

Functional differences of class 1a PI 3'-kinase heterodimers

A dissertation submitted to the University of London in candidature
for the degree of Doctor of Philosophy

by

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ABSTRACT

This thesis describes a study of the class 1a phosphoinositide (PI) 3-kinase enzymes. PI 3-kinases generate lipids which have been implicated in receptor-stimulated signalling in mammalian cells and in a signalling complex which mediates protein trafficking in yeast.

Whilst phosphoinositide 3-phosphate (PtdIns(3)P) is constitutively present in eukaryotic cells, PtdIns (3,4)P₂ and PtdIns(3,4,5)P₃ are almost absent from resting cells, but rise sharply following cell stimulation. These lipids have been implicated in a diverse range of cellular processes, ranging from mitogenesis and oncogenic transformation to insulin triggered changes in glucose transport.

Given the central role of PI 3-kinases in such a variety of events, a study was undertaken to investigate regulation of this enzyme. Class 1a PI 3-kinases are 110-120 kDa proteins that associate with adapter subunits containing *Src* homology-2 (SH2) domains, thereby linking the lipid kinase activity of PI 3-kinase to tyrosine kinase signalling pathways. Various adapter gene products and splice variants have been identified in recent years, and there are at least three genes encoding the catalytic subunit of the class 1a enzyme, but suprisingly little evidence has accumulated for different cellular roles of these isoforms.

The studies described here define clear differences in the intrinsic activity of two of the catalytic isoforms of PI 3-kinase, p110 α and p110 β , and show that other cellular proteins can influence catalytic activity by binding specific domains of the adapter subunit. It is widely assumed that all signalling functions of PI 3-kinases are mediated by their lipid kinase activity. However, they also have a limited protein kinase activity, and the role of this is discussed. In addition, evidence is presented that the catalytic subunit influences enzyme recruitment to signalling complexes following cell stimulation.

Statement

This thesis is an account of research conducted at the Department of Biochemistry and Molecular Biology at University College, London, between September 1995 and May 1999. Except where references are given, this thesis contains my own original work, does not exceed the word limit stipulated by the University and is not substantially the same as any I have submitted for any other degree, diploma or examination.

Some of the work presented in this thesis has been published elsewhere:

Beeton, C.A., Das, P., Waterfield, M.D., Shepherd, P.R., 1999 **The SH3 and BH domains of the p85 α adapter subunit play a critical role in regulating Class 1a phosphoinositide 3-kinase function**, Molecular Cell Biology Research Communications, 1 pp 153-157

Beeton, C.A., Chance, E.M., Shepherd, P.R., 1999 **Differences in the kinetic properties of p110 α and p110 β catalytic subunits class 1a PI 3-kinases suggest different cellular functions**, Manuscript in preparation

Beeton, C.A., Brown, R.A., Shepherd, P.R., 1999 **Role of the p85 α serine 608 phosphorylation in regulating activity of the p110 α and p110 β catalytic subunits of class 1a PI 3-kinases: Use as a novel assay for PI 3-kinase activity**, Manuscript in preparation

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The work presented in this thesis is the result of three and one half years spent in the Department of Biochemistry and Molecular Biology, University College, London. It could not have been carried out without the help and support of many people.

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Abbreviations

ATP	adenosine triphosphate
BH	RacGAP/ breakpoint cluster homology
CHO	chinese hamster ovary cells
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediamine-tetra-acetic acid
ERK	extracellular-signal regulated kinase
FGF	fibroblast growth factor
GAP	GTPase activating protein
GSK-3	glycogen synthase kinase-3
HPLC	high pressure liquid chromatography
IGF	insulin-like growth factor
IRS	insulin receptor substrate
MAPK	mitogen-activated protein kinase
MEK	MAP kinase kinase/ ERK kinase
NIDDM	non-insulin dependent diabetes mellitus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PI	phosphoinositide
PI (3,4)P ₂	phosphatidylinositol 3,4-bisphosphate
PI (3,4,5)P ₃	phosphatidylinositol 3,4,5-trisphosphate
PI3P	phosphatidylinositol 3-phosphate
PI4P	phosphatidylinositol 4-phosphate
PKB	protein kinase B
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate

PMSF	phenyl-methyl-sulphonyl-fluoride
SDS	sodiumdodecyl sulfate
SH2	Src homology 2
SH3	Src homology 3
SHP-2	SHPTP2, SHPTP1D, Syp, SH2-domain containing phosphatase-2
TLC	thin layer chromatography

Introduction

1.1 Initiation of signal transduction cascades by receptor tyrosine kinases

The binding of growth factors and hormones to receptor tyrosine kinases (RTKs) initiates a complex series of signalling cascades which result in cellular responses such as proliferation, differentiation, cell motility and gene transcription. Members of the RTK family include PDGF, FGF, EGF, IGF-1, insulin, NGF, and macrophage colony stimulating factor (M-CSF) receptors. With the exception of the insulin and IGF receptors which are heterotetrameric, most RTKs are monomeric polypeptides in the absence of ligand and contain a single transmembrane domain. Monomeric RTK's dimerize upon ligand binding. All RTKs have an extracellular domain for interaction with a peptide ligand, and a highly conserved intracellular tyrosine kinase domain which catalyses the transfer of phosphate from ATP to tyrosine residues on the receptor or other protein substrate molecules. Ligand binding of RTKs induces activation of the tyrosine kinase. Sites on the receptor are targets for this kinase. Some of these are involved in receptor activation whilst others are involved in creating specific phosphotyrosine binding sites onto which signalling complexes are recruited.

There are two major categories of RTK targets. The first are enzyme families whose activity is directly modulated by phosphorylation or translocation to the plasma membrane including phospholipase C, protein tyrosine phosphatases such as SHPTP2, and rasGAPs (Ras GTPase activating proteins). The second major targets are adapter molecules, which have no catalytic activity but serve to facilitate the formation of multimeric signalling complexes, and these include Grb2, Shc, and the insulin receptor substrate proteins (IRSs). Class 1a PI 3-kinases fall into both of these categories, being recruited either directly to the

receptors of EGF and PDGF, or to the IRS proteins following insulin binding of its receptor. Ligand-induced autophosphorylation of the PDGF receptor results in recruitment of PI 3-kinase activity to the plasma membrane by direct binding of the p85 SH2 domains to tyrosine phosphorylated YMXM motifs on the activated receptor. The insulin receptor is heterotetrameric and insulin binding results in autophosphorylation of the juxtamembrane domains, leading to a rapid increase in tyrosine kinase activity of the β -subunit (Sun et al 1991). Autophosphorylation of the β -subunit of the insulin receptor provides binding sites for the IRS proteins, and signalling molecules including PI 3'-kinases are recruited onto these 'adapter' molecules. PI 3'-kinases are members of the PI kinase family, enzymes which catalyse the phosphorylation of phosphatidylinositol and its derivatives, to generate signalling molecules collectively referred to as phosphoinositides.

1.2 Phosphoinositides

Though phosphatidylinositol represents only a small percentage of total cellular phospholipids, it is unique among membrane lipids in that its head group can be phosphorylated at multiple free hydroxyls. The phosphoinositides comprise several phosphorylated derivatives of phosphatidylinositol, and their levels can be acutely regulated in subcellular compartments by growth factors and hormones, which stimulate the action of kinases, phosphatases and lipase's.

In recent years, a large effort has focused on understanding the function of the D-3 phosphoinositides, particularly PI (3,4)P₂ and PI (3,4,5)P₃, because these were shown to undergo significant transient increases following cell stimulation (Stephens et al 1993). These lipids are products of the class 1a PI 3-kinases, and although this family of enzymes are regulated by multiple growth factors and hormones, in this thesis they have been considered mainly in the context of insulin signalling. One of the major effects of insulin is to stimulate the uptake of glucose from the blood into target tissues, mainly muscle, fat, and liver. The first evidence

that PI 3'-kinase was crucial for insulin signalling pathways came six years ago in studies which showed that insulin stimulated glucose uptake was blocked by chemical inhibitors of PI 3'-kinase, (Clarke et al 1994; Okada et al 1994a), implicating the D3 phosphoinositides. Since then, these findings have been confirmed by other groups using dominant negative p85 constructs (Hara et al 1994; Kotani et al 1995; Sharma et al 1998). In addition, a stimulatory effect with activated p110 constructs has also been observed (Martin et al 1996a). PI 3'-kinase has since been shown to be necessary for a wide range of insulin stimulated responses in the cell including mitogenesis, apoptosis, membrane ruffling and oncogenesis (Shepherd et al 1998).

1.2.1 The phosphoinositide kinase family

Each of the PI kinases is specific for either the D-3, D-4 and D-5 positions of the inositol ring of phosphatidylinositol and their concerted action produces a range of phosphoinositides (see figure 1.1).

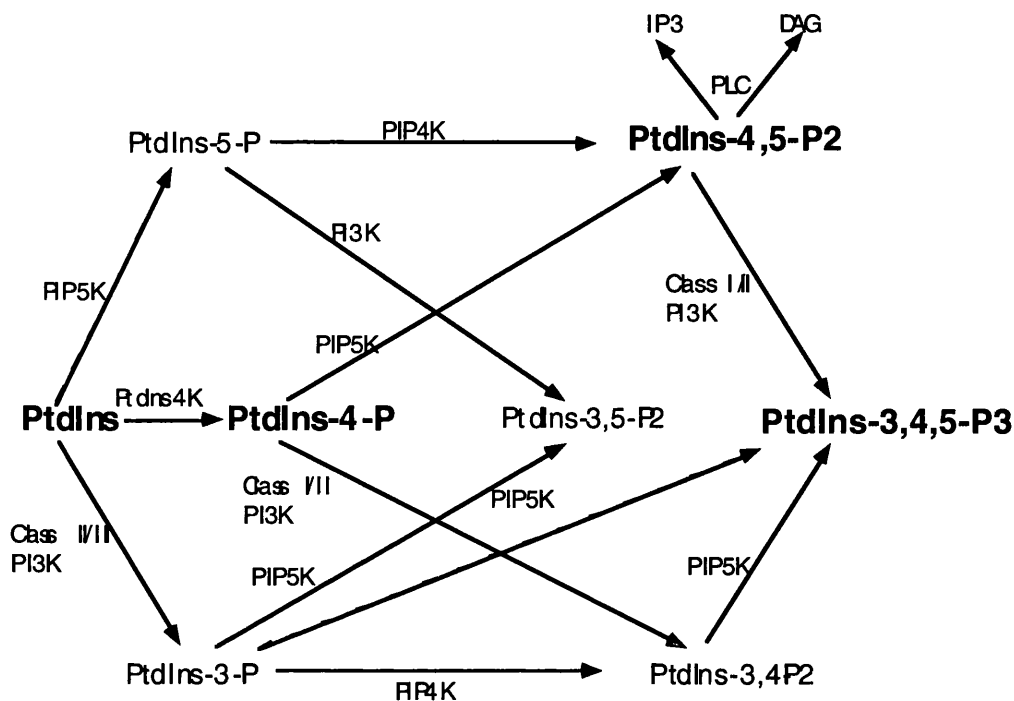


Figure 1.1 The generation of 3-phosphorylated phosphoinositides

Reactions where the enzyme is known are shown with a solid line, predicted reactions with undetermined enzymes are depicted with a dashed line. Some of the phosphatases involved in 3-phosphorylated phosphoinositide metabolism are also known. PI (3,4,5)P3 can be metabolised to PI (4,5)P2 by the action of PTEN. PI (3,4,5)P3 is also metabolised to PI (3,4)P2 by types I and II 5'-phosphatase, SHIP and synaptojanin. PI (3,4)P2 is a substrate for both 3'-phosphatases and 4'-phosphatases. PI 3P is metabolised to PI by type I and II 3'-phosphatases. Abbreviations used are: PtdIns, phosphatidylinositol; Ins, inositol; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; K, kinase.

