced by membrane immunoglobulin in B lymphocytes. *Proc. Natl. Acad. Sci. USA.*, **88**, 2745-2749.

Carvajal, J.J., Pook, M.A., Dos Santos, M., Doudney, K., Hillermann, R., Minogue, S., Williamson, R., Hsuan, J.J. and Chamberlain, S. (1996) The Friedreich Ataxia gene encodes a novel phosphatidylinositol-4-phosphate 5-kinase. *Nature Genetics*, **14**, 157-162.

Carvajal, J.J., Pook, M.A., Doudney, K., Hillermann, R., Wilkes, D., al Mahdawi, S., Williamson, R. and Chamberlain, S. (1995) Friedreich's ataxia: a defect in signal transduction? *Hum. Mol. Genet.*, **4**, 1411-1419.

Castellino, A.M., Parker, G.J., Boronenkov, I.V., Anderson, R.A. and Chao, M.V. (1997) A novel interaction between the juxtamembrane region of the p55 tumor necrosis factor receptor and phosphatidylinositol-4-phosphate 5-kinase. *J. Biol. Chem.*, **272**, 5861-5870.

Chan, D.C., Bedford, M.T. and Leder, P. (1996) Formin binding proteins bear WWP/WW domains that bind proline-rich peptides and functionally resemble SH3 domains. *EMBO J.*, **15**, 1045-1054.

Chang, H.W., Aoki, M., Fruman, D., Auger, K.R., Bellacosa, A., Tsichlis, P.N., Cantley, L.C., Roberts, T.M. and Vogt, P.K. (1997) Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. *Science*, **276**, 1848-1850.

Chapman, E.R., An, S., Edwardson, J.M. and Jahn, R. (1996) A novel function for the second C2 domain of synaptotagmin. Ca^{2+}-triggered dimerization. *J. Biol. Chem.*, **271**, 5844-5849.

Chardin, P., Paris, S., Antonny, B., Robineau, S., Béraud-Dufour, S., Jackson, C.L. and Chabre, M. (1996) A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. *Nature*, **384**, 481-484.

Charriaut Marlangue, C., Otani, S., Creuzet, C., Ben Ari, Y. and Loeb, J. (1991) Rapid activation of hippocampal casein kinase II during long-term potentiation. *Proc. Natl. Acad. Sci. USA.*, **88**, 10232-10236.

Chen, H.I. and Sudol, M. (1995) The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules. *Proc. Natl. Acad. Sci. USA.*, **92**, 7819-7823.

Chen, J.K., Land, W.S., Brauer, A.W., Tanaka, A. and Schreiber, S. (1993) Biased Combinatorial Libraries: novel ligands for the SH3 domain of phosphoinositide 3-kinase. *J. Am. Chem. Soc.*, **115**, 12591-12592.

Chomczinski and Sacchi (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform. *Anal. Biochem.* **162**, 156-159.

Chong, L.D., Traynor, K.A., Bokoch, G.M. and Schwartz, M.A. (1994) The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell*, **79**, 507-513.

Chou, M., Weimin, H., Johnson, J., Graham, L.K., Lee, M.H., Chen, C.-H., Newton, A.C., Schaffhausen, B.S. and Toker, A. (1998) Regulation of protein kinase C zeta by PI-3kinase and PDK-1. *Curr. Biol.*, **8**, 1069-1077.

Cifuentes, M.E., Honkanen, L. and Rebecchi, M.J. (1993) Proteolytic fragments of phosphoinositide-specific phospholipase C-delta 1. Catalytic and membrane binding properties. *J. Biol. Chem.*, **268**, 11586-11593.

Claesson Welsh, L. (1994) Platelet-derived growth factor receptor signals. *J. Biol. Chem.*, **269**, 32023-32026.

Clapham, D.E. (1995) Calcium signaling. *Cell*, **80**, 259-268.

Cocco, L., Gilmour, R.S., Ognibene, A., Letcher, A.J., Manzoli, F.A. and Irvine, R.F. (1987) Synthesis of polyphosphoinositides in nuclei of Friend cells. Evidence for polyphosphoinositide metabolism inside the nucleus which changes with cell differentiation. *Biochem. J.*, **248**, 765-770.

Cochet, C. and Chambaz, E.M. (1986) Catalytic properties of a purified phosphatidylinositol-4-phosphate kinase from rat brain. *Biochem. J.*, **237**, 25-31.

Cochet, C., Filhol, O., Payrastre, B., Hunter, T. and Gill, G.N. (1991) Interaction between the epidermal growth factor receptor and phosphoinositide kinases. *J. Biol. Chem.*, **266**, 637-644.

Cockcroft, S. (1998) Phosphatidylinositol transfer proteins: a requirement in signal transduction and vesicle traffiic. *Bioessays*, **20**, 423-432.

Cohen, G.B., Ren, R. and Baltimore, D. (1995) Modular Binding Domains in Signalling Proteins. *Cell*, **80**, 237-248.

Colman, R.F. (1983) Affinity labeling of purine nucleotide sites in proteins. *Annu. Rev. Biochem.*, **52**, 67-91.

Compartmentalization of phosphatidylinositol 4,5-bisphosphate in low-density membrane domains in the absence of caveolin. *J. Biol. Chem.*, **271**, 26453-26456.

Courtneidge, S.A. and Heber, A. (1987) An 81 kd protein complexed with middle T antigen and pp60c-src: a possible phosphatidylinositol kinase. *Cell*, **50**, 1031-1037.

Cowburn, D. (1997) Peptide recognition by PTB and PDZ domains. *Curr. Opin. Struc. Biol.*, **7**, 835-838.

Craven, S.E. and Bredt, D.S. (1998) PDZ proteins organize synaptic signaling pathways. *Cell*, **93**, 495-498.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M. and Hemmings, B.A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*, **378**, 785-789.

Cross, M.J., Roberts, S., Ridley, A.J., Hodgkin, M.N., Stewart, A., Claesson-Welsh, L. and Wakelam, M.J.O. (1996) Stimulation of stress fibre formation mediated by activation of phospholipase D. *Curr. Biol.*, **6**, 588-597.

Cross, M.J., Stewart, A., Hodgkin, M.N., Kerr, D.J. and Wakelam, M.J. (1995) Wortmannin and its structural analogue demethoxyviridin inhibit stimulated phospholipase A2 activity in Swiss 3T3 cells. Wortmannin is not a specific inhibitor of phosphatidylinositol 3-kinase. *J. Biol. Chem.*, **270**, 25352-25355.

Cunningham, E., Thomas, G.M., Ball, A., Hiles, I. and Cockcroft, S. (1995) Phosphatidylinositol transfer protein dictates the rate of inositol trisphosphate production by promoting the synthesis of PIP2. *Curr. Biol.*, **5**, 775-783.

Currie, R.A., MacLead, B.M.G. and Downes, C.P. (1997) The lipid transfer activity of phosphatidylinositol transfer protein is sufficient to account for enhanced phospholipase C activity in turkey erythrocyte ghosts. *Curr. Biol.*, **7**, 184-190.

Cutler, N.S., Heitman, J. and Cardenas, M.E. (1997) STT4 is an essential phosphatidylinositol 4-kinase that is a target of wortmannin in Saccharomyces cerevisiae. *J. Biol. Chem.*, **272**, 27671-27677.

Damen, J.E., Liu, L., Rosten, P., Humphries, R.K., Jefferson, A.B., Majerus, P.W. and Krystal, G. (1996) The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase. *Proc. Natl. Acad. Sci. USA.*, **93**, 1689-1693.

Das, T., Baek, K.J., Gray, C. and Im, M.J. (1993) Evidence that the Gh protein is a signal mediator from alpha 1-adrenoceptor to a phospholipase C. II. Purification and characterization of a Gh-coupled 69-kDa phospholipase C and reconstitution of alpha 1-adrenoceptor, Gh family, and phospholipase C. *J. Biol. Chem.*, **268**, 27398-27405.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. and Greenberg, M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*, **91**, 231-241.

Daub, H., Weiss, F.U., Wallasch, C. and Ullrich, A. (1996) Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature*, **379**, 557-560.

David, C., McPherson, P.S., Mundigl, O. and de Camilli, P. (1996) A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. *Proc. Natl. Acad. Sci. USA.*, **93**, 331-335.

Davis, J.N., Rock, C.O., Cheng, M., Watson, J.B., Ashmun, R.A., Kirk, H., Kay, R.J. and Roussel, M.F. (1997) Complementation of growth factor receptor-dependent mitogenic signaling by a truncated type I phosphatidylinositol 4-phosphate 5-kinase. *Mol Cell Biol*, **17**, 7398-7406.

De Camilli, P., Emr, S.D., McPherson, P.S. and Novick, P. (1996) Phosphoinositides as regulators in membrane traffic. *Science*, **271**, 1533-1539.

De Camilli, P., Takei, K. and McPherson, P.S. (1995) The function of dynamin in endocytosis. *Curr. Opin. Neurobiol.*, **5**, 559-565.

De Vries, K.J., Heinrichs, A.A., Cunningham, E., Brunink, F., Westerman, J., Somerharju, P.J., Cockcroft, S., Wirtz, K.W. and Snoek, G.T. (1995) An isoform of the phosphatidylinositol-transfer protein transfers sphingomyelin and is associated with the Golgi system. *Biochem. J.*, **310**, 643-649.

Deora, A.A., Win, T., Vanhaesebroeck, B. and Lander, H.M. (1998) A Redox-triggered Ras-Effector Interaction. *J. Biol. Chem.*, **273**, 29923-29928.

Deprez, J., Vertommen, D., Alessi, D.R., Hue, L. and Rider, M.H. (1997) Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. *J. Biol. Chem.*, **272**, 17269-17275.

Desrivieres, S., Cooke, F.T., Parker, P.J. and Hall, M.N. (1998) MSS4, a phosphatidylinositol-4-phosphate 5-kinase required for organization of the actin cytoskeleton in Saccharomyces cerevisiae. *J. Biol. Chem.*, **273**, 15787-15793.

Dhand, R., Hara, K., Hiles, I., Bax, B., Gout, I., Panayotou, G., Fry, M.J., Yonezawa, K., Kasuga, M. and Waterfield, M.D. (1994) PI 3-kinase: structural and functional analysis of intersubunit interactions. *EMBO J..*, **13**, 511-521.

Divecha, N. and Irvine, R.F. (1995) Phospholipid signaling. *Cell*, **80**, 269-278.

Divecha, N., Banfic, H. and Irvine, R.F. (1993) Inositides and the nucleus and inositides in the nucleus. *Cell*, **74**, 405-407.

Divecha, N., Brooksbank, C.E. and Irvine, R.F. (1992) Purification and characterization of phosphatidylinositol 4-phosphate 5-kinases. *Biochem. J.*, **288**, 637-642.

Divecha, N., Truong, O., Hsuan, J.J., Hinchliffe, K.A. and Irvine, R.F. (1995) The cloning and sequence of the C isoform of PtdIns4P 5-kinase. *Biochem. J.*, **309**, 715-719.

Domin, J. and Waterfield, M.D. (1997) Using structure to define the function of phosphoinositide 3-kinase family members. *FEBS-Lett.*, **410**, 91-95.

Domin, J. and Waterfield, M.D. (1997) Using structure to define the function of phosphoinositide 3-kinase family members. *FEBS-Lett.*, **410**, 91-95.

Domin, J., Pages, F., Volinia, S., Rittenhouse, S.E., Zvelebil, M.J., Stein, R.C. and Waterfield, M.D. (1997) Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin

Dove, S.K., Cooke, F.T., Douglas, M.R., Sayers, L.G., Parker, P.J. and Michell, R.H. (1997) Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. *Nature*, **390**, 187-192.

Downes, C.P. and Batty, I.H. (1993) Inositol lipid signalling extacts a transfer fee. *Curr. Biol.*, **3**, 794-796.

Downing, G.J., Kim, S., Nakanishi, S., Catt, K.J. and Balla, T. (1996) Characterization of a soluble adrenal phosphatidylinositol 4-kinase reveals wortmannin sensitivity of type III phosphatidylinositol kinases. *Biochemistry*, **35**, 3587-3594.

Drayer, A.L., Van der Kaay, J., Mayr, G.W. and Van Haastert, P.J. (1994) Role of phospholipase C in Dictyostelium: formation of inositol 1,4,5-trisphosphate and normal development in cells lacking phospholipase C activity. *EMBO J..*, **13**, 1601-1609.

Druker, B.J., Ling, L.E., Cohen, B., Roberts, T.M. and Schaffhausen, B.S. (1990) A completely transformation-defective point mutant of polyomavirus middle T antigen which retains full associated phosphatidylinositol kinase activity. *J. Virol.*, **64**, 4454-4461.

Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R. and Greenberg, M.E. (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science*, **275**, 661-665.

Duerr, E.M., Rollbrocker, B., Hayashi, Y., Peters, N., Meyer Puttlitz, B., Louis, D.N., Schramm, J., Wiestler, O.D., Parsons, R., Eng, C. and von Deimling, A. (1998) PTEN mutations in gliomas and glioneuronal tumors. *Oncogene*, **16**, 2259-2264.

Eberhard, D.A., Cooper, C.L., Low, M.G. and Holz, R.W. (1990) Evidence that the inositol phospholipids are necessary for exocytosis. Loss of inositol phospholipids and inhibition of secretion in permeabilized cells caused by a bacterial phospholipase C and removal of ATP. *Biochem. J.*, **268**, 15-25.