The catalytic domains of PI 3-kinases (discussed in section 1.2.2) share significant homology with PI 4-kinases, and even with enzymes which function only as protein kinases. Amongst the enzymes with substantial homology to PI 3-kinases are VPS34, FRAP/ RAFT-1, and DNA-PK. DNA-dependent protein kinase lacks a conventional protein kinase domain, but has been shown to have a domain with significant homology to PI 3-kinases (Hartley et al 1995b); interestingly, this enzyme is inhibited by wortmannin. Though DNA PK can phosphorylate several proteins, its physiological targets are unknown. Both TOR1 and TOR2 (Target Of Rapamycin) have significant identity with the kinase domains of p110 α , VPS34 and yeast and human PI 4-kinases, and although there were early reports of a lipid

kinase activity associated with these molecules (Cardenas & Heitman 1995), more recent work suggests this is unlikely and the TOR proteins are exclusively protein kinases.

Because of the growth factor induction of D-3 phosphoinositides, a large amount of research effort has focused on PI 3'-kinase. PI 3-kinase activity was first identified as an activity which associated with two viral oncoproteins, polyoma middle T (mT) and pp60^{V-src} (Cantley et al 1991). Subsequent work has identified a major role for PI 3-kinase products in growth regulation, prevention of cell death, oncogenesis and longevity. PI (3,4,5)P₃ was first detected in activated neutrophils (Traynor-Kaplan et al 1988). Since that initial discovery, the cloning of the enzyme family responsible for its production, and the discovery of chemical inhibitors of these enzymes, the lipid products of the PI 3-kinases have been implicated in many cellular responses. Interest in these lipids has recently been further enhanced by the discovery of a retrovirus encoded PI 3-kinase which causes sarcomas in chickens (Chang et al 1997), a mutation in the *C. elegans* gene which increases the lifespan threefold (Morris et al 1996), and the elevated levels of D3 phosphorylated phosphoinositides in various human cancers (Myers et al 1998b; Phillips et al 1998). The targets of the phosphoinositides are only now becoming clear however, and these are discussed in detail in section 1.8.1.

Since the cloning of the 110 kDa catalytic subunit of class 1a PI 3-kinase from bovine cDNA (Fry et al 1992; Hiles et al 1992; Kaplan et al 1987) a number of enzymes possessing PtdIns 3-kinase activity have since been identified, all sharing significant homology in the catalytic domain. It has emerged that there is functional heterogeneity within the group of enzymes with established PI 3-kinase activity, and these have been grouped into classes based on *in vitro* lipid substrate specificity, sequence similarity and mechanisms of regulation.

1.2.2 PI3K subfamilies

The class 1 PI 3-kinases are heterodimers of an adapter and catalytic subunit, in which the adapter protein links them to upstream signalling events. This class of enzyme is further subdivided into two subclasses, A and B. Class 1a PI 3-kinases are 110-120 kDa proteins which *in vivo* are tightly dimerized with SH2 domain containing adapter proteins. By binding phosphotyrosine motifs, the adaptor protein links the class 1a PI 3-kinase to tyrosine kinase signalling pathways. To date, there are three mammalian catalytic subunits, and at least six adapter subunits identified, with homologous molecules found in several species. These are discussed in detail in section 1.3.3.

Class 1b PI 3-kinases do not interact with the adapter subunits which dimerize with the class 1a enzymes, and are stimulated by G-protein $\beta\gamma$ subunits. First identified of this class was the mammalian p110 γ , and a regulatory subunit, termed p101, has since been cloned which does not share homology with any known protein. (Stephens et al 1997).

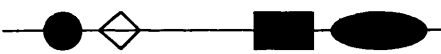

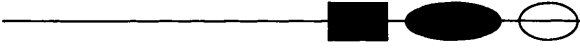

Class 2 PI 3-kinases have an *in vitro* substrate specificity for PtdIns and PtdIns (4)P. These proteins are larger than class 1 proteins, and are defined specifically by a C2 domain at their carboxyl terminus. So far three mammalian isoforms have been identified, PI3K-C2 α , -C2 β and -C2 γ ((Rozycka et al 1998) and references therein) although as these are newly cloned proteins, little is known about their regulation or function. However they have been shown to be activated by growth factors (Brown et al 1999). So far, no adapter proteins have been identified for these enzymes.

The class 3 PI 3-kinases are homologous to the yeast protein Vps34p, and likewise are restricted in substrate specificity to PtdIns. The mammalian homologue is a protein of 150 kDa and is likely to share a housekeeping role in

membrane traffic and post-endocytic sorting of ligand stimulated receptors with its yeast homologue. In yeast, Vps15p, a 170 kDa Ser/ Thr kinase, has been shown to activate and recruit Vps34p to membranes, and a similar, but slightly smaller protein, (150 kDa), has been identified in human and drosophila cells (Linassier et al 1997).

Table 1 summarises the classification of PI3K family members.

Table 1 Classification of phosphoinositide family members

Class	Structural features of catalytic subunit	Subunits: catalytic	Subunits: adapter	In vitro lipid substrates
1a		p110 α,β,δ	p85 α,β p55 α,γ ; p50 α	PI PI4P PI4,5P ₂
1b		p110 δ	p101	
2		PI3K-C2 α,β	?	PI PI4P
3		PI3K (homologue of yeast Vps34p)	?	PI

Structural motifs: adapter binding (circle), Ras binding (diamond), C2 domain (white oval) PIK domain (rectangle), Kinase domain (black oval). PI is Ptd.Ins
Only the mammalian isoforms are shown. For further details see text.

1.2.3 PI 3-Kinase families in insulin signalling

Current evidence indicates that the bulk of the insulin stimulated recruitment of PI 3-kinases into phosphotyrosine based signalling complexes is accounted for by the

class 1a enzymes. Whilst the class 2 PI 3-kinases are widely expressed, and have just been shown to be activated by insulin (Brown et al 1999) they are unlikely to mediate common insulin responses because, at least in vitro, they cannot produce PI (3,4,5)P₃, a key mediator of insulin signalling (PI(4,5)P₂ is not a substrate of this class) (Domin et al 1996). Additionally, whilst many insulin responses are highly sensitive to class 1a PI 3-kinase inhibitors, the class 2 family members are relatively resistant to these compounds.

Again, the class 3 PI 3-kinases are widely expressed, but cannot produce PI (3,4,5)P₃ (PI is the sole substrate), making these enzymes unlikely to play a large role in insulin signalling.

1.3 The class 1a PI 3-kinase adapter subunits

Class 1a PI 3-kinase enzymes are composed of a 110 kDa catalytic subunit (p110) and an associated regulatory subunit. The regulatory proteins are often called p85 proteins, based on the molecular weight of the first two isoforms to be purified and cloned, p85 α and p85 β . These proteins are composed of modular domains, as are the other regulatory subunits since identified.

These adapter subunits were first identified as 85 kDa phosphoproteins which correlated with a PI kinase activity. This activity was shown to be increased in middle-T transformed and PDGF stimulated cells (Kaplan et al 1987). Not until 1991, when two closely related proteins termed p85 α and p85 β were sequenced and cloned from rat brain cDNA libraries was it shown that neither contained sequence motifs common to kinases and could not be demonstrated to possess kinase activity. However, antibodies to the 85 kDa proteins were shown to immunoprecipitate PI 3-kinase from bovine brain preparations, and studies began on determining whether p85's were mediating the interaction between PI 3-kinase activity and activated tyrosine kinase receptors (Otsu et al 1991). Simultaneously,

Carpenter et al (Carpenter et al 1990) showed that PI 3-kinase purified from rat liver was a heterodimer of the previously described 85 kDa protein and a 110 kDa protein.

Sequencing of p85 revealed two proteins, which each contained an SH3 domain and two SH2 domains homologous to domains found in several receptor associated proteins (Escobedo et al 1991; Skolnik et al 1991). These proteins were subsequently shown to be the products of two different genes (Otsu et al 1991). Since then, a total of at least five regulatory subunits of PI 3-kinase have been identified in mammalian cells, two 85 kDa proteins (p85 α and p85 β), two 55 kDa proteins (p55 α (also known as AS53) and p55 γ (also known as p55PIK)) and one 50 kDa (p50 α) protein. These are discussed in section 1.3.2, and are shown schematically in Figure 1.2.

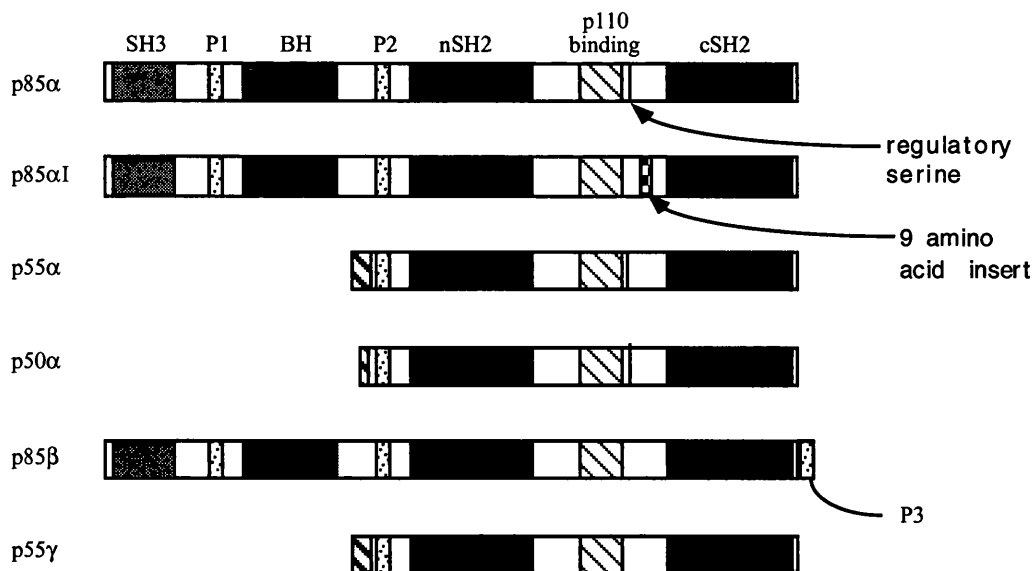


Figure 1.2 Domain structure of class 1a PI 3'-kinase adapter isoforms. Differential splicing of the p85 α gene potentially generates four proteins. Of these, p85 α , p55 α and p50 α are routinely reported in various cell types. p85 α i, in which a sequence of nucleotides containing two serine residues is spliced in place of Asp605, has been partially cloned from human brain. The amino termini of the truncated isoforms comprise a stretch of amino acids, which replace the BH, SH3 and P1 motifs of full length p85 α with a sequence of 33 or 6 amino acids in p55 α and p50 α respectively. A second gene product, p85 β , has the same domain structure as p85 α , except for an additional proline rich motif at the carboxy terminus.. The third gene product, p55 γ , has structural homology with p55 α , and the unique region of p55 α is 60% homologous to the amino terminus of p55 γ . For further details, see text.

1.3.1. Domain structure of the 85 kDa adapter isoforms

p85 α and p85 β , the 85 kDa adapter isoforms of the class 1a PI 3'-kinases, are modular proteins containing an NH₂-terminal SH3 domain, a BH domain, two SH2 domains flanking a site which binds the catalytic subunit and two (p85 α) or three (p85 β) proline rich motifs.

1.3.1.1 The SH2 domains

SH2 domains are noncatalytic modules of about 100 aminoacids, defined by their similarity to a region of Src (Sadowski et al 1986). They are conserved among a whole series of signalling proteins regulated by receptor tyrosine kinases, and most molecules which stably bind RTK's contain SH2 domains (Cohen et al 1995). Different SH2 domains bind to distinct phosphotyrosine containing regions

on the RTK; the SH2 domain of PI 3-kinase, PLC- γ , and GAP do not recognise the same sites on the PDGF receptor (Fantl et al 1992; Kashishian et al 1992). The exact sequence surrounding the phosphotyrosine residue specifies which species of SH2 can bind. Thus, by acting as a ligand for SH2 domains, phosphorylated YXXX motifs on receptors mediate the formation of specific multimeric protein complexes at or near the plasma membrane (for review, see (Koch et al 1991)). Crystal structures of the p85 SH2 domains bound to peptide suggest a conformation in which a 'two-pronged plug' (the YXXX motifs) engage a 'socket' (the SH2 domains) (Waksman et al 1993). Specificity is determined in part by the three residues carboxyl to the phosphotyrosine - for example, a consensus for the SH2 domains of PI 3-kinase is PTyr-(Val/ Met)-X-Met - and in part by the residues around this sequence (Pawson & Schlessinger 1993; Songyang et al 1993). Optimal PI 3'-kinase SH2 binding sites have been identified on a range of molecules including the EGF receptor (Downward et al 1984), the α - and β - PDGF receptors (Kazlauskas & Cooper 1989) the CSF receptor and IRS (Sun et al 1991). Two mechanisms exist by which SH2 domain mediated recruitment may affect the function of the SH2 containing protein. Signalling proteins are relocated in the cell by binding of the SH2 domains to RTK's, as shown by the movement of PI 3-kinase and IRS in response to insulin for example (Inoue et al 1998; Nave et al 1996; Shepherd et al 1997b). It is also probable that binding induces a conformational change which alters the catalytic activity of the interacting proteins.

Potent activation of PI 3-kinase can be achieved by the addition to cytosol of nanomolar concentrations of peptides which mimic the phosphorylated YMXM motifs of the insulin receptor substrate (Herbst et al 1994). The extent of activation achieved with IRS derived peptides containing two YMXM motifs is similar to that caused by cell stimulation with insulin, and 500-1000 fold more effective than peptides containing a mono-YMXM motif. Evidence that full

activation of PI 3-kinase required occupancy of both SH2 domains of p85, as opposed to bivalent binding of a single activating site, came from mono- and di-phosphopeptide binding studies with p85 in which one or both SH2 domains were mutated (Rordorf-Nikolic et al 1995). Briefly, p85 constructs with disabling mutations in either the N-terminal or C-terminal SH2 domain were used to demonstrate a significantly reduced phosphopeptide binding capacity, and a 50% decrease in activation of PI 3-kinase.

Certain SH2 domains, including the carboxy terminal SH2 domain of p85 and the Src SH2 domain, but not those of Lck or Abl, have significant binding affinity for one of the products of PI 3-kinase, PI (3,4,5)P3 (Rameh et al 1995). Lipid binding and phosphopeptide binding are mutually exclusive, as shown by experiments using phenyl phosphate, suggesting a possible negative feedback regulatory mechanism for PI 3-kinase, whereby accumulation of product prevents binding, or causes dissociation from membrane localized phosphoproteins. Another possibility is that PI (3,4,5)P3 recruits a subset of SH2 domain containing proteins to subcellular compartments.

1.3.1.2 The inter-SH2 domain of p85

The SH2 domains of p85 α (and splice variants), p85 β and p55 γ flank a region predicted to adopt a coiled-coil conformation although this has not been formally shown (Panayotou et al 1992). In a definitive study to define PI 3-kinase subunit interactions, GST fusion constructs were used expressing different sections of p85. The inter-SH2 region was shown to be responsible for p110 binding, specifically residues 451-455 (Dhand et al 1994a). In addition, the inter-SH2 domain of p85 α also contains an autoregulatory site, Ser608. This has been shown to be phosphorylated by p110, which causes inhibition of the lipid kinase activity of PI 3-kinase (Dhand et al 1994b). In vitro, the inter-SH2 domain has been shown to bind PI 4P and PI (4,5)P2 (End et al 1993), the major in vivo substrates

of p110, a property which may provide a mechanism for orientating the enzyme at membranes rich in these lipids.

1.3.1.3 The SH3 domain of p85

The SH3 domain is a distinct motif that serves to modulate interactions with the cytoskeleton and membrane. SH3 domains are small, noncatalytic motifs of about 60 amino acids which mediate protein-protein interactions by complexing with proline rich peptide sequences. Despite the fact that at least eight structures for SH3 domains have been reported, some with ligand (Feng et al 1994; Mussacchio et al 1994; Yu et al 1994) and two classes of ligand with high affinity binding have been identified, the biology of SH3 domains is poorly understood. Screening of expression libraries (Cicchetti et al 1992) and mutational analysis have been able to demonstrate some significant and major roles however. The first protein to be identified using a GST SH3 domain fusion construct was 3BP1 which bound to the SH3 domain of Abl via a proline-rich sequence. SH3 domains have since been implicated in suppression of oncogenesis; mutation of the SH3 domain of v-Crk unleashes its transforming potential, and for the tyrosine kinases Abl and Src, deletion of the SH3 domain activates the transforming potential of the proto-oncogene products.

Many proteins which localise to the cytoskeleton have been shown to contain SH3 domains. Microinjection studies (Bar-Sagi et al 1993) were used to demonstrate that PLC- γ is targeted to cytoskeletal microfilaments via its SH3 domain, and both SH3 domains of Grb2 (but not the SH2 domain) are required for its localisation to membrane ruffles. The role of the p85 SH3 domain in cytoskeletal rearrangement is discussed in section 1.11.6.

Grb2 is a small protein that links activated tyrosine kinases (by binding via its SH2 domain) to small G proteins (which it binds with its SH3 domains). In this

way, Grb2 can couple receptor activation to GTP loading of Ras. An SH3 domain of Grb2 has also been shown to bind a proline rich motif of p85 directly (Wang et al 1995). The finding that Rac and Rho proteins control membrane ruffling and formation of stress fibers and focal contacts (Ridley et al 1992) and that these can be regulated by SH3 domain containing proteins, may also link RTK's, via p85, to pathways controlled by GTPases.

1.3.1.4 The BH domain of p85

The domains of p85 α and p85 β which are most divergent in sequence is that with homology to the breakpoint cluster region (BCR) gene; both proteins are approximately 40% homologous to BCR in this region, but have only 57% identity with each other. The BCR protein has homology with rhoGAP, a GTPase activating protein for Rac (Fry 1992). However, the BCR homology (BH) domains of both p85 isoforms lack critical residues for GTP hydrolysis, although p85 α has been shown to bind Rac and Cdc-42. Bokoch and co-workers demonstrated a specific and direct interaction between Rac GTPases and PI 3-kinase which was GTP dependent, but did not determine the location of the Rac binding site (Bokoch et al 1996). In a similar study, Zheng et al demonstrated binding of Cdc-42 to recombinant PI 3-kinase (Zheng et al 1994). Moreover, they show that recombinant Cdc-42 stimulates PI 3-kinase activity in PC12 cell lysates, indicating that although p85 has no GTPase catalytic activity, it may serve as a target of GTPases. Similar results have been reported in vivo. In Rac immunoprecipitates from Swiss 3T3 cells, no PI 3-kinase activity was detected in basal cells, but PDGF induced a significant association as measured by PI 3-kinase assay. A similar stimulation in association was found for Cdc-42 and PI 3-kinase. Both Rac and Cdc-42 association with PI 3-kinase were GTP dependent. In contrast, PI 4P 5-kinase association occurred with either GTP- or GDP-bound Rac and was independent of growth factor stimulation (Tolias et al 1995).

1.3.1.5 The proline rich motifs of p85

The proline-rich domains of the p85 proteins (3 in p85 β , 2 in full length p85 α and 1 in each of the truncated p85 α isoforms) are potential SH3 domain binding motifs. Binding studies using peptides with amino acid composition corresponding to the p85 α proline rich motifs has been shown to bind the SH3 domains of Abl, Fyn and Lck, as well as p85 in vitro (Kapeller et al 1994). These results suggest that in addition to enabling interaction with proteins in multimeric complexes the proline motifs may enable p85 to "self-associate", either by an intra- or inter-molecular mechanism, and this is discussed in more detail in Chaters 5 and 6.

1.3.2 Adapter isoforms cloned

The p85 α gene has been shown to generate at least three isoforms of regulatory subunit. These include full length p85, a splice variant of 55 kDa (termed p55 α or AS53) cloned from rat (Inukai et al 1996; Inukai et al 1997) and human (Antonetti et al 1996), and another splice variant of 50 kDa (termed p50 α or p45 α) cloned from rat (Inukai et al 1997) and mouse (Fruman et al 1996). These splice variants share the 418 carboxyl terminal amino acids of p85 α , but are spliced upstream of the P2 proline rich motif, and therefore lack the BH and SH3 domains of the full length p85. Instead, the amino terminus of p55 α is comprised of a unique sequence of 34 amino acids containing a YMXM motif, whilst the amino terminus of p50 α is replaced with 6 amino acids. Neither of these alternatively spliced regions has significant homology to any protein identified so far, although nine amino acids at the amino terminal of p55 α are almost completely identical to those in the same domain as p55 γ . An additional splice variant of the p85 gene has also been reported, based on a partial cDNA sequence isolated by Antonetti and co-workers (Antonetti et al 1996). In this form, a 24 nucleotide insert between the SH2 domains occurs in a replacement of aspartate 605 with 9 amino acids which add two serine residues in the vicinity of the known

regulatory site, serine 608. However no further information on the abundance or tissue distribution of this splice variant is available.