Eck, M.J., Dhe Paganon, S., Trub, T., Nolte, R.T. and Shoelson, S.E. (1996) Structure of the IRS-1 PTB domain bound to the juxtamembrane region of the insulin receptor. *Cell*, **85**, 695-705.

Ellis, M.V., Carne, A. and Katan, M. (1993) Structural requirements of phosphatidylinositol-specific phospholipase C delta 1 for enzyme activity. *Eur. J. Biochem.*, **213**, 339-347.

Endemann, G., Dunn, S.N. and Cantley, L.C. (1987) Bovine brain contains two types of phosphatidylinositol kinase. *Biochemistry*, **26**, 6845-6852.

Endemann, G.C., Graziani, A. and Cantley, L.C. (1991) A monoclonal antibody distinguishes two types of phosphatidylinositol 4-kinase. *Biochem. J.*, **273**, 63-66.

Eng, S.P. and Lo, C.S. (1990) Mastoparan increases membrane bound phosphatidylinositol kinase and phosphatidylinositol 4-monophosphate kinase activities in Madin-Darby canine kidney cells. *Life Sci.*, **46**, 273-279.

Esch, F.S. and Allison, W.S. (1979) On the subunit stoichiometry of the F1-ATPase and the sites in it that react specifically with p-fluorosulfonylbenzoyl-5'-adenosine. *J. Biol. Chem.*, **254**, 10740-10746.

Escobedo, J.A., Kaplan, D.R., Kavanaugh, W.M., Turck, C.W. and Williams, L.T. (1991a) A phosphatidylinositol-3 kinase binds to platelet-derived growth factor receptors through a specific receptor sequence containing phosphotyrosine. *Mol Cell Biol*, **11**, 1125-1132.

Escobedo, J.A., Navankasattusas, S., Kavanaugh, W.M., Milfay, D., Fried, V.A. and Williams, L.T. (1991b) cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF beta-receptor. *Cell*, **65**, 75-82.

Essen, L.O., Perisic, O., Cheung, R., Katan, M. and Williams, R.L. (1996) Crystal structure of a mammalian phosphoinositide-specific phospholipase C delta. *Nature*, **380**, 595-602.

Fain, J.N. and Berridge, M.J. (1979) Relationship between hormonal activation of phosphatidylinositol hydrolysis, fluid secretion and calcium flux in the blowfly salivary gland. *Biochem. J.*, **178**, 45-58.

Falasca, M., Logan, S.K., Lehto, V.P., Baccante, G., Lemmon, M.A. and Schlessinger, J. (1998) Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO J..*, **17**, 414-422.

Feng, J.F., Rhee, S.G. and Im, M.J. (1996) Evidence that phospholipase delta1 is the effector in the Gh (transglutaminase II)-mediated signaling. *J. Biol. Chem.*, **271**, 16451-16454.

Feng, S., Chen, J.K., Yu, H., Simon, J.A. and Schreiber, S.L. (1994) Two binding orientations for peptides to the Src SH3 domain: Development of a general model for SH3 ligand interactions. *Science*, **266**, 1241-1247.

Fensome, A., Cunningham, E., Prosser, S., Tan, S.K., Swigart, P., Thomas, G., Hsuan, J. and Cockcroft, S. (1996) ARF and PITP restore GTP gamma S-stimulated protein secretion from cytosol-depleted HL60 cells by promoting PIP2 synthesis. *Curr. Biol.*, **6**, 730-738.

Flanagan, C.A. and Thorner, J. (1992) Purification and characterization of a soluble phosphatidylinositol 4-kinase from the yeast Saccharomyces cerevisiae. *J. Biol. Chem.*, **267**, 24117-24125.

Flanagan, C.A., Schnieders, E.A., Emerick, A.W., Kunisawa, R., Admon, A. and Thorner, J. (1993) Phosphatidylinositol 4-kinase: gene structure and requirement for yeast cell viability. *Science*, **262**, 1444-1448.

Flick, J.S. and Thorner, J. (1993) Genetic and biochemical characterization of a phosphatidylinositol-specific phospholipase C in Saccharomyces cerevisiae. *Mol Cell Biol*, **13**, 5861-5876.

Fluckiger, A.C., Li, Z., Kato, R.M., Wahl, M.I., Ochs, H.D., Longnecker, R., Kinet, J.P., Witte, O.N., Scharenberg, A.M. and Rawlings, D.J. (1998) Btk/Tec kinases regulate sustained increases in intracellular Ca^{2+} following B-cell receptor activation. *EMBO J..*, **17**, 1973-1985.

Franke, T.F., Kaplan, D.R., Cantley, L.C. and Toker, A. (1997) Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science*, **275**, 665-668.

Franke, T.F., Yang, S.I., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R. and Tsichlis, P.N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*, **81**, 727-736.

Frech, M., Andjelkovic, M., Ingley, E., Reddy, K.K., Falck, J.R. and Hemmings, B.A. (1997) High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. *J. Biol. Chem.*, **272**, 8474-8481.

Freund, G.G., Wittig, J.G. and Mooney, R.A. (1995) The PI3-kinase serine kinase phosphorylates its p85 subunit and IRS-1 in PI3-kinase/IRS-1 complexes. *Biochem. Biophys. Res. Commun.*, **206**, 272-278.

Fukami, K., Endo, T., Imamura, M. and Takenawa, T. (1994) alpha-Actinin and vinculin are PIP2-binding proteins involved in signaling by tyrosine kinase. *J. Biol. Chem.*, **269**, 1518-1522.

Fukami, K., Furuhashi, K., Inagaki, M., Endo, T., Hatano, S. and Takenawa, T. (1992) Requirement of phosphatidylinositol 4,5-bisphosphate for alpha-actinin function. *Nature*, **359**, 150-152.

Fukuda, M., Kojima, T., Kabayama, H. and Mikoshiba, K. (1996) Mutation of the pleckstrin homology domain of Bruton's tyrosine kinase in immunodeficiency impaired inositol 1,3,4,5-tetrakisphosphate binding capacity. *J. Biol. Chem.*, **271**, 30303-30306.

Garcia, P., Gupta, R., Shah, S., Morris, A.J., Rudge, S.A., Scarlata, S., Petrova, V., McLaughlin, S. and Rebecchi, M.J. (1995) The pleckstrin homology domain of phospholipase C-delta 1 binds with high affinity to phosphatidylinositol 4,5-bisphosphate in bilayer membranes. *Biochemistry*, **34**, 16228-16234.

Garcia-Bustos, J.F., Marini, F., Stevenson, I., Frei, C. and Hall, M.N. (1994) PIK1, an essential phosphatidylinositol 4-kinase associated with the yeast nucleus. *EMBO J.*, **13**, 2352-2361.

Gasman, S., Chasserot Golaz, S., Hubert, P., Aunis, D. and Bader, M.F. (1998) Identification of a potential effector pathway for the trimeric Go protein associated with secretory granules. Go stimulates a granule-bound phosphatidylinositol 4-kinase by activating RhoA in chromaffin cells. *J. Biol. Chem.*, **273**, 16913-16920.

Gaullier, J.M., Simonsen, A., D'Arrigo, A., Bremnes, B., Stenmark, H. and Aasland, R. (1998) FYVE fingers bind PtdIns(3)P [letter; comment]. *Nature*, **394**, 432-433.

Gehrmann, T., Vereb, G., Schmidt, M., Klix, D., Meyer, H.E., Varsanyi, M. and Heilmeyer, L.M., Jr. (1996) Identification of a 200 kDa polypeptide as type 3 phosphatidylinositol 4-kinase from bovine brain by partial protein and cDNA sequencing. *Biochim. Biophys. Acta.*, **1311**, 53-63 .

Gilmore, A.P. and Burridge, K. (1996) Regulation of vinculin binding to talin and actin by phosphatidyl-inositol-4-5-bisphosphate. *Nature*, **381**, 531-535.

Glenn, D.E. and Burgoyne, R.D. (1996) Botulinum neurotoxin light chains inhibit both $Ca^{(2+)}$-induced and GTP analogue-induced catecholamine release from permeabilised adrenal chromaffin cells. *FEBS-Lett.*, **386**, 137-140.

Gout, I., Dhand, R., Hiles, I.D., Fry, M.J., Panayotou, G., Das, P., Truong, O., Totty, N.F., Hsuan, J., Booker, G.W. and et al. (1993) The GTPase dynamin binds to and is activated by a subset of SH3 domains. *Cell*, **75**, 25-36.

Gratacap, M.P., Payrastre, B., Viala, C., Mauco, G., Plantavid, M. and Chap, H. (1998) Phosphatidylinositol 3,4,5-trisphosphate-dependent stimulation of phospholipase C-gamma2 is an early key event in FcgammaRIIA-mediated activation of human platelets. *J. Biol. Chem.*, **273**, 24314-24321.

Graziani, A., Ling, L.E., Endemann, G., Carpenter, C.L. and Cantley, L.C. (1992) Purification and characterization of human erythrocyte phosphatidylinositol 4-kinase. Phosphatidylinositol 4-kinase and phosphatidylinositol 3-monophosphate 4-kinase are distinct enzymes. *Biochem. J.*, **284**, 39-45.

Grondin, P., Plantavid, M., Sultan, C., Breton, M., Mauco, G. and Chap, H. (1991) Interaction of pp60c-src, phospholipase C, inositol-lipid, and diacyglycerol kinases with the cytoskeletons of thrombin-stimulated platelets. *J. Biol. Chem.*, **266**, 15705-15709.

Guan, K.L. and Dixon, J.E. (1991) Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.*, **192**, 262-267.

Guo, J. and Yu, F.X. (1997) Cloning and characterization of human homologue of Drosophila retinal degeneration B: a candidate gene for degenerative retinal diseases. *Dev Genet*, **20**, 235-245.

Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science*, **279**, 509-514.

Hamilton, B.A., Smith, D.J., Mueller, K.L., Kerrebrock, A.W., Bronson, R.T., van Berkel, V., Daly, M.J., Kruglyak, L., Reeve, M.P., Nemhauser, J.L., Hawkins, T.L., Rubin, E.M. and Lander, E.S. (1997) The vibrator mutation causes neurodegeneration via reduced expression of PITP alpha: positional complementation cloning and extragenic suppression. *Neuron*, **18**, 711-722.

Hamm, H.E. and Gilchrist, A. (1996) Heterotrimeric G proteins. *Curr. Opin. Cell Biol.*, **8**, 189-196.

Han, J., Luby-Phelps, K., Das, B., Shu, Y.X., Mosteller, R.D., Krishna, U.M., Falck, J.R., White, M.A. and Broek, D. (1998) Role of Substrates and Products of PI 3-kinase in Regulating Activation of Rac-related Guanosine Triphosphatases by Vav. *Science*, **279**, 558-560.

Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**, 42-52.

Harlow, E. and Lane, D. (1988) *Antibodies: a laboratory manual.* Cold Spring Harbour Laboratory, NY.

Hartley, K.O., Gell, D., Smith, G.C., Zhang, H., Divecha, N., Connelly, M.A., Admon, A., Lees Miller, S.P., Anderson, C.W. and Jackson, S.P. (1995) DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. *Cell*, **82**, 849-856.

Hartwig, J.H., Bokoch, G.M., Carpenter, C.L., Janmey, P.A., Taylor, L.A., Toker, A. and Stossel, T.P. (1995) Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell*, **82**, 643-653.

Hawkins, P.T., Eguinoa, A., Qiu, R.G., Stokoe, D., Cooke, F.T., Walters, R., Wennstrom, S., Claesson-Welsh, L., Evans, T., Symons, M. and Stephens, L. (1995) PDGF stimulates an increase in GTP-Rac via activation of Phosphoinositide 3-kinase. *Curr. Biol.*, **5**, 393-403.

Hay, J.C. and Martin, T.F. (1993) Phosphatidylinositol transfer protein required for ATP-dependent priming of $Ca^{(2+)}$-activated secretion. *Nature*, **366**, 572-575.

Hay, J.C., Fisette, P.L., Jenkins, G.H., Fukami, K., Takenawa, T., Anderson, R.A. and Martin, T.F. (1995) ATP-dependent inositide phosphorylation required for $Ca^{(2+)}$-activated secretion. *Nature*, **374**, 173-177.

Helms, J.B., de Vries, K.J. and Wirtz, K.W. (1991) Synthesis of phosphatidylinositol 4,5-bisphosphate in the endoplasmic reticulum of Chinese hamster ovary cells. *J. Biol. Chem.*, **266**, 21368-21374.

Hempel, W.M., Schatzman, R.C. and DeFranco, A.L. (1992) Tyrosine phosphorylation of phospholipase C-gamma 2 upon cross-linking of membrane Ig on murine B lymphocytes. *J. Immunol.*, **148**, 3021-3027.

Hiles, I.D., Otsu, M., Volinia, S., Fry, M.J., Gout, I., Dhand, R., Panayotou, G., Ruiz, L.F., Thompson, A., Totty, N.F. and et, a.l. (1992) Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell*, **70**, 419-429.

Hinchliffe, K.A., Irvine, R.F. and Divecha, N. (1996) Aggregation-dependent, integrin-mediated increases in cytoskeletally associated PtdInsP2 (4,5) levels in human platelets are controlled by translocation of PtdIns 4-P 5-kinase C to the cytoskeleton. *EMBO J.*., **15**, 6516-6524.