The p85 α gene products have been shown to be differentially expressed, with p55 α being highly transcribed in brain and skeletal muscle (Antonetti et al 1996), and p50 α has been shown to be most abundant in liver, but is also abundant in brain and kidney (Inukai et al 1997), as determined from Northern blotting. Thus the current data suggests that the p85 α gene undergoes tissue specific alternative splicing. Whilst both Antonetti et al and Inukai et al identified these splice variants by expression cloning using IRS1 as a probe, p55 α was found to coprecipitate with IRS1 to a much lesser extent than p85 α from skeletal muscle following insulin stimulation. p50 α however, has been demonstrated to have the highest affinity for phosphorylated IRS in vivo (Inukai et al 1997).

The third gene encodes a product with domain homology to p55 α , which has been termed p55 γ . This protein was also isolated by screening expression libraries with phosphorylated IRS1, and is composed of a unique 30 residue amino terminus, and two SH2 domains which show significant homology with those of p85 α and p85 β (Chuang et al 1994). The domain structure makes p55 γ comparable with p55 α . p55 γ is expressed early in development, and in adult mouse is expressed most abundantly in brain, adipocytes, heart and kidney.

1.3.3 Role of individual adapter isoforms

The molecular basis of the unique pattern of responses to activation and recruitment of PI 3-kinase activity following stimulation by different growth factors, hormones and oncogenic stimuli, and the tissue specificity of these responses, is largely undefined. However, as more isoforms of the PI 3-kinase adapter subunit are being defined, so too are some differences amongst them emerging.

As described above, the differently spliced products of the p85 α gene are subject to tissue specific expression, which imparts specificity at one level. In terms of insulin signalling, both of the truncated products of this gene are highly expressed in muscle and liver, major sites of insulin action in vivo. Inukai et al have shown that the p85 α gene products have different PI 3-kinase activity elevating responses to insulin. In both PC12 and HepG2 cells, the degree of PI 3-kinase activation associated with p50 α was revealed to be twice that associated with full length p85 α or p55 α following insulin stimulation (Inukai et al 1997). In the same study it was also shown that PI 3-kinase associated with p50 α had a higher affinity for IRS1 following insulin stimulation than the other isoforms. In mice with targeted disruption of full length p85 α , upregulation of p50 α compensates for the loss. Indeed, the upregulation of p50 α appears to confer hypersensitivity to insulin, with elevated glucose uptake in response to insulin compared to wildtype mice (Terauchi et al 1999), implicating an important role for this isoform in insulin mediated glucose uptake.

All five regulatory subunit isoforms have been identified in rat brain by in situ hybridisation (Shin et al 1998), and expression appears to be ubiquitous, but some differences in signalling have been determined. Following insulin stimulation, for example, p55 γ but not p85 α or p55 α , were shown to be heavily tyrosine phosphorylated, indicating that the functions of these adapter subunits function differently (Inukai et al 1996). Since then, work by Shepherd et al has shown some significant differences in the interaction of these isoforms with phosphotyrosine complexes in human skeletal muscle, although the data is complicated by the identification of five bands which cross react with monoclonal antibodies to the SH2 domain of p85 α , at 46, 48, 53 and 54 kDa. Antibodies raised against the amino terminal of p50 α identified the 46 kDa protein only. The most prominent differences concerned the proportion of the total amount of each

isoform which could be immunoprecipitated with antiphosphotyrosine antibodies. Full length p85 α , and especially p55 α , p55 γ , and p50 α were readily detectable in antiphosphotyrosine immunoprecipitates. Indeed high levels of p55 α and p55 γ were found at these complexes under basal conditions, and only increased marginally in response to insulin stimulation. However, insulin stimulated almost 20-fold the amount of 48 kDa variant (p50 α) in antiphosphotyrosine immunoprecipitates (Shepherd et al 1997b). Overall these studies indicate profound differences in recruitment and activation of class 1a PI 3'-kinases. This is interesting since all of these isoforms contain the core unit (SH2 domains) for binding phosphotyrosine motifs, with four of the variants sharing identical SH2 domains.

Whilst there is some controversy regarding insulin stimulated tyrosine phosphorylation of p85 α , it has emerged that p55 γ is significantly phosphorylated on Tyr 341 in response to cell stimulation with insulin, and this adapter has also been shown to coimmunoprecipitate with the insulin receptor β subunit (Pons et al 1995). The physiological implication of p55 γ tyrosine phosphorylation is not known, but it does not correlate with elevated p110 activity. As in the case of p85 α , occupancy of both SH2 domains of p55 γ is necessary for stimulating associated PI 3-kinase activity. Both p55 γ and p55 α contain an amino terminal YXXM motif, which theoretically at least could be phosphorylated and bind an SH2 domain, though phosphorylation of this site has so far not been demonstrated.

p85 α and p85 β share almost identical domain homology, with the exception of an additional proline rich motif at the carboxy terminus of p85 β , but specific sequence differences suggest that the roles of these two isoforms will not be redundant. The region in which the sequences of these isoforms are most divergent is within the BH domain, suggesting differential interactions with small

G proteins, although this has not yet been shown conclusively. Phosphorylation of Ser608 on p85 α by p110 α is well documented in the literature as a mechanism for inhibiting the lipid kinase activity of PI 3-kinase ((Dhand et al 1994b) and others), but this does not appear to occur in p85 β (Reif et al 1993). The data concerning insulin stimulated recruitment of p85 β is conflicting. In human skeletal muscle, insulin stimulation has been shown to cause a 2-fold increase in p85 β at phosphotyrosine complexes (Shepherd et al 1997b). In a study of 3T3-L1 adipocytes and CHO-IR cells, Baltensperger and co-workers showed that PI 3-kinase was recruited to IRS1 irrespective of the isoform of regulatory subunit. However, the intrinsic activity of IRS1 associated PI 3-kinase was shown to be stimulated several fold when associated with p85 α , but no stimulation was observed when associated with p85 β (Baltensperger et al 1994). It was also shown that the basal level of PI 3-kinase activity associated with the β isoform was elevated compared to p85 α .

Hartley et al have demonstrated, using monoclonal antibodies, that in vivo the β -isoform of p85, but not the α - isoform, interacts with Cbl, thus identifying another potential difference between these isoforms (Hartley et al 1995a). c-cbl is the cellular homologue of v-cbl, a virus which induces certain leukaemia's in neonatal mice (Andoniou et al 1994). Furthermore, c-cbl resides on a human chromosome near a translocation breakpoint found in about 10% of human leukaemia's. Cbl contains an elongated proline rich region, making it a possible target for SH3 containing proteins and a number of potential motifs which could serve as ligands for SH2 domains. p120^{Cbl} was originally identified as a signal transduction molecule when it was cloned as a Nck SH3 domain-binding protein (Rivero-Lezcano et al 1994). Cbl has also been shown to associate with Grb2 via the amino terminal SH3 domain (Donovan et al 1994), to form a complex with Crk proteins following TCR activation by binding to their SH2 domains (Reedquist et al 1996) and to associate with PI 3-kinase activity (Meisner et al 1998), primarily

via SH2 domains on p85 and pY motifs on Cbl. Although Cbl immunoprecipitates from stimulated cells contain a large number of proteins, immunoblotting of p85 immunoprecipitates from T cell and B cell lines reveals only a single tyrosine phosphorylated band, suggesting Cbl functions as a docking protein for the formation of multimeric signalling complexes in these cell types (Hartley et al 1995a).

1.4 The PI 3-kinase catalytic isoforms

1.4.1 Domain structure

Three class 1a PI 3-kinase catalytic subunits have been cloned, designated p110 α , p110 β , and p110 δ , with 50% overall identity. Each contains an amino terminal adapter binding motif, a domain which binds the G protein Ras, a PIK domain found in all PI 3- and PI 4-kinases, and a carboxy terminal catalytic domain. In the kinase and PIK domains, identity is approximately 70%, and these proteins diverge most in the ras binding region, where identity is only 52%. In vitro, these proteins are all capable of phosphorylating PI, PI 4P and PI (4,5)P₂, although agonist stimulation of these enzymes in vivo predominantly increases levels of PI (3,4)P₂ and PI (3,4,5)P₃; kinetic studies suggest PI (3,4)P₂ is produced as the result of phosphatase action on PI (3,4,5)P₃, indicating PI (4,5)P₂ is the major in vivo target (Stephens et al 1991). As with all phosphoinositide kinases, class 1a PI 3-kinases contain several key residues conserved in classical protein kinases. These confer a serine kinase activity which is inseparable from their lipid kinase activities (Vanhaesebroeck et al 1997b). This serine kinase activity targets within the heterodimer, though p85/ p110 complexes have also been shown to phosphorylate IRS1 (Freund et al 1995; Lam et al 1994). All three class 1a catalytic subunits are inhibited by nanomolar concentrations of wortmannin, and by LY294002 which inhibits at micromolar concentrations, both in vitro and in vivo (Vlahos et al 1994).

1.4.1.1 p85 binding domain

The region of p110 which binds the adapter subunit was identified by Dhand et al using GST fusion proteins encompassing various regions of p110 α (Dhand et al 1994a). These constructs were expressed in Sf9 cells, and their ability to bind p85 α and p85 β was assessed. The study demonstrated that the first 128 residues of p110 are necessary for adapter binding, and amino acids 20-108 comprised the minimum structural element required for p85 binding.

1.4.1.2 Ras binding domain

The Ras family of proteins are regulators of cell growth and proliferation, which interact with target molecules via an 'effector region' to exert a biological effect. The first Ras effector protein identified was p120^{GAP} which interacts only with GTP bound Ras. This protein stimulates the GTPase activity of Ras, making it a negative regulator (Trahey & McCormick 1986). PI 3-kinase was shown to be a direct target of Ras (p21^{ras}) by Rodriguez-Viciana et al, who demonstrated that Ras proteins immobilised on beads bound high levels of PI 3-kinase activity when loaded with GTP (Rodriguez-Viciana et al 1994). The same study also showed that expression of activated Ras stimulated synthesis of PI (3,4)P₂ and PI (3,4,5)P₃ in COS cells, and that the interaction was likely to be direct, since Coomassie staining of PI 3-kinase precipitated with agarose immobilised Ras showed no other proteins. Ras activation was shown to occur independently of p85. The exact site on PI 3-kinase that binds Ras was determined by expression of p110 deletion mutants in baculovirus (Rodriguez-Viciana et al 1996). For both p110 α and β isoforms of PI 3-kinase, the site of interaction was mapped to amino acids 133-314, and a peptide of these residues was shown to be able to interact with Ras in a GTP dependent manner. Dominant negative forms of Ras can inhibit D-3 phosphoinositide synthesis, and PI 3-kinase stimulated DNA synthesis has been shown to be Ras dependent in some circumstances (McIlroy et al 1997). Furthermore, Ras effector domain mutants which cannot bind PI 3-kinase have

pinpointed Ras activated PI 3-kinase in cellular effects such as inhibition of anoikis. This is mediated by the role of PI 3-kinase and PKB in sensing the adhesion status of a cell, and becoming inactivated when a cell detaches from the matrix thereby committing it to apoptosis. Stimulation of PI 3-kinase in suspended cells by expression of p110-CAAX or Ras blocks entry into apoptosis (Khwaja et al 1997; Rodriguez-Viciana et al 1997).

1.4.1.3 PIK domain

The PIK domain was defined following the comparison of a yeast PI 4-kinase with other PI kinases (Flanagan et al 1993). This domain is conserved in all classes of PI 3- and PI 4-kinases, but not protein kinases, suggesting a role involving the lipid substrate. Thus far, little effort has focused on this domain, and no function can categorically be allocated to it.

1.4.1.4 Catalytic domain

The catalytic domains of PI 3-kinases share significant homology with PI 4-kinases, as well as with enzymes which function only as protein kinases, and in fact at least two of the class 1a PI 3-kinase catalytic isoforms, α and δ , have a demonstrated protein kinase activity.

Based on the sequence similarity between p110 and other known kinases, a putative core of essential amino acids has been defined. These are residues which have similar spatial characteristics to those found in other kinases, such as cAMP dependent protein kinase: in p110 α , the residues are G842, K863, D916, N921, and the DFG sequence at 933-935 (Stephens et al 1993). These theoretical assignments are supported by studies of p110 point mutants, in which protein and lipid kinase activities were simultaneously abolished (Dhand et al 1994b).

1.4.2 Roles of the catalytic isoforms

As described in earlier sections, all three catalytic isoforms can phosphorylate PI, PI 4P and PI (4,5)P₂. The major substrates of p110 protein kinase activity identified so far are within the heterodimer itself, or the IRS proteins. p110 α has been shown to phosphorylate p85 α at Ser 608, which results in down regulation of p110 lipid kinase activity. The protein kinase of p110 δ similarly downregulates lipid kinase activity, but the mechanism involves p110 autophosphorylation rather than intersubunit phosphorylation (Vanhaesebroeck et al 1997b). Lam et al first demonstrated IRS1 phosphorylation by PI 3-kinase, and that this could be stimulated by insulin (Lam et al 1994). In a further study, Freund and co-workers demonstrated that this was manganese dependent, (unlike other IRS kinases which were dependent on magnesium), and that increased phosphorylation of IRS correlated with decreased phosphorylation of p85 (Freund et al 1995). This suggests a potential mechanism by which PI 3-kinase could be activated at IRS, since p85 phosphorylation has been shown to downregulate the lipid kinase activity of p110. To date, the phosphorylation sites targeted by PI 3-kinase on IRS have not been mapped. The catalytic isoforms involved in this phosphorylation have not been determined either.

Although few studies have been concerned with the specific functional properties of the class 1a PI 3-kinase catalytic isoforms, their sequence variations and expression patterns suggest they do not serve entirely redundant functions. In terms of insulin signalling, p110 δ is unlikely to be a significant player, since its expression is restricted to haematopoietic cells (Vanhaesebroeck et al 1997b). p110 α and p110 β are widely expressed, and therefore are more likely to mediate insulin stimulated responses. One study of insulin stimulated glucose transport in 3T3-L1 adipocytes (Wang et al 1998) has suggested translocation of p110 β , but not p110 α , is responsive to insulin. Wang et al demonstrated that levels of p85 α were elevated in GLUT4 containing vesicles following stimulation by insulin, and

a parallel gain in p110 β was observed, but there were no changes in levels of p110 α . Similarly, preliminary data from Funaki et al implicates p110 β as the insulin responsive isoform in adipocytes (Funaki et al 1998). A 20-fold stimulation of the β - isoform was observed in response to insulin, and it was further shown that overexpression of p110 β in 3T3-L1 adipocytes enhanced insulin sensitivity in terms of stimulating glucose transport, whereas overexpression of p110 α only elevated glucose transport in basal cells.

Another functional difference between p110 α and p110 β is the surprising finding that the $\beta\gamma$ subunits of heterodimeric G-proteins can activate p110 β but have no effect on p110 α . This mode of stimulation was previously thought to be restricted to class 1b PI 3'-kinases (Kurosu et al 1997).

1.5 Activators of PI 3-kinases

1.5.1 Receptor Tyrosine Kinases

The function of RTKs in terms of PI 3-kinase activation is two fold. Firstly, recruitment of PI 3-kinase increases the proximity of the enzyme to its lipid substrate at the plasma membrane. Secondly, binding of RTKs or the IRS proteins has been shown to induce conformational changes in PI 3-kinase which activate its lipid kinase activity, as described in section 1.3.1.1.

Panayotou et al have shown that binding of peptides of the phosphorylated PDGF receptor to the SH2 domains resulted in a conformational change in the inter-SH2 region of p85 α causing activation of p110 α (Panayotou et al 1992). Similarly, it has been shown that association with IRS activates PI 3-kinase (Backer et al 1992), and that this activation is optimal when both SH2 domains of p85 α are occupied by phosphotyrosine motifs (Rordorf-Nikolic et al 1995). This suggests that the elevation of the levels of D-3 phosphoinositides during insulin stimulation

is not solely dependent on recruitment of the enzyme to its substrate, but that an activation event is also involved.

1.5.2 Polyoma middle-T

Polyomavirus is a small double stranded closed circular DNA virus endemic in wild mouse populations where it has few effects (for review see (Dilworth 1995)). In closely related species such as hamsters and rats, the virus can occasionally be incorporated into genomic DNA, causing transformation and multiple tumor formation. The transforming effect is caused by middle-T, a 55 kDa protein associated with membranes which has tyrosine kinase activity, and closely related to proteins of the simian virus 40 and BK virus in humans. Middle-T binds a number of host cell regulatory proteins and alters their function. For example, binding of middle-T to PP2A prevents dephosphorylation and hence inactivation of the MAP kinase signal transduction pathway (Sontag et al 1993). The 14-3-3 family of proteins (see section 1.7) have also been shown to interact with middle-T (Pallsa et al 1994), as has SHC (Campbell et al 1994). PI kinase activity was shown to be present in middle-T immunoprecipitates by Whitman et al, who demonstrated a close correlation between middle-T transformation and PI kinase activity (Whitman et al 1985). Phosphorylation of Tyr315 on middle-T creates an SH2 binding site for the p85 subunit of PI 3-kinase (Talmage et al 1989), and this has been linked with many of the discrete physiological changes associated with middle-T transformation including elevated glucose transport (Young et al 1995), induction of MAPK (Urich & El Shemerly 1995) and increased expression of early response genes (Srinivas et al 1994).

1.5.3 Small GTPases

As discussed in section 1.3.1.3, Rac1 and Cdc-42 interact with PI 3-kinase in a GTP dependent manner elevating the lipid kinase activity of the enzyme (Tolias et al 1995). The interaction of these GTPases with PI 3-kinase has been shown to be

growth factor dependent and a likely downstream event is growth factor induced changes in cytoskeletal arrangement, leading to membrane ruffling (Hawkins et al 1995).

1.5.4 Dimerization of PI 3-kinase

Another mechanism for activation of signalling proteins is oligomerization, as happens with Raf (Farrar et al 1996). Dimerization of PI 3-kinase has been demonstrated by Layton, Harpur and co-workers, who have shown activation of PI 3-kinase is increased by dimerization following their binding to peptides containing multiple diphosphotyrosine motifs (Layton et al 1998). This dimerization is likely to result from interactions involving either the SH3 or BH domains of p85, since it does not occur in low molecular mass isoforms of the adapter subunit. In vivo relevance for such a mechanism is possible since both the PDGF receptor (Claessonwelsch et al 1988), and IRS1 (Backer et al 1992) contain two diphosphotyrosine motifs.

1.6 Regulation of class 1a PI 3-kinases in insulin signalling

1.6.1 The IRS proteins

Though direct recruitment of PI 3-kinase to the insulin receptor has been shown, the major mechanism for insulin stimulated recruitment is via the insulin receptor substrate proteins. This was shown by Backer *et al*, who demonstrated that IRS-1 undergoes rapid tyrosine phosphorylation by the insulin receptor during insulin stimulation, and can then form a stable complex containing the p85 of PI 3-kinase. IRS-1/ PI 3-kinase complex formation is blocked *in vitro* by the addition of synthetic peptides containing phosphorylated (but not unphosphorylated) YXXM motifs, which suggested that binding occurred through the p85 SH2 domains (Backer et al 1992). Furthermore, overexpression of IRS-1 potentiates the activation of PI 3-kinase in insulin stimulated cells, and tyrosyl phosphorylated IRS-1 activates PI 3-kinase *in vitro*.

The first evidence for an insulin receptor specific substrate came from White, Kahn and co-workers, (White et al 1985) who demonstrated the insulin stimulated tyrosine phosphorylation of a 185 kDa cytosolic protein using phosphotyrosine immunoblotting. Originally referred to as pp185, based on its migration during SDS-PAGE, it was subsequently purified from rat liver and 3T3-L1 adipocytes, and its cDNA cloned (Sun et al 1991). A whole family of IRS molecules have since been identified, with the same overall domain structure, and sharing highly homologous amino terminal PH and phosphotyrosine binding (PTB) domains.

1.6.1.1 IRS-1 and IRS-2

The best characterised of the insulin receptor substrates are IRS-1 and IRS-2. Although originally isolated as specific insulin receptor substrates, it has emerged that these proteins have a broader role, as proximal substrates in growth hormone and cytokine receptor signalling. Indeed, phosphorylation of IRS-1 has been shown to generate recognition motifs for the SH2 domains of several proteins, including PI 3-kinase (Y460, Y608, Y939, Y987), the ubiquitous adapter protein Grb2 (Y895) and the protein tyrosine phosphatase SHPTP2 (Y1172) (Sugimoto et al 1994; Sun et al 1993). In addition, there are over 30 potential serine/ threonine phosphorylation sites in motifs recognised by various kinases.

Various lines of evidence indicate an important role for IRS-1 in mitogenesis. Antisense IRS-1 cDNA, or injection of IRS-1 blocking antibodies, reduce the mitogenic response to insulin (Waters et al 1993); conversely, overexpression of IRS-1 increases insulin stimulated thymidine incorporation into CHO-IR cell DNA two-fold (Sun et al 1992). In the case of the PDGF receptor, the mitogenic effect has been shown to depend on p110 α , but not p110 β (Vanhaesebroeck et al 1999). Insulin has also been shown to induce Ras mediated mitogenesis, although

tyrosine phosphorylated IRS-1 does not result in Ras activation (Pruett et al 1995). Since tyrosine phosphorylation of Shc and subsequent Ras activation are insufficient for mitogenesis in cells lacking IRS-1, it appears that pathways coupled by IRS-1 are in existence to bring about mitogenesis.