Hinchliffe, K.A., Irvine, R.F. and Divecha, N. (1998) Regulation of PtdIns4P 5-kinase C by thrombin-stimulated changes in its phosphorylation state in human platelets. *Biochem. J.*, **329**, 115-119.

Hirai, H. and Varmus, H.E. (1990) Mutations in src homology regions 2 and 3 of activated chicken c-src that result in preferential transformation of mouse or chicken cells. *Proc. Acad. Sci. USA.*, **87**, 8592-8596 .

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene*, **77**, 51-59.

Hokin, M.R. and Hokin, L.E. (1953) *J. Biol. Chem.*, 967-977.

Homma, K., Terui, S., Minemura, M., Qadota, H., Anraku, Y., Kanaho, Y. and Ohya, Y. (1998) Phosphatidylinositol-4-phosphate 5-kinase localized on the plasma membrane is essential for yeast cell morphogenesis. *J. Biol. Chem.*, **273**, 15779-15786.

Homma, Y. and Emori, Y. (1995) A dual functional signal mediator showing RhoGAP and phospholipase C-delta stimulating activities. *EMBO J.*, **14**, 286-291.

Hong, Z. and Verma, D.P. (1994) A phosphatidylinositol 3-kinase is induced during soybean nodule organogenesis and is associated with membrane proliferation. *Proc. Natl. Acad. Sci. USA.*, **91**, 9617-9621.

Hope, H.R. and Pike, L.J. (1996) Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains. *Mol Biol Cell*, **7**, 843-851.

Hou, W.M., Zhang, Z.L. and Tai, H.H. (1988) Purification and characterization of phosphatidylinositol kinase from porcine liver microsomes. *Biochim. Biophys. Acta.*, **959**, 67-75 .

Hsuan, J.J. (1993) Oncogene regulation by growth factors. *Anticancer Res.*, **13**, 2521-2532.

Hsuan, J.J. and Tan, S.H. (1997) Growth factor-dependent phosphoinositide signalling. *Int. J. Biochem. Cell Biol.*, **29**, 415-435.

Hsuan, J.J., Minogue, S. and dos Santos, M. (1998) Phosphoinositide 4- and 5-kinases and the cellular roles of phosphatidylinositol 4,5-bisphosphate. *Adv. Cancer Res.*, **74**, 167-216.

Hsuan, J.J., Totty, N.F., Truong, O. and Waterfield, M.D. (1994) Purification of phosphatidylinositol 3-kinase by SDS-agarose gel electrophoresis and HPEC. *Prot. Pept. Lett.*, **1**, 25-23.

Hu, P., Margolis, B., Skolnik, E.Y., Lammers, R., Ullrich, A. and Schlessinger, J. (1992) Interaction of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Mol Cell Biol*, **12**, 981-990.

Hu, P., Mondino, A., Skolnik, E.Y. and Schlessinger, J. (1993) Cloning of a novel, ubiquitously expressed human phosphatidylinositol 3-kinase and identification of its binding site on p85. *Mol Cell Biol*, **13**, 7677-7688.

Hu, Q., Klippel, A., Muslin, A.J., Fantl, W.J. and Williams, L.T. (1995) Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science*, **268**, 100-102.

Hug, H. and Sarre, T.F. (1993) Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.*, **291**, 329-343.

Hunter, T. (1995) When is a lipid kinase not a lipid kinase? When it is a protein kinase. *Cell*, **83**, 1-4.

Husebye, E.S. and Flatmark, T. (1988) Phosphatidylinositol kinase of bovine adrenal chromaffin granules: kinetic properties and inhibition by low concentrations of Ca^{2+}. *Biochim. Biophys. Acta.*, **968**, 261-265.

Husebye, E.S. and Flatmark, T. (1989) Purification and kinetic properties of a soluble phosphatidylinositol-4-phosphate kinase of the bovine adrenal medulla with emphasis on its inhibition by calcium ions. *Biochim. Biophys. Acta.*, **1010**, 250-257.

Hwang, S.C., Jhon, D.Y., Bae, Y.S., Kim, J.H. and Rhee, S.G. (1996) Activation of phospholipase C-gamma by the concerted action of tau proteins and arachidonic acid. *J. Biol. Chem.*, **271**, 18342-18349.

Inokuchi, S. and Imboden, J.B. (1990) Antigen receptor-mediated regulation of sustained polyphosphoinositide turnover in a human T cell line. Evidence for a receptor-regulated pathway for production of phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.*, **265**, 5983-5989.

Irvine, R. and Cullen, P. (1996) Inositol phosphates - whither bound? Intracellular signalling. *Curr. Biol.*, **6**, 537-540.

Ishihara, H., Shibasaki, Y., Kizuki, N., Katagiri, H., Yazaki, Y., Asano, T. and Oka, Y. (1996) Cloning od cDNAs encoding two isoforms of 68-kDa type I phosphatidylinositol 4-phosphate 5-kinase. *J. Biol. Chem.*, **271**, 23611-23614.

Ishihara, H., Shibasaki, Y., Kizuki, N., Wada, T., Yazaki, Y., Asano, T. and Oka, Y. (1998) Type I phosphatidylinositol-4-phosphate 5-kinases. Cloning of the third isoform and deletion/substitution analysis of members of this novel lipid kinase family. *J. Biol. Chem.*, **273**, 8741-8748.

Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N. and Narumiya, S. (1996) The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J.*, **15**, 1885-1893.

Itoh, T., Ijuin, T. and Takenawa, T. (1998) A novel phosphatidylinositol-5-phosphate 4-kinase (phosphatidylinositol-phosphate kinase IIgamma) is phosphorylated in the endoplasmic reticulum in response to mitogenic signals. *J. Biol. Chem.*, **273**, 20292-20299.

Jackson, P. and Baltimore, D. (1989) N-terminal mutations activate the leukemogenic potential of the myristoylated form of c-abl. *EMBO J.*, **8**, 449-456.

Jackson, S.P. (1997) DNA-dependent protein kinase. *Int. J. Biochem. Cell Biol.*, **29**, 935-938.

Jackson, S.P., Schoenwaelder, S.M., Matzaris, M., Brown, S. and Mitchell, C.A. (1995) Phosphatidylinositol 3,4,5-trisphosphate is a substrate for the 75 kDa inositol polyphosphate 5-phosphatase and a novel 5-phosphatase which forms a complex with the p85/p110 form of phosphoinositide 3-kinase. *EMBO J.*, **14**, 4490-4500.

James, S.R., Downes, C.P., Gigg, R., Grove, S.J., Holmes, A.B. and Alessi, D.R. (1996) Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. *Biochem. J.*, **315**, 709-713.

Janmey, P.A. and Stossel, T.P. (1987) Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. *Nature*, **325**, 362-364.

Janmey, P.A. and Stossel, T.P. (1987) Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. *Nature*, **325**, 362-364.

Janmey, P.A., Iida, K., Yin, H.L., Stossel, T.P., Lamb, J., Allen, P.G. and Matsudaira, P.T. (1987) Polyphosphoinositide micelles and polyphosphoinositide-containing vesicles

dissociate endogenous gelsolin-actin complexes and promote actin assembly from the fast-growing end of actin filaments blocked by gelsolin: Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate Phosphoinositide-binding peptides derived from the sequences of gelsolin and villin. *J. Biol. Chem.*, **262**, 12228-12236.

Jeggo, P.A. (1998) DNA breakage and repair. *Adv. Genet.*, **38**, 185-218.

Jenkins, G.H., Fisette, P.L. and Anderson, R.A. (1994) Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J. Biol. Chem.*, **269**, 11547-11554.

Jenkins, G.H., Subrahmanyam, G. and Anderson, R.A. (1991) Purification and reconstitution of phosphatidylinositol 4-kinase from human erythrocytes. *Biochim. Biophys. Acta.*, **1080**, 11-18.

Ji, Q.S., Ermini, S., Baulida, J., Sun, F.L. and Carpenter, G. (1998) Epidermal growth factor signaling and mitogenesis in Plcg1 null mouse embryonic fibroblasts. *Mol Biol Cell*, **9**, 749-757.

Jimenez, C., Jones, D.R., Rodriguez Viciana, P., Gonzalez Garcia, A., Leonardo, E., Wennstrom, S., von Kobbe, C., Toran, J.L., L, R.B., Calvo, V., Copin, S.G., Albar, J.P., Gaspar, M.L., Diez, E., Marcos, M.A., Downward, J., Martinez, A.C., Merida, I. and Carrera, A.C. (1998) Identification and characterization of a new oncogene derived from the regulatory subunit of phosphoinositide 3-kinase. *EMBO J.*, **17**, 743-753.

Jockusch, B.M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G. and Winkler, J. (1995) The molecular architecture of focal adhesions. *Annu. Rev. Cell Dev. Biol.*, **11**, 379-416.

Johnson, R.M., Connelly, P.A., Sisk, R.B., Pobiner, B.F., Hewlett, E.L. and Garrison, J.C. (1986) Pertussis toxin or phorbol 12-myristate 13-acetate can distinguish between epidermal growth factor- and angiotensin-stimulated signals in hepatocytes. *Proc. Natl. Acad. Sci. USA.*, **83**, 2032-2036.

Jones, G.A. and Carpenter, G. (1993) The regulation of phospholipase C-gamma 1 by phosphatidic acid. Assessment of kinetic parameters. *J. Biol. Chem.*, **268**, 20845-20850.

Kaech, S.M., Whitfield, C.W. and Kim, S.K. (1998) The LIN-2/LIN-7/LIN-10 complex mediates basolateral membrane localization of the C. elegans EGF receptor LET-23 in vulval epithelial cells. *Cell*, **94**, 761-771.

Kamada, Y., Qadota, H., Python, C.P., Anraku, Y., Ohya, Y. and Levin, D.E. (1996) Activation of yeast protein kinase C by Rho1 GTPase. *J. Biol. Chem.*, **271**, 9193-9196.

Kamps, M.P., Taylor, S.S. and Sefton, B.M. (1984) Direct evidence that oncogenic tyrosine kinases and cyclic AMP-dependent protein kinase have homologous ATP-binding sites. *Nature*, **310**, 589-592.

Kanoh, H., Banno, Y., Hirata, M. and Nozawa, Y. (1990) Partial purification and characterization of phosphatidylinositol kinases from human platelets. *Biochim. Biophys. Acta.*, **1046**, 120-126.

Kasurinen, J., van, P.P., Wirtz, K.W. and Somerharju, P. (1990) Affinity of phosphatidylcholine molecular species for the bovine phosphatidylcholine and phosphatidylinositol transfer proteins. Properties of the sn-1 and sn-2 acyl binding sites. *Biochemistry*, **29**, 8548-8554.

Katan, M. (1996) The control of inositol lipid hydrolysis. *Cancer Surveys*, **27**: 199-211.

Kauffmann Zeh, A., Rodriguez Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J. and Evan, G. (1997) Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature*, **385**, 544-548.

Kauffmann Zeh, A., Thomas, G.M., Ball, A., Prosser, S., Cunningham, E., Cockcroft, S. and Hsuan, J.J. (1995) Requirement for phosphatidylinositol transfer protein in epidermal growth factor signaling. *Science*, **268**, 1188-1190 .

Kauffmann-Zeh, A., Klinger, R., Endemann, G., Waterfield, M.D., Wetzker, R. and Hsuan, J.J. (1994) Regulation of human type II phosphatidylinositol kinase activity by epidermal growth factor-dependent phsophorylation and receptor association. *J.Biol.Chem.*, **269**, 31243-31251.

Kavanaugh, W.M., Turck, C.W. and Williams, L.T. (1995) PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. *Science*, **268**, 1177-1179.

Kearns, B.G., Alb, J.G.J. and Bankaitis, V.A. (1998) Phosphatidylinositol transfer proteins: the long and winding road to physiological function. *Trends Cell Biol..*, **8**, 276-282.

Keith, C.T. and Schreiber, S.L. (1995) PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints. *Science*, **270**, 50-51.

Kim, D., Jun, K.S., Lee, S.B., Kang, N.-G., Min, D.S., Kim, Y.-H., Ryu, S.H., Suh, P.-G. and Shin, H.-S. (1997) Phospholipase C isozymes selectively couple to specific neurotransmitter receptors. *Nature*, **389**, 290-293.

Kim, H.K., Kim, J.W., Zilberstein, A., Margolis, B., Kim, J.G., Schlessinger, J. and Rhee, S.G. (1991) PDGF stimulation of inositol phospholipid hydrolysis requires PLC-gamma 1 phosphorylation on tyrosine residues 783 and 1254. *Cell*, **65**, 435-441.

King, C.E., Hawkins, P.T., Stephens, L.R. and Michell, R.H. (1989) Determination of the steady-state turnover rates of the metabolically active pools of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in human erythrocytes. *Biochem. J.*, **259**, 893-896.

Klarlund, J.K., Guilherme, A., Holik, J.J., Virbasius, J.V., Chawla, A. and Czech, M.P. (1997) Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains. *Science*, **275**, 1927-1930.

Klippel, A., Kavanaugh, W.M., Pot, D. and Williams, L.T. (1997) A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol Cell Biol*, **17**, 338-344.

Klippel, A., Reinhard, C., Kavanaugh, W.M., Apell, G., Escobedo, M.A. and Williams, L.T. (1996) Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. *Mol Cell Biol*, **16**, 4117-4127.