Despite the high structural similarity, the physiological roles of IRS-1 and IRS-2 do not overlap as significantly as was initially thought. Indeed, whilst IRS-1 is preferentially located at intracellular membranes, IRS-2 is predominantly cytosolic. Results from studies undertaken by several independent groups taken together show that whilst IRS-1 is essential and sufficient for many responses elicited by insulin, it may not be the most important with respect to glucose transport. In the obese diabetic (*ob/ob*) mouse, Kerouz et al have shown there are multiple alterations in the initial stages of insulin signalling. IRS-1 and IRS-2 were shown to be differentially regulated in response to insulin, specifically in differences in decreases in phosphorylation, docking of p85 α and p55 α , and subsequent activation of PI 3-kinase in muscle and liver (Kerouz et al 1997). Inoue et al studied the time-course of compartmentalisation and phosphorylation of these two insulin receptor substrates following insulin stimulation of 3T3-L1 adipocytes, in both whole cells and isolated membranes (Inoue et al 1998). Experiments with insulin stimulated intracellular membranes (IM) suggest that translocation of IRS-1 from IM to cytosol is due to a decrease in the ability of the IM to associate with IRS. This is likely to be regulated by an insulin induced serine/ threonine phosphorylation of an IM component, since exclusion of okadaic acid from the homogenisation buffer prevented the dissociation of IRS-1. The dissociation of IRS-2 from IM appears to be regulated differently, because it occurs even in the absence of phosphatase inhibitors. The data indicated that IRS-1 mediated signalling is more stable, whereas IRS-2 mediated signalling is quite transient, reflected by the time in which tyrosine phosphorylation of each IRS was maintained. Ogihara and co-workers have since implicated PI 3-kinase in the

rapid dephosphorylation of IRS-2. Like Inoue et al, they found that following insulin stimulation, IRS-2 dephosphorylated more quickly than IRS-1. Though dephosphorylation was blocked by sodium orthovanadate (a tyrosine phosphatase inhibitor), a role for PI 3-kinase was suggested since wortmannin, or overexpression of a dominant negative p85, also inhibits IRS-2 phosphorylation (Ogihara et al 1997).

Studies in which IRS gene expression is disrupted implicate IRS-2 as the more critical *in vivo* insulin signalling molecule in terms of Non Insulin Dependent Diabetes Mellitus (NIDDM). Development of type 2 (non insulin dependent) diabetes is characterised by a breakdown in the regulation of glucose homeostasis. Although it has been shown that there is a marked decrease in insulin stimulated glucose transport in the isolated adipocytes of mice with a targeted disruption of the IRS-1 gene (Araki et al 1994), diabetes does not develop *in vivo* because increased insulin secretion can compensate for the mild resistance which results (Tamemoto et al 1994). In these mice, growth retardation is the predominant phenotype (Araki et al 1994; Tamemoto et al 1994). Both Tamemoto et al, and Araki et al noted that to a certain extent, the insulin signalling pathways were rescued by a protein of Mr ~10 kDa larger than IRS-1.

This protein was subsequently identified as IRS-2, formerly identified in myeloid cells and named 4PS (Patti et al 1995). Withers and co-workers have since shown that dysfunction of IRS-2 may contribute to the pathophysiology of type 2 diabetes, since IRS-2^{-/-} mice show marked defects in glucose homeostasis, but minimal growth defects. In mice with targeted disruption for IRS-2, insulin receptor expression and phosphorylation were normal, as were the levels of PI 3-kinase proteins. The increase in PI 3-kinase activity associated with IRS-1 following insulin stimulation in the liver and muscle of these mice was reduced by 50%, in part the result of an elevated basal association. This suggests a defect in

the ability of cells to regulate both basal and insulin stimulated PI 3-kinase in cells lacking IRS-2 (Withers et al 1998), and overt diabetes develops in these mice at ten weeks. Analysis of the pancreases of these animals showed specifically a reduction in β -cell mass. In addition, in control animals, immunofluorescence localises IRS-2 with insulin in the islets, suggesting it is expressed in β -cells but not non- β -cells. In all, studies of mice with targeted gene disruption of IRS proteins show that while IRS-1 may be the predominant isoform in terms of ubiquitous expression, IRS-2 is the most likely candidate of the two for the pathogenesis of NIDDM.

1.6.1.2 IRS-3

The roles of the recently cloned IRS-3 and IRS-4 in insulin signalling are as yet unclear. However, it is known that IRS-3 undergoes tyrosine phosphorylation in response to insulin, and binds PI 3-kinase (Smith-Hall et al 1997). It has also been shown that IRS-3, rather than IRS-2, rescues most of the non-glucose uptake pathways in adipocytes from IRS-1 deficient mice. In a high fat diet induced rat model of insulin resistance, mRNA and protein levels of IRS-3 are elevated, but those of IRS-1 and IRS-2 are decreased, in epididymal adipocytes. GLUT4 translocation in adipocytes has been shown to be stimulated by recruitment of PI 3-kinase to low density microsomes, but IRS-3 localises predominantly to the plasma membrane, a factor likely to contribute to the inability of the third isoform of IRS to rescue decreases in IRS-1 and IRS-2 signal transduction.

1.6.1.3 IRS-4

IRS-4, a 160 kDa protein, was isolated from human embryonic kidney cells (Kuhne et al 1995). cDNA cloning and amino acid sequencing showed it to be a novel member of the IRS family. IRS-4 is of similar length (1257 aa) to both IRS-1 and IRS-2 but shares only a limited sequence identity; the protein shares only 27 and 29% identity respectively. However, the 120 amino acid PH domain of IRS-4

is approximately 50% identical to those of IRS-1, -2 and -3. The 101 amino acid PTB domain shares over 60% identity with IRS-1 and -2, and 40% identity with IRS-3 (Lavan et al 1997). The crystal structure of IRS1 bound to a peptide which mimics part of the insulin receptor shows that 15 residues of the PTB domain are involved in the binding event (Eck et al 1996) and 13 of these are conserved in IRS-4. Both PI 3-kinase and Grb2 coimmunoprecipitate with IRS-4, and this is stimulated by insulin (Fantin et al 1999).

1.7 Cellular mechanisms causing attenuation of PI 3-kinase activation by IRS proteins

Mechanisms for attenuating insulin stimulated PI 3'-kinase are likely to be important in downregulating insulin signalling, but improper attenuation may lead to insulin resistance. The capacity of insulin to activate PI 3-kinase has been shown to be inhibited by a variety of mechanisms, several of which act by altering the phosphorylation state of the IRS proteins, correlating with a decreased ability of the insulin receptor to tyrosine phosphorylate these molecules. The IRS proteins contain multiple consensus sequences for a number of protein kinases, and the migration of IRS-1 on SDS-PAGE at an electrophoretic mobility corresponding to 185 kDa, compared to its predicted molecular mass of 130 kDa, is consistent with a high level of serine/ threonine phosphorylation observed in the basal state (Sun et al 1991). Mutational analysis of several serine residues suggest these sites may be involved in negative modulation of insulin stimulated PI 3-kinase activation (Mothe & VanObberghen 1996).

SHP-2 is a cytoplasmic protein tyrosine phosphatase containing two SH2 domains which bind the YIDL and YASI motifs in the COOH-terminal of IRS-1 (Hof et al 1998). Several reports have shown a critical role for SHP-2 in insulin stimulated MAP-kinase activation. Myers et al used a mutant IRS-1 molecule lacking the SHP-2 binding motifs to demonstrate that in the absence of this phosphatase, IRS-

1 phosphorylation, PI 3-kinase binding to IRS-1, and activation of protein synthesis in response to insulin, were elevated (Myers et al 1998a). This suggests that the interaction of IRS-1 and SHP-2 attenuates some insulin mediated metabolic events, perhaps by dephosphorylation of YMXM motifs on IRS-1.

14-3-3 β protein is another IRS-1 binding molecule which is likely to modulate PI 3-kinase in insulin signalling. The 14-3-3 proteins are modulators for protein kinase C, oncogene products including Raf-1 and polyoma middle-T, and cell cycle progression proteins. Kosaki and co-workers showed by immunoprecipitation that the association of IRS-1 and 14-3-3 can be increased 2.5-fold following insulin stimulation in 3T3-L1 adipocytes. By sequential immunoprecipitation, they showed this association reduces the lipid kinase activity of bound PI 3-kinase activity by 75% (Kosaki et al 1998). The binding of 14-3-3 is dependent on serine phosphorylation of RXRXXS motifs on IRS-1, and there are four of these in the PTB domain of IRS-1, with others located in the region containing YXXM motifs. Since the data was normalised for p85 protein associated, it is unlikely that the mechanism of inhibition is simply a blocking access of phosphorylated YMXM motifs on IRS-1 by 14-3-3. Importantly, the insulin stimulated association of IRS-1 with 14-3-3 is inhibited by wortmannin. As insulin induced phosphorylation of IRS-1 by PI 3-kinase has been shown to occur, (Tanti et al 1994) it is tempting to speculate that a negative feedback mechanism is in operation, wherein the insulin stimulated association of PI 3-kinase with IRS-1 is attenuated by a PI 3-kinase induced association of 14-3-3 with IRS-1.

Activation of protein kinase C has been shown to inhibit insulin stimulated pathways in several systems, and this is potentiated by overexpression of particular PKC isoforms (Chin et al 1993). PKC activation by PMA in HEK 293 cells is sufficient to inhibit insulin stimulated tyrosine phosphorylation of IRS-1,

mediated by PKC stimulated phosphorylation of residue Ser612 of IRS-1 (De Fea & Roth 1997b). Moreover, a similar IRS-1 kinase activity was shown to be elevated in the liver of *ob/ob* mice. In liver extracts of these mice, PKC activation, and concomitant serine phosphorylation of IRS-1 resulted in a 50% inhibition of recruitment of PI 3-kinase activity; the recruitment of PI 3-kinase activity to a S612A IRS-1 mutant was unaffected.

PKC mediated inhibition of PI 3-kinase association with IRS-1 is dependent on activation of ERK1 and 2, and catalytically active ERK coimmunoprecipitates with IRS-1 from PMA treated cells. This was shown using a reversible inhibitor of MEK1, the enzyme that directly activates ERK1 and 2 (De Fea & Roth 1997a). While insulin stimulated IRS-1 associated PI 3-kinase activity 6-fold, pre-treatment with PMA inhibited this by 70%. Inhibition of MEK1 restored the PI 3-kinase activity associated with IRS-1 in PMA treated cells to normal levels.

In obese humans, and animal models of obesity, TNF α - has been shown to play a key role in abnormal signalling and systemic insulin resistance (Korthauer et al 1993). Myeloid 32D cells, which lack endogenous IRS-1, are resistant to TNF mediated inhibition of IR signalling, but become acutely sensitive when IRS-1 is overexpressed (Hotamisligil et al 1996b). This indicates that TNF- α attenuates insulin signalling through a mechanism involving the IRS-1 molecule. Kanety et al have shown that in TNF treated Fao cells, there is a 60% reduction in insulin stimulated IRS-1 tyrosine phosphorylation, with no change in the tyrosine kinase activity of the insulin receptor (Kanety et al 1995), and this was associated with impaired PI 3-kinase association, indicating IRS-1 is central to TNF mediated inhibition of insulin resistance. Though TNF activates protein kinase C in several cell lines (Gorospe et al 1993) pre-treatment of cells with staurosporine, a potent non-specific inhibitor of PKC, did not decrease the TNF mediated inhibition of insulin induced tyrosine phosphorylation. Other kinases whose activity has been

shown to be upregulated by TNF are the MAP kinase isoforms, which may be candidates for conveying the TNF mediated effect (White & Kahn 1994).