Knighton, D.R., Zheng, J.H., Ten Eyck, L.F., Ashford, V.A., Xuong, N.H., Taylor, S.S. and Sowadski, J.M. (1991) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science*, **253**, 407-414.

Kohn, A.D., Summers, S.A., Birnbaum, M.J. and Roth, R.A. (1996) Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J. Biol. Chem.*, **271**, 31372-31378.

Kolanus, W., Nagel, W., Schiller, B., Zeitlmann, L., Godar, S., Stockinger, H. and Seed, B. (1996) Alpha L beta 2 integrin/LFA-1 binding to ICAM-1 induced by cytohesin-1, a cytoplasmic regulatory molecule. *Cell*, **86**, 233-242.

Kozak, M. (1991) An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell. Biol.*, **115**, 887-903.

Kular, G., Loubtchenkov, M., Swigart, P., Whatmore, J., Ball, A., Cockcroft, S. and Wetzker, R. (1997) Co-operation of phosphatidylinositol transfer protein with

phosphoinositide 3-kinase γ in the formylmethionyl-leucylphenylalanine-dependent production of phosphatidylinositol 3,4,5-trisphosphate in human neutrophils. *Biochem. J.*, **325**, 299-301.

Labeit, S. and Kolmerer, B. (1995) The complete primary structure of human nebulin and its correlation to muscle structure. *J Mol Biol*, **248**, 308-315.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Lam, K., Carpenter, C.L., Ruderman, N.B., Friel, J.C. and Kelly, K.L. (1994) The phosphatidylinositol 3-kinase serine kinase phosphorylates IRS-1. Stimulation by insulin and inhibition by Wortmannin. *J. Biol. Chem.*, **269**, 20648-20652.

Lassing, I. and Lindberg, U. (1985) Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. *Nature*, **314**, 472-474.

Lassing, I. and Lindberg, U. (1988) Specificity of the interaction between phosphatidylinositol 4,5-bisphosphate and the profilin-actin complex. *J. Cell Biochem.*, **37**, 255-267.

Lawton, M.A., Yamamoto, R.T., Hanks, S.K. and Lamb, C.J. (1989) Molecular cloning of plant transcripts encoding protein kinase homologs. *Proc. Natl. Acad. Sci. USA.*, **86**, 3140-3144.

Le Good, J.A., Ziegler, W.H., Parekh, D.B., Alessi, D.R., Cohen, P. and Parker, P.J. (1998) Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science*, **281**, 2042-2045.

Lee, C., Fisher, S.K., Agranoff, B.W. and Hajra, A.K. (1991) Quantitative analysis of molecular species of diacylglycerol and phosphatidate formed upon muscarinic receptor activation of human SK-N-SH neuroblastoma cells. *J. Biol. Chem.*, **266**, 22837-22846.

Lee, M.H. and Bell, R.M. (1991) Mechanism of protein kinase C activation by phosphatidylinositol 4,5-bisphosphate. *Biochemistry*, **30**, 1041-1049.

Lee, S.B. and Rhee, S.G. (1995) Significance of PIP2 hydrolysis and regulation of phospholipase C isozymes. *Curr. Opin. Cell Biol.*, **7**, 183-189.

Lee, S.B. and Rhee, S.G. (1996) Molecular cloning, splice variants, expression, and purification of phospholipase C-delta 4. *J. Biol. Chem.*, **271**, 25-31.

Leevers, S.J., Paterson, H.F. and Marshall, C.J. (1994) Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature*, **369**, 411-414.

Leevers, S.J., Weinkove, D., MacDougall, L.K., Hafen, E., Waterfield, M.D., Vanhaesebroeck, B. and Panayotou, G. (1996) The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth p60 is an adaptor for the Drosophila phosphoinositide 3-kinase, Dp110. *EMBO J.*, **15**, 6584-6594.

Lehto, V.P., Wasenius, V.M., Salven, P. and Saraste, M. (1988) Transforming and membrane proteins [letter]. *Nature*, **334**, 388.

Lemmon, M.A., Ferguson, K.M., O'Brien, R., Sigler, P.B. and Schlessinger, J. (1995) Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain. *Proc. Acad. Sci. USA.*, **92**, 10472-10476.

Levin, D.E., Fields, F.O., Kunisawa, R., Bishop, J.M. and Thorner, J. (1990) A candidate protein kinase C gene, PKC1, is required for the S. cerevisiae cell cycle. *Cell*, **62**, 213-224.

Li, C., Ullrich, B., Zhang, J.Z., Anderson, R.G., Brose, N. and Sudhof, T.C. (1995a) $Ca^{(2+)}$-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature*, **375**, 594-599.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H. and Parsons, R. (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*, **275**, 1943-1947.

Li, T., Tsukada, S., Satterthwaite, A., Havlik, M.H., Park, H., Takatsu, K. and Witte, O.N. (1995b) Activation of Bruton's tyrosine kinase (BTK) by a point mutation in its pleckstrin homology (PH) domain. *Immunity*, **2**, 451-460.

Li, Y.S., Porter, F.D., Hoffman, R.M. and Deuel, T.F. (1989) Separation and identification of two phosphatidylinositol 4-kinase activities in bovine uterus. *Biochem-Biophys-Res-Commun*, **160**, 202-209.

Liang, M. and Garrison, J.C. (1992) Epidermal growth factor activates phospholipase C in rat hepatocytes via a different mechanism from that in A431 or rat1hER cells. *Mol. Pharmacol.*, **42**, 743-752.

Likos, J.J. and Colman, R.F. (1981) Affinity labeling of rabbit muscle pyruvate kinase by a new fluorescent nucleotide alkylating agent 5'-[*p*-(fluorosulfonyl)benzoyl]-1,N6-ethenoadenosine. *Biochemistry*, **20**, 491-499.

Lin, H.C. and Gilman, A.G. (1996) Regulation of dynamin I GTPase activity by G protein betagamma subunits and phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.*, **271**, 27979-27982.

Linassier, C., MacDougall, L.K., Domin, J. and Waterfield, M.D. (1997) Molecular cloning and biochemical characterization of a Drosophila phosphatidylinositol-specific phosphoinositide 3-kinase. *Biochem. J.*, **321**, 849-856.

Ling, L.E., Druker, B.J., Cantley, L.C. and Roberts, T.M. (1992) Transformation-defective mutants of polyomavirus middle T antigen associate with phosphatidylinositol 3-kinase (PI 3-kinase) but are unable to maintain wild-type levels of PI 3-kinase products in intact cells. *J Virol*, **66**, 1702-1708.

Ling, L.E., Schulz, J.T. and Cantley, L.C. (1989) Characterization and purification of membrane-associated phosphatidylinositol-4-phosphate kinase from human red blood cells. *J. Biol. Chem.*, **264**, 5080-5088.

Lisanti, M.P., Scherer, P.E., Vidugiriene, J., Tang, Z., Hermanowski Vosatka, A., Tu, Y.H., Cook, R.F. and Sargiacomo, M. (1994) Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. *J. Cell. Biol.*, **126**, 111-126.

Liscovich, M. and Cantley, L.C. (1995) Signal transduction and membrane traffic: The PITP/phosphoinositide connection. *Cell*, **81**, 1-20.

Liscovitch, M., Chalifa, V., Pertile, P., Chen, C.S. and Cantley, L.C. (1994) Novel function of phosphatidylinositol 4,5-bisphosphate as a cofactor for brain membrane phospholipase D. *J. Biol. Chem.*, **269**, 21403-21406.

Liu, N., Fukami, K., Yu, H. and Takenawa, T. (1996) A new phospholipase C delta 4 is induced at S-phase of the cell cycle and appears in the nucleus. *J. Biol. Chem.*, **271**, 355-360.

Liu, Y., Casey, L. and Pike, L.J. (1998) Compartmentalization of phosphatidylinositol 4,5-bisphosphate in low-density membrane domains in the absence of caveolin. *Biochem. Biophys. Res. Commun.*, **245**, 684-690.

Loijens, J.C. and Anderson, R.A. (1996) Type I phosphatidylinositol-4-phosphate 5-kinases are distinct members of this novel lipid kinase family. *J. Biol. Chem.*, **271**, 32937-32943.

Loijens, J.C., Boronenkov, I.V., Parker, G.J. and Anderson, R.A. (1996) The phosphatidylinositol 4-phosphate 5-kinase family. *Adv. Enzyme. Regul.*, **36**, 115-140.

Lomasney, J.W., Cheng, H.-F., Wang, L.-P., Kuan, Y.-S., Liu, S.-M., Fesik, S.W. and Kim, K. (1996) Phosphatidylinositol 4,5-bisphosphate binding to the pleckstrin homology domain of Phospholipase C-delta 1 enhances enzyme activity. *J. Biol. Chem.*, **271**, 25316-25326.

Lopez Ilasaca, M., Crespo, P., Pellici, P.G., Gutkind, J.S. and Wetzker, R. (1997) Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. *Science*, **275**, 394-397.

Loyet, K.M., Kowalchyk, J.A., Chaudhary, A., Chen, J., Prestwich, G.D. and Martin, T.F.J. (1998) Specific binding of phosphatidylinositol 4,5-bisphosphate to calcium-dependent activator protein for secretion (CAPS), a potential phosphoinositide effector protein for regulated exocytosis. *J. Biol. Chem.*, **273**, 8337-8343.

MacDougall, L.K., Domin, J. and Waterfield, M.D. (1995) A family of phosphoinositide 3-kinases in Drosophila identifies a new mediator of signal transduction. *Curr. Biol.*, **5**, 1404-1415.

Maehama, T. and Dixon, J.E. (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.*, **273**, 13375-13378.

Mariot, P., O'Sullivan, A.J., Brown, A.M. and Tatham, P.E. (1996) Rho guanine nucleotide dissociation inhibitor protein (RhoGDI) inhibits exocytosis in mast cells. *EMBO J.*, **15**, 6476-6482.

Martin, T.F. (1997) Phosphoinositides as spatial regulators of membrane traffic. *Curr. Opin. Neurobiol.*, **7**, 331-338.

Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J.*, **15**, 2208-2216.

Matsumoto, M., Nakagawa, T., Inoue, T., Nagata, E., Tanaka, K., Takano, H., Minowa, O., Kuno, J., Sakakibara, S., Yamada, M., Yoneshima, H., Miyawaki, A., Fukuuchi, Y., Furuichi, T., Okano, H., Mikoshiba, K. and Noda, T. (1996) Ataxia and epileptic seizures in mice lacking type 1 inositol 1,4,5-trisphosphate receptor. *Nature*, **379**, 168-171.

McNamee, H.P., Ingber, D.E. and Schwartz, M.A. (1993) Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. *J. Cell. Biol.*, **121**, 673-678.

McPherson, P.S., Garcia, E.P., Slepnev, V.I., David, C., Zhang, X., Grabs, D., Sossin, W.S., Bauerfeind, R., Nemoto, Y. and De Camilli, P. (1996) A presynaptic inositol-5-phosphatase. *Nature*, **379**, 353-357.

Meisenhelder, J., Suh, P.G., Rhee, S.G. and Hunter, T. (1989) Phospholipase C-gamma is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. *Cell*, **57**, 1109-1122.

Mellor, H. and Parker, P.J. (1998) The extended protein kinase C superfamily. *Biochem. J.*, **332**, 281-292.

Mewes, H.W., Albermann, K., Bahr, M., Frishman, D., Gleissner, A., Hani, J., Heumann, K., Kleine, K., Maierl, A., Oliver, S.G., Pfeiffer, F. and Zollner, A. (1997) Overview of the yeast genome. *Nature*, **387**, 7-65.

Meyers, R. and Cantley, L.C. (1997) Cloning and characterization of a wortmannin-sensitive human phosphatidylinositol 4-kinase. *J. Biol. Chem.*, **272**, 4384-4390.

Michell, R.H. (1975) Inositol phospholipids and cell surface receptor function. *Biochim Biophys Acta*, **415**, 81-47.

Michell, R.H., Kirk, C.J., Jones, L.M., Downes, C.P. and Creba, J.A. (1981) The stimulation of inositol lipid metabolism that accompanies calcium mobilization in stimulated cells: defined characteristics and unanswered questions. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, , **296**, 123-138.

Milligan, S.C., Alb, J.G., Elegina, R.B., Bankaitis, V.A. and Hyde, D.R. (1997) The phosphatidylinositol transfer protein domain of Drosophila retinal degeneration B protein is essential for photoreceptor cell survival and recovery from light stimulation. *J. Cell. Biol.*, **139**, 351-363.

Mills, I.G., Jones, A.T. and Clague, M.J. (1998) Involvement of the endosomal early autoantigen EEA1 in homotypic fusion of early endosomes. *Curr. Biol.*, **8**, 881-884.

Mizushima, S. and Nagata, S. (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Research*, **18**, 5322.

Moarefi, I., LaFevre Bernt, M., Sicheri, F., Huse, M., Lee, C.H., Kuriyan, J. and Miller, W.T. (1997) Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. *Nature*, **385**, 650-653.

Mohammadi, M., Honegger, A.M., Rotin, D., Fischer, R., Bellot, F., Li, W., Dionne, C.A., Jaye, M., Rubinstein, M. and Schlessinger, J. (1991) A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. *Mol. Cell Biol.*, **11**, 5068-5078.

Molz, L., Chen, Y.W., Hirano, M. and Williams, L.T. (1996) Cpk is a novel class of Drosophila PtdIns 3-kinase containing a C2 domain. *J. Biol. Chem.*, **271**, 13892-13899.

Monaco, M.E. and Gershengorn, M.C. (1992) Subcellular organization of receptor-mediated phosphoinositide turnover. *Endocr. Rev.*, **13**, 707-718.

Morgan, S.J., Smith, A.D. and Parker, P.J. (1990) Purification and characterization of bovine brain type I phosphatidylinositol kinase. *Eur. J. Biochem.*, **191**, 761-767.

Moritz, A., De Graan, P.N., Ekhart, P.F., Gispen, W.H. and Wirtz, K.W. (1990) Purification of a phosphatidylinositol 4-phosphate kinase from bovine brain membranes. *J. Neurochem.*, **54**, 351-354.

Moritz, A., De Graan, P.N.E., Gispen, W.H. and Wirtz, K.W.A. (1992) Phosphatidic acid is a specific activator of phosphatidylinositol-4-phosphate kinase. *J. Biol. Chem.*, **267**, 7207-7210.

Moriya, S., Kazlauskas, A., Akimoto, K., Hirai, S., Mizuno, K., Takenawa, T., Fukui, Y., Watanabe, Y., Ozaki, S. and Ohno, S. (1996) Platelet-derived growth factor activates protein kinase C epsilon through redundant and independent signaling pathways involving phospholipase C gamma or phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA.*, **93**, 151-155.

Morris, J.Z., Tissenbaum, H.A. and Ruvkun, G. (1996) A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. *Nature*, **382**, 536-539.

Muller, E., Hegewald, H., Jaroszewicz, K., Cumme, G.A., Hoppe, H. and Frunder, H. (1986) Turnover of phosphomonoester groups and compartmentation of polyphosphoinositides in human erythrocytes. *Biochem. J.*, **235**, 775-783.

Musacchio, A., Cantley, L.C. and Harrison, S.C. (1996) Crystal structure of the breakpoint cluster region-homology domain from phosphoinositide 3-kinase p85 alpha subunit. *Proc. Natl. Acad. Sci. USA.*, **93**, 14373-14378.

Nakagawa, T., Goto, K. and Kondo, H. (1996) Cloning and characterization of a 92 kDa soluble phosphatidylinositol 4-kinase. *Biochem. J.*, **320**, 643-649.

Nakagawa, T., Goto, K. and Kondo, H. (1996) Cloning, expression, and localization of 230-kDa phosphatidylinositol 4-kinase. *J. Biol. Chem.*, **271**, 12088-12094.

Nakanishi, H., Brewer, K.A. and Exton, J.H. (1993) Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.*, **268**, 13-16.

Nakanishi, S., Catt, K.J. and Balla, T. (1995) A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. *Proc. .Natl. Acad. Sci. USA.*, **92**, 5317-5321.

Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., Matsuda, Y. and et al. (1992) Wortmannin, a microbial product inhibitor of myosin light chain kinase. *J. Biol. Chem.*, **267**, 2157-2163.

Nakaoka, H., Perez, D.M., Baek, K.J., Das, T., Husain, A., Misono, K., Im, M.J. and Graham, R.M. (1994) Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function. *Science*, **264**, 1593-1596.

Nakashima, S., Banno, Y., Watanabe, T., Nakamura, Y., Mizutani, T., Sakai, H., Zhao, Y., Sugimoto, Y. and Nozawa, Y. (1995) Deletion and site-directed mutagenesis of EF-hand domain of phospholipase C-delta 1: effects on its activity. *Biochem. Biophys. Res. Commun.*, **211**, 365-369.

Nalefski, E.A., McDonagh, T., Somers, W., Seehra, J., Falke, J.J. and Clark, J.D. (1998) Independent folding and ligand specificity of the C2 calcium-dependent lipid binding domain of cytosolic phospholipase A2. *J. Biol. Chem.*, **273**, 1365-1372.

Neer, E.J. (1995) Heterotrimeric G proteins: organizers of transmembrane signals. *Cell*, **80**, 249-257.

Nickels, J.T., Jr., Buxeda, R.J. and Carman, G.M. (1992) Purification, characterization, and kinetic analysis of a 55-kDa form of phosphatidylinositol 4-kinase from Saccharomyces cerevisiae. *J. Biol. Chem.*, **267**, 16297-16304 .

Nishikawa, K., Toker, A., Wong, K., Marignani, P.A., Johannes, F.J. and Cantley, L.C. (1998) Association of protein kinase Cmu with type II phosphatidylinositol 4-kinase and type I phosphatidylinositol-4-phosphate 5-kinase. *J. Biol. Chem.*, **273**, 23126-23133.

Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*, **308**, 693-698.

Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature*, **334**, 661-665.

Nishizuka, Y. (1992) Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. *Science*, **258**, 607-614.

Noh, D.Y., Shin, S.H. and Rhee, S.G. (1995) Phosphoinositide-specific phospholipase C and mitogenic signaling. *Biochim Biophys Acta*, **1242**, 99-113.

Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A. and Takai, Y. (1995) A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in Saccharomyces cerevisiae. *EMBO J.*, **14**, 5931-5938.

Norris, F.A. and Majerus, P.W. (1994) Hydrolysis of phosphatidylinositol 3,4-bisphosphate by inositol polyphosphate 4-phosphatase isolated by affinity elution chromatography. *J. Biol. Chem.*, **269**, 8716-8720.

Norris, F.A., Atkins, R.C. and Majerus, P.W. (1997a) The cDNA cloning and characterization of inositol polyphosphate 4-phosphatase type II. Evidence for conserved alternative splicing in the 4-phosphatase family. *J. Biol. Chem.*, **272**, 23859-23864.

Norris, F.A., Atkins, R.C. and Majerus, P.W. (1997b) Inositol polyphosphate 4-phosphatase is inactivated by calpain-mediated proteolysis in stimulated human platelets. *J. Biol. Chem.*, **272**, 10987-10989.

Norris, F.A., Auethavekiat, V. and Majerus, P.W. (1995) The isolation and characterization of cDNA encoding human and rat brain inositol polyphosphate 4-phosphatase. *J. Biol. Chem.*, **270**, 16128-16133.

Obermeier, A., Halfter, H., Wiesmuller, K.H., Jung, G., Schlessinger, J. and Ullrich, A. (1993) Tyrosine 785 is a major determinant of Trk--substrate interaction. *EMBO J.*, **12**, 933-941.

Ohara Imaizumi, M., Fukuda, M., Niinobe, M., Misonou, H., Ikeda, K., Murakami, T., Kawasaki, M., Mikoshiba, K. and Kumakura, K. (1997) Distinct roles of C2A and C2B domains of synaptotagmin in the regulation of exocytosis in adrenal chromaffin cells. *Proc. Natl. Acad. Sci. USA.*, **94**, 287-291.

Ohashi, M., de Vries, K.J., Frank, R., Snoek, G., Bankaitis, V., Wirtz, K. and Huttner, W.B. (1995) A role for phosphatidylinositol transfer protein in secretory vesicle formation. *Nature*, **377**, 544-547.

Olivos Glander, I.M., Janne, P.A. and Nussbaum, R.L. (1995) The oculocerebrorenal syndrome gene product is a 105-kD protein localized to the Golgi complex. *Am. J. Hum. Genet.*, **57**, 817-823.

Ono, F., Nakagawa, T., Saito, S., Owada, Y., Sakagami, H., Goto, K., Suzuki, M., Matsuno, S. and Kondo, H. (1998) A novel class II phosphoinositide 3-kinase predominantly expressed in the liver and its enhanced expression during liver regeneration. *J. Biol. Chem.*, **273**, 7731-7736.

Osborne, M.A., Zenner, G., Lubinus, M., Zhang, X., Songyang, Z., Cantley, L.C., Majerus, P., Burn, P. and Kochan, J.P. (1996) The inositol 5'-phosphatase SHIP binds to immunoreceptor signaling motifs and responds to high affinity IgE receptor aggregation. *J. Biol. Chem.*, **271**, 29271-29278.

Otsu, M., Hiles, I., Gout, I., Fry, M.J., Ruiz, L.F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N. and et, a.l. (1991) Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60c-src complexes, and PI3-kinase. *Cell*, **65**, 91-104.

Ozaki, K., Tanaka, K., Imamura, H., Hihara, T., Kameyama, T., Nonaka, H., Hirano, H., Matsuura, Y. and Takai, Y. (1996) Rom1p and Rom2p are GDP/GTP exchange proteins (GEPs) for the Rho1p small GTP binding protein in Saccharomyces cerevisiae. *EMBO J.*, **15**, 2196-2207.

Pages, F., Ragueneau, M., Rottapel, R., Truneh, A., Nunes, J., Imbert, J. and Olive, D. (1994) Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signalling. *Nature*, **369**, 327-329.

Palmer, R.H., Dekker, L.V., Woscholski, R., Le Good, J.A., Gigg, R. and Parker, P.J. (1995) Activation of PRK1 by phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. A comparison with protein kinase C isotypes. *J. Biol. Chem.*, **270**, 22412-22416.

Panayotou, G., Bax, B., Gout, I., Federwisch, M., Wroblowski, B., Dhand, R., Fry, M.J., Blundell, T.L., Wollmer, A. and Waterfield, M.D. (1992) Interaction of the p85 subunit of PI 3-kinase and its N-terminal SH2 domain with a PDGF receptor phosphorylation site: structural features and analysis of conformational changes. *EMBO J.*, **11**, 4261-4272.

Park, D., Jhon, D.Y., Lee, C.W., Lee, K.H. and Rhee, S.G. (1993) Activation of phospholipase C isozymes by G protein beta gamma subunits. *J. Biol. Chem.*, **268**, 4573-4576.

Parker, P.J., Hemmings, B.A. and Gierschik, P. (1994) PH domains and phospholipases--a meaningful relationship? *Trends Biochem. Sci.*, **19**, 54-55.

Parton, R.G. (1996) Caveolae and caveolins. *Curr. Opin. Cell Biol.*, **8**, 542-548.

Patki, V., Lawe, D.C., Corvera, S., Virbasius, J.V. and Chawla, A. (1998) A functional PtdIns(3)P-binding motif [letter]. *Nature*, **394**, 433-434.

Pawson, T. (1995) Protein modules and signalling networks. *Nature*, **373**, 573-580.

Payne, W.E. and Fitzgerald Hayes, M. (1993) A mutation in PLC1, a candidate phosphoinositide-specific phospholipase C gene from Saccharomyces cerevisiae, causes aberrant mitotic chromosome segregation. *Mol Cell Biol*, **13**, 4351-4364.

Payrastre, B., Nievers, M., Boonstra, J., Breton, M., Verkleij, A.J. and Van Bergen en Henegouwen, P.M. (1992) A differential location of phosphoinositide kinases, diacylglycerol kinase, and phospholipase C in the nuclear matrix. *J. Biol. Chem.*, **267**, 5078-5084.

Payrastre, B., Plantavid, M., Breton, M., Chambaz, E. and Chap, H. (1990) Relationship between phosphoinositide kinase activities and protein tyrosine phosphorylation in plasma membranes from A431 cells. *Biochem. J.*, **272**, 665-670.

Payrastre, B., van Bergen en Henegouwen, P.M., Breton, M., den Hartigh, J.C., Plantavid, M., Verkleij, A.J. and Boonstra, J. (1991) Phosphoinositide kinase, diacylglycerol kinase, and phospholipase C activities associated to the cytoskeleton: effect of epidermal growth factor. *J. Cell. Biol.*, **115**, 121-128.

Pearson, R.B. and Kemp, B.E. (1991) Protein Kinase phosphorylation Site Sequences and Consensus: Tabulations. *Methods Enzymol.*, **200**, 62-85.

Pertile, P. and Cantley, L.C. (1995) Type II phosphatidylinositol 4-kinase is recruited to CD4 in response to CD4 cross-linking. *Biochim Biophys Acta*, **1248**, 129-134.

Pettitt, T.R., Martin, A., Horton, T., Liossis, C., Lord, J.M. and Wakelam, M.J. (1997) Diacylglycerol and phosphatidate generated by phospholipases C and D, respectively, have distinct fatty acid compositions and functions. Phospholipase D-derived diacylglycerol does not activate protein kinase C in porcine aortic endothelial cells. *J. Biol. Chem.*, **272**, 17354-17359.

Pettitt, T.R., Zaqqa, M. and Wakelam, M.J. (1994) Epidermal growth factor stimulates distinct diradylglycerol species generation in Swiss 3T3 fibroblasts: evidence for a potential phosphatidylcholine-specific phospholipase C-catalysed pathway. *Biochem. J.*, **3**, 655-660.

Pike, L.J. (1992) Phosphatidylinositol 4-kinases and the role of polyphosphoinositides in cellular regulation. *Endocr. Rev.*, **13**, 692-706.

Pike, L.J. and Eakes, A.T. (1987) Epidermal growth factor stimulates the production of phosphatidylinositol monophosphate and the breakdown of polyphosphoinositides in A431 cells. *J. Biol. Chem.*, **262**, 1644-1651.

Pike, L.J. and Miller, J.M. (1998) Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. *J. Biol. Chem.*, **273**, 22298-22304.

Pike, L.J., Casey, L. and Liu, Y. (1996) Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains. *J. Biol. Chem.*, **271**, 26453-26456.