1.8 Lipid products as signalling molecules

The inositol containing phospholipids are minor membrane components which can be used to convey diverse signals from stimulants such as neurotransmitters, hormones, growth factors and stress. PI (4,5)P₂ is the major substrate for two groups of agonist dependent enzymes: the phospholipase C superfamily cleave this phospholipid to generate diacylglycerol and I (1,4,5)P₃, both of which are well documented second messengers particularly in PKC activation, and the PI 3-kinase family, which can use the lipid as a substrate for PI (3,4,5)P₃. In addition, PI (4,5)P₂ can function as a signalling molecule in its own right, with a significant role in mediating actin assembly (Downes & Currie 1998).

1.8.1 Transmission of the PI 3-kinase signal

The ability to chemically synthesise phosphoinositides (Wang & Chen 1996) and the development of new strategies to clone lipid binding proteins (Klarlund et al 1997) has greatly improved our understanding of the contribution of each PI kinase makes to cell function.

PI3P is constitutive to both mammalian and yeast cells, and is most likely produced *in vivo* by the action of class III PI 3-kinase. This class has substantial homology to the yeast protein Vps34, which has been shown to be essential for correct endocytic sorting (Backer & McIlroy 1998; Stack & Emr 1994). PI3P has recently been shown to interact with FYVE finger domain modules (a type of RING zinc finger), and the FYVE finger containing protein EEA1 determines the subcellular localisation of early endosomes (Gaulhier et al 1998; Simonsen et al 1998). The discovery of FYVE-containing proteins as targets of PI3P has therefore provided a lead in the mechanism by which PI 3-kinases regulate vesicle trafficking.

PI (3,4)P₂ levels are also subject to acute regulation by extracellular signals, (Stephens et al 1991) suggesting a role in signalling. Whilst PI (3,4)P₂ was until recently considered to be the product of dephosphorylation of PI (3,4,5)P₃, it has subsequently been shown that class II PI 3-kinases can phosphorylate PI4P, although the contribution of this pathway to cellular levels of this lipid is unknown. PKB, a serine/ threonine protein kinase, is the best characterised PI (3,4)P₂ target. It is now becoming clear that some of the major functions of D-3 phosphoinositides are mediated by their PH domain binding abilities. Some recent studies have addressed the binding of PH domains to D-3 phosphoinositides, and it is emerging that different PH domains have specificity for distinct phosphoinositides. PKB contains a PH domain, which has in vitro binding preferences for phosphoinositides in the order PI (3,4)P₂ > PI (3,4,5)P₃ > PI (4,5)P₂ (Franke et al 1997; Frech et al 1997). Phosphoinositide binding activates PKB, and in vivo frequently correlates with PI (3,4)P₂ rather than PI (3,4,5)P₃ synthesis (Franke et al 1997). Full activation of PKB is also dependent on its phosphorylation state, and the kinases responsible, PDK1 and PDK2, are phosphoinositide dependent, as discussed in section 1.9.

The PKC isoforms ϵ and ζ are also significantly activated by PI (3,4)P₂ both by direct interaction with the phosphoinositide and indirectly by PDK1. Salim et al have also demonstrated that dynamin PH domain binding to PI (4,5)P₂ is tight, but almost non-existent in the case of PI (3,4,5)P₃ (Salim et al 1996), raising the possibility that PI 3-kinase may negatively regulate dynamin GTPase activity.

PI (3,5)P₂ is a newly identified molecule whose levels rise in mammalian cells following hypo-osmotic shock. No targets for this lipid have yet been found. In vivo, the most likely mode of synthesis is by the consecutive action of class III PI

3-kinase on PtdIns, followed by D-5 phosphorylation by PI3P 5-kinase (Whiteford et al 1997).

Class 1 enzymes are the only PI 3-kinases which can utilize PI (4,5)P₂ as a substrate, and insulin stimulated D-3 phosphoinositide synthesis mainly elevates levels of PI (3,4,5)P₃. However, PI (4,5)P₂ independent mechanisms of PI (3,4,5)P₃ synthesis have also been described (Tolias et al 1998; Zhang et al 1997). In addition to activating PKB, PDK1 and some PKC isoforms, usually to a lesser extent than described for PI (4,5)P₂, several other targets for PI (3,4,5)P₃ have been identified. The PH domain of Bruton's tyrosine kinase (Btk) has a high affinity for PI (3,4,5)P₃, but binds PI(3,4)P₂ and PI(4,5)P₂ poorly (Salim et al 1996). The high affinity interaction was dependent on Arg28, of Btk which is frequently mutated in X-linked agammaglobulinaemia. Overexpression of activated class 1a p110 results in activation (via autophosphorylation) of Btk (Li et al 1997), as measured by its ability to regulate PLC γ 2. Furthermore, the PH domain of PLC has been shown to bind PI (3,4,5)P₃, which localises the enzyme to the plasma membrane from where it can regulate intracellular calcium release (Rameh et al 1998).

The general receptor for phosphoinositides Grp1 contains a PH domain which is highly selective for PI (3,4,5)P₃. Grp1 also contains a domain which functions as a guanine nucleotide exchange factor for the small G proteins Arf1 and Arf5 (for review see (Moss & Vaughan 1998), which suggests PI (3,4,5)P₃ may regulate Grp1 activity by recruiting it to membranes where Arf is localized (Klarlund et al 1998). (Arf is a component of coatamer, a complex of seven proteins one of which, α -COP is also a target for PI (3,4,5)P₃ (Chaudhary et al 1998)). Infact many exchange factors for small G proteins contain PH domains (Mussachio et al 1993), and growth factor stimulation of GTP binding to small G proteins is frequently PI 3-kinase dependent, suggesting the mechanism described for Arf

regulation may be widespread. Indeed, it is reported that the activity of Vav towards Rac, Cdc42 and RhoA is stimulated by PI (3,4,5)P3 binding to Vav, but inhibited by PI (4,5)P2 (Han et al 1998). In addition to targetting some certain PH domains, PI (3,4,5)P2 has also been shown to bind SH2 domains. PI (3,4,5)P3 can compete with phosphotyrosine motifs to bind the SH2 domains of p85 (Rameh et al 1995), and can also bind the SH2 domains of PLC stimulating its phospholipase activity towards PI (4,5)P2 (Rameh et al 1998).

Centaurin- α was cloned in a screen for Ins (1,3,4,5)P4 binding activities, and this binding event can be could be competed with PI (3,4,5)P3 (Hammonds-Odie et al 1996). Centaurin- α is a 48 kDa protein with homology to ARF GAP suggesting a role in vesicle trafficking. It is largely basic, and therefore suitable for binding negatively charged phospholipids. Whilst it is highly soluble with no membrane spanning motifs, a 30 amino acid stretch has considerable hydrophobic character, and indeed centaurin- α is found to partition between soluble and membrane compartments, consistent with it playing a role in regulation of the cytoskelton or vesicle movement.

PKB is phosphorylated by two protein kinases which are PI (3,4,5)P3 dependent (Kohn et al 1996b; Marte & Downward 1997). 3-phosphoinositide dependent kinase 1 (PDK1) phosphorylates the Thr308 site (Alessi et al 1997a), and PDK2 (as yet uncloned) which phosphorylates Ser473 on PKB. Purification and cloning of PDK1 have revealed a protein with a PH domain which binds PI (3,4,5)P3 (Alessi et al 1997b; Frech et al 1997). PDK1 is constitutively active, not dependent on insulin stimulation, nor inhibited by wortmannin. The 3-phosphoinositide dependence is conferred since PDK1 will only phosphorylate PKB-bound to PI (3,4,5)P3 (Alessi et al 1997b). The phosphorylation of PKB by PDK2 is also PI 3-kinase dependent, as is translocation of PKB to the plasma membrane (Andjelkovic et al 1997).

PI 3'-kinase activation was implicated in growth factor mediated membrane ruffling since it is inhibited by wortmannin (Kotani et al 1994). In addition, activated PI 3-kinase can promote actin rearrangement in a similar pattern to that observed for activated Rac or Rho (Reif et al 1996). This places PI 3'-kinase downstream of the insulin receptor and upstream of Rac. PI 3-kinase induced Rac activation has now been shown to be dependent on PI (3,4,5)P3. Missy et al have demonstrated that Rac1, and to a lesser extent RhoA, selectively bound PI (3,4,5)P3, and that this binding event had a potent GDP releasing activity (Missey et al 1998).

1.9 Molecules linking PI 3-kinase to downstream signalling

1.9.1 Protein kinase B

Protein kinase B (also known as c-Akt) is a 57 kDa ser/ thr kinase homologous to the retrovirus v-Akt (for review, see (Coffer et al 1998)). Three isoforms have been identified, PKB- α and β which are the predominant isoforms in insulin target tissues (Walker et al 1998) and PKB- γ which has a more restricted pattern of expression (predominantly brain and testes). Nevertheless, all tissues contain at least one isoform of PKB, and homologues have been identified in *C. elegans* (Waterston et al 1992) and *D. melanogaster* (Franke et al 1994).

Phosphoinositide bound PKB can be activated by phosphorylation on Thr308 by PDK1, and on Ser473 by PDK2 (Alessi et al 1997b; Franke et al 1997; Klippel et al 1997). Though PKB is largely cytosolic, its PH domain is likely to function as a PI (3,4,5)P3 receptor, targeting the molecule to the plasma membrane. It is probable that the high affinity of PDK1 for PI (3,4,5)P3 will also localise this protein to the plasma membrane. In addition, it has been shown that the PH domain mediates interaction between PKB molecules, and that this may be important in regulation (Walker et al 1998). Activated PKB can then detach from

the membrane, and phosphorylate its cellular targets. In several studies, deletion of the amino terminal PH domain on this protein blocks the ability of some agonists (such as PDGF) to stimulate PKB activity *in vivo* which would be consistent with this model (Franke et al 1995). It is interesting then that deletion of the PH domain of PKB does not block its activation by insulin (Kohn et al 1995).

Active versions of PKB have been constructed using various approaches, including myristoylation sequences to target PKB to membranes, and mutation of Thr308 and Ser473 to aspartate. Whilst both of these approaches mimic wild type phosphorylated PKB to a certain extent, and *in vitro* these proteins are catalytically active, activation is usually only partial. In some *in vivo* cases the behaviour of these appears dominant negative. Plasma membrane targeted PKB remains membrane bound, while PKB_{DD} tends to be constitutively cytosolic. These spatial problems, combined with the absence of any effective PKB inhibitors so far, has complicated research in this area. In addition, not all pathways downstream of PKB are inhibited by wortmannin (Moule et al 1997).