Pitcher, J.A., Touhara, K., Payne, E.S. and Lefkowitz, R.J. (1995) Pleckstrin homology domain-mediated membrane association and activation of the beta-adrenergic receptor kinase requires coordinate interaction with G beta gamma subunits and lipid. *J. Biol. Chem.*, **270**, 11707-11710.

Pons, S., Asano, T., Glasheen, E., Miralpeix, M., Zhang, Y., Fisher, T.L., Myers, M.G., Jr., Sun, X.J. and White, M.F. (1995) The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase. *Mol Cell Biol*, **15**, 4453-4465.

Ponting, C.P. and Parker, P.J. (1996) Extending the C2 domain family: C2s in PKCs delta, episilon, eta, theta, phospholipases, GAPs and perforin. *Protein Science*, **5**, 162-166.

Porter, F.D., Li, Y.S. and Deuel, T.F. (1988) Purification and characterization of a phosphatidylinositol 4-kinase from bovine uteri. *J. Biol. Chem.*, **263**, 8989-8995.

Prasad, K.V., Kapeller, R., Janssen, O., Repke, H., Duke Cohan, J.S., Cantley, L.C. and Rudd, C.E. (1993) Phosphatidylinositol (PI) 3-kinase and PI 4-kinase binding to the CD4-p56lck complex: the p56lck SH3 domain binds to PI 3-kinase but not PI 4-kinase. *Mol. Cell Biol.*, **13**, 7708-7717.

Price, L.S., Norman, J.C., Ridley, A.J. and Koffer, A. (1995) The small GTPases Rac and Rho as regulators of secretion in mast cells. *Curr. Biol.*, **5**, 68-73.

Rambourg, A., Clermont, Y., Nicaud, J.M., Gaillardin, C. and Kepes, F. (1996) Transformations of membrane-bound organelles in sec 14 mutants of the yeasts Saccharomyces cerevisiae and Yarrowia lipolytica. *Anat. Rev.*, **245**, 447-458.

Rameh, L.E., Arvidsson, A., Carraway, K.L., 3rd, Couvillon, A.D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M.P., Ravichandran, K.S., Burakoff, S.J., Wang, D.S., Chen, C.S. and Cantley, L.C. (1997a) A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. *J. Biol. Chem.*, **272**, 22059-22066.

Rameh, L.E., Chen, C.S. and Cantley, L.C. (1995) Phosphatidylinositol (3,4,5)P3 interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins. *Cell*, **83**, 821-830.

Rameh, L.E., Rhee, S.-G., Spokes, K., Kazulauskas, A., Cantley, L.C. and Cantley, L.G. (1998) Phosphoinositide 3-kinase regulates phospholipase C-gamma mediated calcium signalling. *J. Biol. Chem.*, **273**, 23750-23757.

Rameh, L.E., Tolias, K.F., Duckworth, B.C. and Cantley, L.C. (1997) A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. *Nature*, **390**, 192-196.

Rao, V.D., Misra, S., Boronenkov, I.V., Anderson, R.A. and Hurley, J.H. (1998) Structure of type IIbeta phosphatidylinositol phosphate kinase: a protein kinase fold flattened for interfacial phosphorylation. *Cell*, **94**, 829-839.

Rapoport, I., Miyazaki, M., Boll, W., Duckworth, B., Cantley, L.C., Shoelson, S. and Kirchhausen, T. (1997) Regulatory interactions in the recognition of endocytic sorting signals by AP-2 complexes. *EMBO J.*, **16**, 2240-2250.

Reif, K., Gout, I., Waterfield, M.D. and Cantrell, D.A. (1993) Divergent regulation of phosphatidylinositol 3-kinase P85 alpha and P85 beta isoforms upon T cell activation. *J. Biol. Chem.*, **268**, 10780-10788.

Ren, X.-D., Bokoch, G.M., Traynor-Kaplan, A., Jenkins, G.H., Anderson, R.A. and Schwartz, M.A. (1996) Physical association of the small GTPase rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. *Mol Biol Cell*, **7**, 435-442.

Rickles, R.J., Botfield, M.C., Zhou, X.M., Henry, P.A., Brugge, J.S. and Zoller, M.J. (1995) Phage display selection of ligand residues important for Src homology 3 domain binding specificity. *Proc. Natl. Acad. Sci. USA.*, **92**, 10909-10913.

Rittinger, K., Walker, P.A., Eccleston, J.F., Nurmahomed, K., Owen, D., Laue, E., Gamblin, S.J. and Smerdon, S.J. (1997) Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP. *Nature*, **388**, 693-697.

Rizo, R. and Sudhof, T.C. (1998) C2 Domains. *J. Biol. Chem.*, **273**, 15879-15882.

Rodriguez Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D. and Downward, J. (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature*, **370**, 527-532.

Rodriguez Viciana, P., Warne, P.H., Khwaja, A., Marte, B.M., Pappin, D., Das, P., Waterfield, M.D., Ridley, A. and Downward, J. (1997) Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell*, **89**, 457-467.

Rodriguez Viciana, P., Warne, P.H., Vanhaesebroeck, B., Waterfield, M.D. and Downward, J. (1996) Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. *EMBO J.*, **15**, 2442-2451.

Ron, D., Luo, J. and Mochly Rosen, D. (1995) C2 region-derived peptides inhibit translocation and function of beta protein kinase C in vivo. *J. Biol. Chem.*, **270**, 24180-24187.

Rubio, I., Rodriguez Viciana, P., Downward, J. and Wetzker, R. (1997) Interaction of Ras with phosphoinositide 3-kinase gamma. *Biochem. J.*, **326**, 891-895.

Russo, M.W., Lukas, T.J., Cohen, S. and Staros, J.V. (1985) Identification of residues in the nucleotide binding site of the epidermal growth factor receptor/kinase. *J. Biol. Chem.*, **260**, 5205-5208.

Ryves, W.J., Evans, A.T., Olivier, A.R., Parker, P.J. and Evans, F.J. (1991) Activation of the PKC-isotypes alpha, beta 1, gamma, delta and epsilon by phorbol esters of different biological activities. *FEBS-Lett.*, **288**, 5-9.

Sakisaka, T., Itoh, T., Miura, K. and Takenawa, T. (1997) Phosphatidylinositol 4,5-bisphosphate phosphatase regulates the rearrangement of actin filaments. *Mol Cell Biol*, **17**, 3841-3849.

Salim, K., Bottomley, M.J., Querfurth, E., Zvelebil, M.J., Gout, I., Scaife, R., Margolis, R.L., Gigg, R., Smith, C.I., Driscoll, P.C., Waterfield, M.D. and Panayotou, G. (1996) Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J.*, **15**, 6241-6250.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A laboratory manual.* Cold Spring Harbour Press, NY.

Saraste, M., Hyvönen, M. (1995) Pleckstrin homology domains: a fact file. *Curr. Biol.*, **5**, 403-408.

Saraste, M., Sibbald, P.R. and Wittinghofer, A. (1990) The P-loop--a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.*, **15**, 430-434.

Savitsky, K., Bar Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., Uziel, T., Sfez, S. and et al. (1995) A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science*, **268**, 1749-1753.

Scharenberg, A.M., El Hillal, O., Fruman, D.A., Beitz, L.O., Li, Z., Lin, S., Gout, I., Cantley, L.C., Rawlings, D.J. and Kinet, J.P. (1998) Phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3)/Tec kinase-dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals. *EMBO J.*, **17**, 1961-1972.

Schekman, R. and Orci, L. (1996) Coat proteins and vesicle budding. *Science*, **271**, 1526-1533.

Schiavo, G., Gu, Q.M., Prestwich, G.D., Sollner, T.H. and Rothman, J.E. (1996) Calcium-dependent switching of the specificity of phosphoinositide binding to synaptotagmin [published erratum appears in Proc Natl Acad Sci U S A 1997 Feb 4;94(3):1047]. *Proc. Natl. Acad. Sci. USA.*, **93**, 13327-13332.

Scholz, G., Barritt, G.J. and Kwok, F. (1991) Purification and chemical modification of a phosphatidylinositol kinase from sheep brain. *Eur-J-Biochem*, **201**, 249-255.

Schu, P.V., Takegawa, K., Fry, M.J., Stack, J.H., Waterfield, M.D. and Emr, S.D. (1993) Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science*, **260**, 88-91.

Scott, G.K., Dodson, J.M., Montgomery, P.A., Johnson, R.M., Sarup, J.C., Wong, W.L., Ullrich, A., Shepard, H.M. and Benz, C.C. (1991) p185HER2 signal transduction in breast cancer cells. *J. Biol. Chem.*, **266**, 14300-14305.

Seidel-Dugan, C., Meyer, B.E., Thomas, S.M. and Brugge, J.S. (1992) Effects of SH2 and SH3 deletions on the functional activities of wild-type and transforming variants of c-Src. *Mol. Cell Biol.*, **12**, 1835-1845.

Serunian, L.A., Auger, K.R. and Cantley, L.C. (1991) Identification and quantification of polyphosphoinositides produced in response to platelet-derived growth factor stimulation. *Methods Enzymol.*, **198**, 78-87.

Serunian, L.A., Haber, M.T., Fukui, T., Kim, J.W., Rhee, S.G., Lowenstein, J.M. and Cantley, L.C. (1989) Polyphosphoinositides produced by phosphatidylinositol 3-kinase are poor substrates for phospholipases C from rat liver and bovine brain. *J. Biol. Chem.*, **264**, 17809-17815.

Shao, X., Li, C., Fernandez, I., Zhang, X., Sudhof, T.C. and Rizo, J. (1997) Synaptotagmin-syntaxin interaction: the C2 domain as a Ca^{2+}-dependent electrostatic switch. *Neuron*, **18**, 133-142.

Sheetz, M.P. and Singer, S.J. (1974) Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. USA.*, **71**, 4457-4461.

Shepherd, P.R., Reaves, B.J. and Davidson, H.W. (1996) Phosphoinositide 3-kinases and membrane traffic. *Trends Cell Biol.*, **6**, 92-97.

Shibasaki, Y., Ishihara, H., Kizuki, N., Asano, T., Oka, Y. and Yazaki, Y. (1997) Massive actin polymerization induced by phosphatidylinositol-4-phosphate 5-kinase in vivo. *J. Biol. Chem.*, **272**, 7578-7581.

Shoelson, S.E., Sivaraja, M., Williams, K.P., Hu, P., Schlessinger, J. and Weiss, M.A. (1993) Specific phosphopeptide binding regulates a conformational change in the PI 3-kinase SH2 domain associated with enzyme activation. *EMBO J.*, **12**, 795-802.

Sidorenko, S.P., Law, C.L., Klaus, S.J., Chandran, K.A., Takata, M., Kurosaki, T. and Clark, E.A. (1996) Protein kinase C mu (PKC mu) associates with the B cell antigen receptor complex and regulates lymphocyte signaling. *Immunity*, **5**, 353-363.

Sikorski, R.S. and Hieter, P. (1989) A system of Shuttle Vectors and Yeast host Strains Designed for Efficient Manipulation of DNA in Saccharomyces cerevisiae. *Genetics*, **122**, 19-26.

Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature*, **387**, 569-572.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J.M., Brech, A., Callaghan, J., Toh, B.H., Murphy, C., Zerial, M. and Stenmark, H. (1998) EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature*, **394**, 494-498.

Simske, J.S., Kaech, S.M., Harp, S.A. and Kim, S.K. (1996) LET-23 receptor localization by the cell junction protein LIN-7 during C. elegans vulval induction. *Cell*, **85**, 195-204.

Sjaastad, M.D. and Nelson, W.J. (1997) Integrin-mediated calcium signaling and regulation of cell adhesion by intracellular calcium. *Bioessays*, **19**, 47-55.

Smith, C.D. and Chang, K.J. (1989) Regulation of brain phosphatidylinositol-4-phosphate kinase by GTP analogues. A potential role for guanine nucleotide regulatory proteins. *J. Biol. Chem.*, **264**, 3206-3210.

Smith, C.D. and Wells, W.W. (1984) Characterization of a phosphatidylinositol 4-phosphate-specific phosphomonoesterase in rat liver nuclear envelopes. *Arch. Biochem. Biophys.*, **235**, 529-537.

Smith, C.D., Wen, D., Mooberry, S.L. and Chang, K.J. (1992) Inhibition of phosphatidylinositol 4-phosphate kinase by heparin. A possible mechanism for the antiproliferative effects of heparin. *Biochem. J.*, **281**, 803-808.

Smith, D.J., Zhu, Y., Zhang, J., Cheng, J.F. and Rubin, E.M. (1995) Construction of a panel of transgenic mice containing a contiguous 2-Mb set of YAC/P1 clones from human chromosome 21q22.2. *Genomics*, **27**, 425-434.

Snoek, G.T., Westerman, J., Wouters, F.S. and Wirtz, K.W. (1993) Phosphorylation and redistribution of the phosphatidylinositol-transfer protein in phorbol 12-myristate 13-acetate- and bombesin-stimulated Swiss mouse 3T3 fibroblasts. *Biochem. J.*, **291**, 649-656.

Soler, C., Alvarez, C.V., Beguinot, L. and Carpenter, G. (1994) Potent SHC tyrosine phosphorylation by epidermal growth factor at low receptor density or in the absence of receptor autophosphorylation sites. *Oncogene*, **9**, 2207-2215.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., Neel, B.G., Birge, R.B., Fajardo, J.E., Chou, M.M., Hanafusa, H., Schaffhausen, B. and Cantley, L. (1993) SH2 domains recognise specific phosphopeptide sequences. *Cell*, **72**, 767-778.

Spiegel, S., Foster, D. and Kolesnick, R. (1996) Signal transduction through lipid second messengers. *Curr. Opin. Cell Biol.*, **8**, 159-167.

Stack, J.H. and Emr, S.D. (1994) Vps34p required for yeast vacuolar protein sorting is a multiple specificity kinase that exhibits both protein kinase and phosphatidylinositol-specific PI 3-kinase activities. *J. Biol. Chem.*, **269**, 31552-31562.

Stack, J.H., Horazdovsky, B. and Emr, S.D. (1995) Receptor-mediated protein sorting to the vacuole in yeast: roles for a protein kinase, a lipid kinase and GTP-binding proteins. *Annu. Rev. Cell Dev. Biol.*, **11**, 1-33.

Stauffer, T.P., Ahn, S. and Meyer, T. (1998) Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P_2 concentration monitored in living cells. *Curr. Biol.*, **8**, 343-346.

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D.H. and Tavtigian, S.V. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.*, **15**, 356-362.

Stenmark, H., Aasland, R., Toh, B.H. and D'Arrigo, A. (1996) Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. *J. Biol. Chem.*, **271**, 24048-24054.

Stephens, L., Jackson, T.R. and Hawkins, P.T. (1993) Activation of phosphatidylinositol 4,5-bisphosphate supply by agonists and non-hydrolysable GTP analogues. *Biochem. J.*, **296**, 481-488.

Stephens, L.R., Eguinoa, A., Erdjument Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A.S., Thelen, M., Cadwallader, K., Tempst, P. and Hawkins, P.T. (1997) The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell*, **89**, 105-114.

Stephens, L.R., Hughes, K.T. and Irvine, R.F. (1991) Pathway of phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature*, **351**, 33-39.

Stevenson, J.M., Perera, I.Y. and Boss, W.F. (1998) A phosphatidylinositol 4-kinase pleckstrin homology domain that binds phosphatidylinositol 4-monophosphate. *J. Biol. Chem.*, **273**, 22761-22767.

Stoffler, H.E., Ruppert, C., Reinhard, J. and Bahler, M. (1995) A novel mammalian myosin I from rat with an SH3 domain localizes to Con A-inducible, F-actin-rich structures at cell-cell contacts. *J. Cell. Biol.*, **129**, 819-830.

Stokoe, D., Stephens, L.R., Copeland, T., Gaffney, P.R., Reese, C.B., Painter, G.F., Holmes, A.B., McCormick, F. and Hawkins, P.T. (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science*, **277**, 567-570.

Stossel, T.P. (1993) On the crawling of animal cells. *Science*, **260**, 1086-1094.

Stoyanov, B., Volinia, S., Hanck, T., Rubio, I., Loubtchenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhand, R., Nurnberg, B. et, a.l. (1995) Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science*, **269**, 690-693.

Stoyanova, S., Bulgarelli Leva, G., Kirsch, C., Hanck, T., Klinger, R., Wetzker, R. and Wymann, M.P. (1997) Lipid kinase and protein kinase activities of G-protein-coupled phosphoinositide 3-kinase gamma: structure-activity analysis and interactions with wortmannin. *Biochem. J.*, **324**, 489-495.

Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature*, **306**, 67-69.

Sudhof, T.C. (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature*, **375**, 645-653.

Sultan, C., Plantavid, M., Bachelot, C., Grondin, P., Breton, M., Mauco, G., Levy Toledano, S., Caen, J.P. and Chap, H. (1991) Involvement of platelet glycoprotein IIb-IIIa (alpha IIb-beta 3 integrin) in thrombin-induced synthesis of phosphatidylinositol 3',4'-bisphosphate. *J. Biol. Chem.*, **266**, 23554-23557.

Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R. and Yamada, K.M. (1998) Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science*, **280**, 1614-1617.

Tang, W., Bunting, M., Zimmerman, G.A., McIntyre, T.M. and Prescott, S.M. (1996) Molecular cloning of a novel human diacylglycerol kinase highly selective for arachidonate-containing substrates. *J. Biol. Chem.*, **271**, 10237-10241.

Tapon, N. and Hall, A. (1997) Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. *Curr. Opin. Cell Biol.*, **9**, 86-92.

Teitelbaum, I., Strasheim, A. and Berl, T. (1990) Epidermal growth factor-stimulated phosphoinositide hydrolysis in cultured rat inner medullary collecting tubule cells. Regulation by G protein, calcium, and protein kinase C. *J. Clin. Invest.*, **85**, 1044-1050.

Terui, T., Kahn, R.A. and Randazzo, P.A. (1994) Effects of acid phospholipids on nucleotide exchange properties of ADP-ribosylation factor 1. Evidence for specific interaction with phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.*, **269**, 28130-28135.

Thom, D., Powell, A.J., Lloyd, C.W. and Rees, D.A. (1977) Rapid isolation of plasma membranes in high yield from cultured fibroblasts. *Biochem. J.*, **168**, 187-194.

Thomas, G. and Hall, M.N. (1997) TOR signalling and control of cell growth. *Curr. Opin. Cell Biol.*, **9**, 782-787.

Thomas, G.M., Cunningham, E., Fensome, A., Ball, A., Totty, N.F., Truong, O., Hsuan, J.J. and Cockcroft, S. (1993) An essential role for phosphatidylinositol transfer protein in phospholipase C-mediated inositol lipid signaling. *Cell*, **74**, 919-928.

Thompson, D.M., Cochet, C., Chambaz, E.M. and Gill, G.N. (1985) Separation and characterization of a phosphatidylinositol kinase activity that co-purifies with the epidermal growth factor receptor. *J. Biol. Chem.*, **260**, 8824-8830.

Thorner, J. (1996) Genetic and biochemical analysis of 4-phoshoinositide synthesis and turnover in the yeast *Saccharomyces cerevisiae*. (meeting abstract). The Role of Phosphoinositides in Cell Signalling. University of Wisconsin, Madison WI. June 20-23 1996.

Toker, A. and Cantley, L.C. (1997) Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature*, **387**, 673-676.

Toker, A., Bachelot, C., Chen, C.S., Falck, J.R., Hartwig, J.H., Cantley, L.C. and Kovacsovics, T.J. (1995) Phosphorylation of the platelet p47 phosphoprotein is mediated by the lipid products of phosphoinositide 3-kinase. *J. Biol. Chem.*, **270**, 29525-29531.

Toker, A., Meyer, M., Reddy, K.K., Falck, J.R., Aneja, R., Aneja, S., Parra, A., Burns, D.J., Ballas, L.M. and Cantley, L.C. (1994) Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. *J. Biol. Chem.*, **269**, 32358-32367.

Tolias, K.F., Cantley, L.C. and Carpenter, C.L. (1995) Rho family GTPases bind to phosphoinositide kinases. *J. Biol. Chem.*, **270**, 17656-17659.

Tolias, K.F., Couvillon, A.D., Cantley, L.C. and Carpenter, C.L. (1998) Characterization of a Rac1- and RhoGDI-associated lipid kinase signaling complex. *Mol Cell Biol*, **18**, 762-770.

Totty, N.F., Waterfield, M.D. and Hsuan, J.J. (1992) Accelerated high-sensitivity microsequencing of proteins and peptides using a miniature reaction cartridge. *Protein Sci*, **1**, 1215-1224.

Touhara, K., Inglese, J., Pitcher, J.A., Shaw, G. and Lefkowitz, R.J. (1994) Binding of G protein beta gamma-subunits to pleckstrin homology domains. *J. Biol. Chem.*, **269**, 10217-10220.

Tran, D., Gascard, P., Berthon, B., Fukami, K., Takenawa, T., Giraud, F. and Claret, M. (1993) Cellular distribution of polyphosphoinositides in rat hepatocytes. *Cell signal.*, **5**, 565-581.

Traynor Kaplan, A.E., Harris, A.L., Thompson, B.L., Taylor, P. and Sklar, L.A. (1988) An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. *Nature*, **334**, 353-356.

Traynor Kaplan, A.E., Thompson, B.L., Harris, A.L., Taylor, P., Omann, G.M. and Sklar, L.A. (1989) Transient increase in phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol trisphosphate during activation of human neutrophils. *J. Biol. Chem.*, **264**, 15668-15673.

Tsunoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M. and Zuker, C.S. (1997) A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. *Nature*, **388**, 243-249.

Ui, M., Okada, T., Hazeki, K. and Hazeki, O. (1995) Wortmannin as a unique probe for an intracellular signalling protein, phosphinositide 3-kinase. *Trends Biochem. Sci.*, **20**, 303-307.

Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell*, **61**, 203-212.

Urumow, T. and Wieland, O.H. (1988) Evidence for a cholera-toxin-sensitive G-protein involved in the regulation of phosphatidylinositol 4-phosphate kinase of rat liver membranes. *Biochim. Biophys. Acta.*, **972**, 232-238.

Urumow, T. and Wieland, O.H. (1990) Purification and partial characterization of phosphatidylinositol-4-phosphate kinase from rat liver plasma membranes. Further evidence for a stimulatory G-protein. *Biochim. Biophys. Acta.*, **1052**, 152-158.

Valius, M. and Kazlauskas, A. (1993) Phospholipase C-gamma 1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. *Cell*, **73**, 321-334.

Van Aelst, L. and D'Souza Schorey, C. (1997) Rho GTPases and signaling networks. *Genes Dev*, **11**, 2295-2322.

Van Dongen, C.J., Zwiers, H. and Gispen, W.H. (1984) Purification and partial characterization of the phosphatidylinositol 4-phosphate kinase from rat brain. *Biochem. J.*, **223**, 197-203.

van Paridon, P.A., Gadella, T.W., Jr., Somerharju, P.J. and Wirtz, K.W. (1988) Properties of the binding sites for the sn-1 and sn-2 acyl chains on the phosphatidylinositol transfer protein from bovine brain. *Biochemistry*, **27**, 6208-6214.

Vanhaesebroeck, B., Leevers, S.J., Panayotou, G. and Waterfield, M.D. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem. Sci.*, **22**, 267-272.

Vanhaesebroeck, B., Welham, M.J., Kotani, K., Stein, R., Warne, P.H., Zvelebil, M.J., Higashi, K., Volinia, S., Downward, J. and Waterfield, M.D. (1997) P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc. Natl. Acad. Sci. USA.*, **94**, 4330-4335.

Varnai, P. and Balla, T. (1998) Visualisation of phosphoinositides that bind plectrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo[^{3}H]inositol-labeled phosphoinositide pools. *J. Cell. Biol.*, **143**, 501-510.

Venkateswarlu, K., Oatey, P.B., Tavare, J.M. and Cullen, P.J. (1998) Insulin-dependent translocation of ARNO to the plasma membrane of adipocytes requires phosphatidylinositol 3-kinase. *Curr. Biol.*, **8**, 463-466.

Vihtelic, T.S., Goebl, M., Milligan, S., O'Tousa, J.E. and Hyde, D.R. (1993) Localization of Drosophila retinal degeneration B, a membrane-associated phosphatidylinositol transfer protein. *J. Cell. Biol.*, **122**, 1013-1022.

Vihtelic, T.S., Hyde, D.R. and O'Tousa, J.E. (1991) Isolation and characterization of the Drosophila *retinal degeneration B (rdgB)* gene. *Genetics*, **127**, 761-768.

Virbasius, J.V., Guilherme, A. and Czech, M.P. (1996) Mouse p170 is a novel phosphatidylinositol 3-kinase containing a C2 domain. *J. Biol. Chem.*, **271**, 13304-13307.

Volinia, S., Dhand, R., Vanhaesebroeck, B., MacDougall, L.K., Stein, R., Zvelebil, M.J., Domin, J., Panaretou, C. and Waterfield, M.D. (1995) A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system. *EMBO J.*, **14**, 3339-3348.

Wahl, M.I., Jones, G.A., Nishibe, S., Rhee, S.G. and Carpenter, G. (1992) Growth factor stimulation of phospholipase C-gamma 1 activity. Comparative properties of control and activated enzymes. *J. Biol. Chem.*, **267**, 10447-10456.

Walker, D.H., Dougherty, N. and Pike, L.J. (1988) Purification and characterization of a phosphatidylinositol kinase from A431 cells. *Biochemistry*, **27**, 6504-6511.

Walsh, J.P., Caldwell, K.K. and Majerus, P.W. (1991) Formation of phosphatidylinositol 3-phosphate by isomerization from phosphatidylinositol 4-phosphate. *Proc. Natl. Acad. Sci. USA.*, **88**, 9184-9187.

Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakizuka, A. and Narumiya, S. (1996) Protein kinase N (PKN) and PKN-related protein rhophilin as targets of small GTPase Rho. *Science*, **271**, 645-648.

Waterfield, M.D., Mayes, E.L., Stroobant, P., Bennet, P.L., Young, S., Goodfellow, P.N., Banting, G.S. and Ozanne, B. (1982) A monoclonal antibody to the human epidermal growth factor receptor. *J. Cell Biochem.*, **20**, 149-161.

Waugh, M.G., Lawson, D., Tan, S.K. and Hsuan, J.J. (1998) Phosphatidylinositol 4-phosphate synthesis in immunoisolated caveolae- like vesicles and low buoyant density non-caveolar membranes. *J. Biol. Chem.*, **273**, 17115-17121.

Waugh, M.G., Lawson, D. and Hsuan, J.J. (1999) Epidermal Growth Factor Receptor activation is localised within Low Buoyant Density, Non-Caveolar, Membrane Domains. *Biochem. J.*, in press.

Weiss, A. and Littman, D.R. (1994) Signal transduction by lymphocyte antigen receptors. *Cell*, **76**, 263-274.

Welters, P., Takegawa, K., Emr, S.D. and Chrispeels, M.J. (1994) AtVPS34, a phosphatidylinositol 3-kinase of Arabidopsis thaliana, is an essential protein with homology to a calcium-dependent lipid binding domain. *Proc. Natl. Acad. Sci. USA.*, **91**, 11398-11402.

Westerman, J., de, V.K., Somerharju, P., Timmermans, H.J., Snoek, G.T. and Wirtz, K.W. (1995) A sphingomyelin-transferring protein from chicken liver. Use of pyrene-labeled phospholipid. *J. Biol. Chem.*, **270**, 14263-14266.

Wetzker, R., Klinger, R., Hsuan, J., Fry, M.J., Kauffmann Zeh, A., Muller, E., Frunder, H. and Waterfield, M. (1991) Purification and characterization of phosphatidylinositol 4-kinase from human erythrocyte membranes. *Eur-J-Biochem*, **200**, 179-185.

Whiteford, C.C., Brearley, C.A. and Ulug, E.T. (1997) Phosphatidylinositol 3,5-bisphosphate defines a novel PI 3-kinase pathway in resting mouse fibroblasts. *Biochem. J.*, **323**, 597-601.

Whitman, M., Kaplan, D., Roberts, T. and Cantley, L. (1987) Evidence for two distinct phosphatidylinositol kinases in fibroblasts. Implications for cellular regulation. *Biochem. J.*, **247**, 165-174.

Wiedemann, C., Schäfer, T. and Burger, M.M. (1996) Chromaffin granule-associated phosphatidylinositol 4-kinase activity is required for stimulated secretion. *EMBO J.*, **15**, 2094-2101.

Wirtz, K.W. (1991) Phospholipid transfer proteins. *Annu. Rev. Biochem.*, **60**, 73-99.

Wirtz, K.W. (1997) Phospholipid transfer proteins revisited. *Biochem. J.*, **324**, 353-360.

Wong, K. and Cantley, L.C. (1994) Cloning and characterization of a human phosphatidylinositol 4-kinase. *J. Biol. Chem.*, **269**, 28878-28884.

Wong, K., Meyers, R. and Cantley, L.C. (1997) Subcellular localisation of phosphatidylinositol 4-kinase isoforms. *J. Biol. Chem.*, **272**, 13236-13241.

Woodard, C., Alcorta, E. and Carlson, J. (1992) The rdgB gene of Drosophila: a link between vision and olfaction. *J. Neurogenet.*, **8**, 17-31.

Woscholski, R., Finan, P.M., Radley, E., Totty, N.F., Sterling, A.E., Hsuan, J.J., Waterfield, M.D. and Parker, P.J. (1997) Synaptojanin is the major constitutively active

phosphatidylinositol- 3,4,5-trisphosphate 5-phosphatase in rodent brain. *J. Biol. Chem.*, **272**, 9625-9628.

Woscholski, R., Kodaki, T., McKinnon, M., Waterfield, M.D. and Parker, P.J. (1994) A comparison of demethoxyviridin and wortmannin as inhibitors of phosphatidylinositol 3-kinase. *FEBS-Lett.*, **342**, 109-114.

Wymann, M.P., Bulgarelli Leva, G., Zvelebil, M.J., Pirola, L., Vanhaesebroeck, B., Waterfield, M.D. and Panayotou, G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol Cell Biol*, **16**, 1722-1733.

Xu, W., Harrison, S.C. and Eck, M.J. (1997) Three-dimensional structure of the tyrosine kinase c-Src. *Nature*, **385**, 595-602.

Yagisawa, H., Hirata, M., Kanematsu, T., Watanabe, Y., Ozaki, S., Sakuma, K., Tanaka, H., Yabuta, N., Kamata, H., Hirata, H. and et al. (1994) Expression and characterization of an inositol 1,4,5-trisphosphate binding domain of phosphatidylinositol-specific phospholipase C-delta 1. *J. Biol. Chem.*, **269**, 20179-20188.

Yamakawa, A. and Takenawa, T. (1988) Purification and characterization of membrane-bound phosphatidylinositol kinase from rat brain. *J. Biol. Chem.*, **263**, 17555-17560.

Yamakawa, A., Nishizawa, M., Fujiwara, K.T., Kawai, S., Kawasaki, H., Suzuki, K. and Takenawa, T. (1991) Molecular cloning and sequencing of cDNA encoding the phosphatidylinositol kinase from rat brain [retracted by Yamakawa A, Nishizawa M, Fujiwara KT, Kawai S, Kawasaki H, Suzuki K, Takenawa T. In: *J. Biol. Chem.* 1992 Dec 15;267(35):25620]. *J. Biol. Chem.*, **266**, 17580-17583.

Yamakawa, A., Nishizawa, M., Fujiwara, K.T., Kawai, S., Kawasaki, H., Suzuki, K. and Takenawa, T. (1992) Molecular cloning and sequencing of cDNA encoding the phosphatidylinositol kinase from rat brain [retraction of Yamakawa A, Nishizawa M, Fujiwara KT, Kawai S, Kawasaki H, Suzuki K, Takenawa T. In: *J. Biol. Chem.* 1991 Sep 15;266(26):17580-3]. *J. Biol. Chem.*, **267**, 25620.

Yamamoto, A., DeWald, D.B., Boronenkov, I.V., Anderson, R.A., Emr, S.D. and Koshland, D. (1995) Novel PI(4)P 5-kinase homologue, Fab1p, essential for normal vacuole function and morphology in yeast. *Mol Biol Cell*, **6**, 525-539.

Yamamoto, K., Graziani, A., Carpenter, C., Cantley, L.C. and Lapetina, E.G. (1990) A novel pathway for the formation of phosphatidylinositol 3,4-bisphosphate.

Phosphorylation of phosphatidylinositol 3-monophosphate by phosphatidylinositol-3-monophosphate 4-kinase. *J. Biol. Chem.*, **265**, 22086-22089.

Yang, L., Camoratto, A.M., Baffy, G., Raj, S., Manning, D.R. and Williamson, J.R. (1993) Epidermal growth factor-mediated signaling of G(i)-protein to activation of phospholipases in rat-cultured hepatocytes. *J. Biol. Chem.*, **268**, 3739-3746.

Yang, L.J., Rhee, S.G. and Williamson, J.R. (1994) Epidermal growth factor-induced activation and translocation of phospholipase C-gamma 1 to the cytoskeleton in rat hepatocytes. *J. Biol. Chem.*, **269**, 7156-7162.

Yauch, R.L., Berditchevski, F., Harler, M.B., Reicher, J. and Hemler, M.E. (1998) Highly Stoichiometric, Stable and Specific Association of Integrin alpha-3 beta-1 with CD151 Provides a Major Link to Phosphatidylinositol 4-kinase and may Regulate Cell Migration. *Mol Cell Biol*, 2751-2765.

Yokoo, T., Matsui, Y., Yagisawa, H., Nojima, H., Uno, I. and Tohe, A. (1993) The putative phosphoinositide-specific phospholipase C gene, PLC1, of the yeast Saccharomyces cerevisiae is important for cell growth. *Proc. Natl. Acad. Sci. USA.*, **90**, 1804-1808.

Yonezawa, N., Homma, Y., Yahara, I., Sakai, H. and Nishida, E. (1991) A short sequence responsible for both phosphoinositide binding and actin binding activities of cofilin. *J. Biol. Chem.*, **266**, 17218-17221.

Yoshida, S., Ikeda, E., Uno, I. and Mitsuzawa, H. (1992) Characterization of a staurosporine- and temperature-sensitive mutant, stt1, of Saccharomyces cerevisiae: STT1 is allelic to PKC1. *Mol. Gen. Genet.*, **231**, 337-344.

Yoshida, S., Ohya, Y., Goebl, M., Nakano, A. and Anraku, Y. (1994) A novel gene, STT4, encodes a phosphatidylinositol 4-kinase in the PKC1 protein kinase pathway of Saccharomyces cerevisiae. *J. Biol. Chem.*, **269**, 1166-1172.

Yoshida, S., Ohya, Y., Nakano, A. and Anraku, Y. (1994) Genetic interactions among genes involved in the STT4-PKC1 pathway of Saccharomyces cerevisiae. *Mol. Gen. Genet.*, **242**, 631-640.

Yu, F.X., Johnston, P.A., Sudhof, T.C. and Yin, H.L. (1990) gCap39, a calcium ion- and polyphosphoinositide-regulated actin capping protein. *Science*, **250**, 1413-1415.

Yu, H., Chen, J.K., Feng, S., Dalgarno, D.C., Brauer, A.W. and Schreiber, S.L. (1994) Structural basis for the binding of proline-rich peptides to SH3 domains. *Cell*, **76**, 933-945.

Zhang, X., Hartz, P.A., Philip, E., Racusen, L.C. and Majerus, P.W. (1998) Cell lines from kidney proximal tubules of a patient with Lowe syndrome lack OCRL inositol polyphosphate 5-phosphatase and accumulate phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.*, **273**, 1574-1582.

Zhang, X., Loijens, J.C., Boronenkov, I.V., Parker, G.J., Norris, F.A., Chen, J., Thum, O., Prestwich, G.D., Majerus, P.W. and Anderson, R.A. (1997) Phosphatidylinositol-4-phosphate 5-kinase isozymes catalyze the synthesis of 3-phosphate-containing phosphatidylinositol signaling molecules. *J. Biol. Chem.*, **272**, 17756-17761.

Zheng, J., Knighton, D.R., ten Eyck, L.F., Karlsson, R., Xuong, N., Taylor, S.S. and Sowadski, J.M. (1993) Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. *Biochemistry*, **32**, 2154-2161.

Zheng, Y., Bagrodia, S. and Cerione, R.A. (1994) Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. *J. Biol. Chem.*, **269**, 18727-18730.

Zhou, K., Takegawa, K., Emr, S.D. and Firtel, R.A. (1995a) A phosphatidylinositol (PI) kinase gene family in Dictyostelium discoideum: biological roles of putative mammalian p110 and yeast Vps34p PI 3-kinase homologs during growth and development. *Mol Cell Biol*, **15**, 5645-5656.

Zhou, S., Margolis, B., Chaudhuri, M., Shoelson, S.E. and Cantley, L.C. (1995b) The phosphotyrosine interaction domain of SHC recognizes tyrosine-phosphorylated NPXY motif. *J. Biol. Chem.*, **270**, 14863-14866.

Zoller, M.J. and Taylor, S.S. (1979) Affinity labeling of the nucleotide binding site of the catalytic subunit of cAMP-dependent protein kinase using p-fluorosulfonyl- [^{14}C]benzoyl 5'-adenosine. Identification of a modified lysine residue. *J. Biol. Chem.*, **254**, 8363-8638.

Zoller, M.J., Nelson, N.C. and Taylor, S.S. (1981) Affinity labeling of cAMP-dependent protein kinase with p-fluorosulfonylbenzoyl adenosine. Covalent modification of lysine 71. *J. Biol. Chem.*, **256**, 10837-10842.

Zugaza, J.L., Waldron, R.T., Sinnett Smith, J. and Rozengurt, E. (1997) Bombesin, vasopressin, endothelin, bradykinin, and platelet-derived growth factor rapidly activate

protein kinase D through a protein kinase C-dependent signal transduction pathway. *J. Biol. Chem.*, **272**, 23952-23960.

Zvelebil, M.J., MacDougall, L., Leevers, S., Volinia, S., Vanhaesebroeck, B., Gout, I., Panayotou, G., Domin, J., Stein, R., Pages, F. and et al. (1996) Structural and functional diversity of phosphoinositide 3-kinases. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.,* , **351**, 217-223.

Acknowlegements

This thesis is the result of help, stimulation and encouragement from many people - too many to mention them all. In particular members of the Ludwig Institute between 1994 and 1998: Andrea Kaufmann-Zeh, Stefano Volinia, Ivan Gout, George Panayotou, Marketa Zvelebil, Bart Vanhaesbroek, Phil Gates, Mike Howell, Caroline Hill, Elly Tanaka, Jan Domin, Francoise Pages, Sally Leevers, Parmjit Jat, Oahn Truong, Alistair Stirling, Nick Totty, Damian Holliman, Meridith Layton, Kay Mistry, Anne Ridley, Ritu Garg, and Geoff Scrace.

I would also like to formally acknowlege the assistance of Jeremy Thorner and Bo Qing Wang (University of Berkeley, CA) who collaborated on the PtdIns 4Kβ yeast work and who supplied Figure 6.9 along with ideas and unpublished results. Maria dos Santos performed many microinjections and provided advice on immunofluorescence. Rob Stein performed the HPLC analysis of PtdIns*P*K IIα reaction products (Figure 3.5).

I thank the members of the Protein Biochemistry group; Siow Khoon Tan, Yvonne Fulwood, Mark Waugh and Steve Hayes, for your support and good humour during the last few years. Maria do Ceu Gomes dos Santos has been endlessly reliable and helpful, thank you!

A special acknkowlegement is reserved for Justin Hsuan who, with his knowledge and enthusiasm for science, has taught me more than I thought was possible.