Nevertheless, it is known that PKB is a multifunctional mediator of PI 3-kinase signal transduction. Acute activation of PKB has been shown to be sufficient to stimulate the phosphorylation of PHAS-1 (phosphorylated heat and acid stable protein) and to induce its dissociation from eIF-4E, a key step in the regulation of protein synthesis (Kohn et al 1998). Activation of mTOR, and subsequently p70 s6k, may also lead to increased translation of a specific subset of mRNA's. In a similar study, an activated PKB expressed in 3T3-L1 fibroblasts was shown to cause spontaneous differentiation into adipocytes, constitutive glucose transport and increased GLUT4 translocation to the cell surface. In addition, this activated PKB induced constitutive lipogenesis and prevented insulin induced glycogen synthesis (Kohn et al 1996a). Through its inhibition of GSK3, activated PKB also

downregulates eIF2B, another translation initiation factor. Glucose metabolism and MAPK are also regulated by PKB (Coffer et al 1998)).

1.9.2 Glycogen Synthase Kinase

Inactivation of GSK-3 results in dephosphorylation and subsequently activation of glycogen synthase, forming part of the mechanism by which insulin stimulates glycogen synthesis. Various lines of evidence have implicated insulin stimulated PKB in a linear pathway to GSK-3 inhibition (Brady et al 1998). Phosphorylation of Ser9 on GSK-3 α , and of Ser21 on GSK-3 β inactivates these enzymes, and insulin stimulated GSK-3 phosphorylation is inhibited by wortmannin but not rapamycin (Cross et al 1994). Several kinases have been shown to phosphorylate this site *in vitro*, including PKB, PKC, MAPKAPK1 and p70s6k. PKB has also been shown phosphorylate GSK-3 *in vivo* (Cross et al 1995). A dominant-negative PKB mutant (PKB-CAAX) is an efficient inhibitor of insulin stimulated GSK-3 inactivation. In addition, the transforming construct gagPKC causes translocation of GSK-3 to the plasma membrane from the cytosol (van Weeren et al 1998).

1.9.3 p70 s6 protein kinase

p70 s6k causes ribosomal protein s6 phosphorylation to contribute to increased mRNA translation, and has been shown to be downstream of PKB (Reif et al 1997). The activation of this protein requires PI 3-kinase and involves multiple phosphorylation events, including two which are analogous to those which activate PKB. In fact, PDK1 can phosphorylate p70s6k at Thr229, a site equivalent to Thr308 in PKB. The picture emerging for activation of p70s6k is similar to that for PKB in other aspects. In the proposed model for PKB activation, PI (3,4,5)P3 binding simultaneously localises this kinase to the plasma membrane and induces a conformational change which exposes the Thr308 site for phosphorylation by PDK1 (Gold et al 1994). Conformational inhibition in

p70s6k is released by MAPK action in the carboxyl terminus autoinhibitory domain of p70s6k. Thr389 is then phosphorylated by an unidentified PI (3,4,5)P3 dependent kinase, possibly mTOR. Both of these steps are necessary before PDK1 can phosphorylate p70s6k, resulting in a synergistic activation of the enzyme.

1.9.4 Protein kinase C

The PKC family has been subdivided into three groups: Ca²⁺/ DAG dependent, DAG dependent and atypical PKC's (Kochs et al 1993) (Mellor & Parker 1998). Several studies have provided evidence that the phosphoinositides, particularly PI (3,4)P2 and PI (3,4,5)P3, can activate certain isoforms of PKC (Toker et al 1994). *In vitro* however, PKC proteins have been shown to be promiscuous in terms of both substrate and activating agents, and only recently have studies began to address *in vivo* activation of PKC by D-3 phosphoinositides. Both PKC-ε (Moriya et al 1996) and PKC-λ have been shown to be activated by PI 3-kinase in agonist stimulated cells. In addition, overexpression of PI 3-kinase enhances PKC activation, whilst overexpression of a dominant negative p85 suppresses activation (Akimoto et al 1996). Toker et al studied the phosphorylation of pleckstrin to determine the contribution of phosphoinositides to activation of PKC in thrombin activated platelets, a process shown to be inhibited by wortmannin (Toker et al 1995). They found that the addition of synthetic PI (3,4)P2 and PI (3,4,5)P3 to permeabilized platelets could induce pleckstrin phosphorylation, which was not wortmannin sensitive, and also that in wortmannin treated agonist stimulated platelets, phorbol ester treatment could rescue aggregation, implicating PKC activation by phosphoinositides. The most likely *in vivo* targets of PI (3,4)P2 and PI (3,4,5)P3 are the calcium independent and atypical PKC's, which have been implicated in insulin stimulated GLUT4 translocation (Bandyopadhyay et al 1997; Kotani et al 1998; Standaert et al 1997).

1.9.5 Proteins involved in actin polymerization

D3 phosphoinositides also bind to a number of proteins involved in actin assembly, and actin rearrangement to form membrane ruffles is observed following insulin stimulation in most cell types. Although in many cases PI (4,5)P₂ is the most significant phosphoinositide regulating actin assembly, PI 3-kinase products have also been implicated in this process. For example, the ruffling effect is blocked by wortmannin and dominant negative p85 constructs (Wennstrom et al 1994). Also, N-formyl peptide induced actin polymerization correlates with elevated PI (3,4,5)P₃ (Eberle et al 1990) and it has been shown that the affinity of profilin with D-3 phosphoinositides is substantially higher than that of PI (4,5)P₂ (Lu et al 1996). Actin capping proteins such as profilin and gelsolin are inhibited by PI (3,4)P₂, enabling actin polymerization to occur, and under some conditions F-actin can induce actin polymerization by increasing the exchange of actin bound nucleotides.

1.9.6 Serine phosphatases

Insulin has been shown to regulate several protein phosphatases as well as kinases. Ragolia et al have shown that insulin regulates Na⁺/K⁺-ATPase activity in muscle cells by promoting dephosphorylation of the α -subunit by activating protein phosphatase-1 (Ragolia et al 1997). Activation of this phosphatase following insulin stimulation is inhibited by nanomolar concentrations of wortmannin, indicating that PP1 is a downstream target of PI 3-kinase. Similar results have been reported from studies in 3T3-L1 adipocytes (Brady et al 1998), and in this cell type, insulin stimulation of PP1 activity resulted in activation of glycogen synthase. Inactivation of protein phosphatase-2A by insulin has also been demonstrated (Begum & Ragolia 1996). Begum and Ragolia showed that in rat skeletal muscle cells, insulin leads to a rapid inactivation of PP2A with a concomitant 3-fold increase in tyrosine phosphorylation in the catalytic subunit of this enzyme, and that this inhibition could partly be overcome with wortmannin.

1.10 Investigating cellular functions of PI 3-kinases

1.10.1 Chemical inhibitors

Wortmannin, a fungal metabolite, was first shown to inhibit cellular responses to receptor stimulation *in vitro* in 1987 (Arcaro & Wymann 1993; Baggiolini et al 1987) and this was followed by the discovery that wortmannin added to rat adipocytes inhibited insulin stimulated glucose uptake (Okada et al 1994b). The identification of PI 3-kinase as the target of wortmannin (Arcaro & Wymann 1993) has helped implicate these enzymes in four apparently distinct cellular functions: mitogenic and metabolic signalling, inhibition of apoptosis, intracellular vesicle trafficking/ secretion and regulation of cytoskeletal elements. It has been shown that wortmannin reacts covalently with Lys-802 of the p110 α , making inhibition irreversible. This lysine residue is situated in the conserved core of the catalytic domain, and is crucial to the phosphate transfer reaction (Wymann et al 1996). Moreover, this residue is shared by all PI 3-kinases as well as a host of related molecules. Though the IC₅₀ value for the inhibition by wortmannin is in the low nanomolar range for classes 1a, 1b and 3 PI 3-kinases in mammalian cells, and addition to cells blocks ligand stimulated synthesis of PI(3,4)P₂ and PI(3,4,5)P₃, it only causes a 70% decrease in levels of PI 3P synthesis. In addition, wortmannin has been shown to inhibit a novel PI 4-kinase (Nakanishi et al 1995) so affecting cellular levels of PI (4,5)P₂, as well as DNA PK (Hartley et al 1995b) and mTOR (Shepherd et al 1997a). Results based solely on studies performed with these reagents must therefore be interpreted with caution. The compound LY294002 similarly inhibits PI 3-kinase (Vlahos et al 1994). Despite their limitations, both wortmannin and LY294002 are cell permeable, and great progress has been made in identifying pathways in which PI 3-kinases are involved.

1.10.2 Molecular approaches

In a similar but more definitive approach, pathways requiring PI 3-kinase have been probed using molecular reagents: dominant negative and constitutively active PI 3-kinase molecules have been used to determine whether this class of enzyme is necessary and/ or sufficient to elicit a particular response. Constitutively active p110 constructs have been shown to mimic some of the effects of insulin action. For example, in the elucidation of the pathway leading from insulin receptor activation to the inhibition of glucose-6-phosphatase gene transcription, expression of activated p110 is sufficient to almost entirely replicate the effect of insulin (Dickens et al 1998). Approaches which utilize activated p110 constructs are likely to prove valuable in studies of the pathological roles of PI 3'-kinases, since they will override mechanisms of downregulation which occur via the p85 subunit.

Studies in which cells are transfected with p85 constructs which lack the p110 binding motif (commonly termed Δ p85), which acts to dominantly block growth factor stimulation of class 1a PI 3'-kinases, are commonly used to show dependence on this enzyme downstream of receptor-induced activation. For example, inhibition of PI (3,4,5)P₃ synthesis in insulin stimulated CHO-IR cells transfected with Δ p85 has been linked with reduced glucose transport and GLUT1 translocation (Hara et al 1994), and Burgering and Coffey used a Δ p85 construct to demonstrate a role for PI 3-kinase in PDGF stimulated PKB and p70 S6K activation (Burgering & Coffey 1995). Sharma *et al* used an similar approach to interfere with complex formation between PI 3-kinase and its binding targets. In the studies described, adenovirus mediated overexpression of the p85-NSH2 domain blocked insulin stimulated glucose transport, DNA synthesis and glycogen synthase activity in 3T3-L1 adipocytes (Sharma et al 1998).

Recently, understanding of different PI 3-kinase isoforms has been furthered by gene targeting experiments. While most $p85\alpha$ - $p55\alpha$ - $p50\alpha$ $^{-/-}$ animals die within days of birth, several studies have recently been published which overcome this difficulty. The involvement of p85 in apoptosis, in a PI 3-kinase independent pathway, was shown by Yin *et al*, in MEF cells derived from mice with a targeted disruption of the $p85\alpha$ gene locus (Yin *et al* 1998). The RAG2 $^{-/-}$ deficient blastocyte complementation system (Chen *et al* 1993) can be used to circumvent the problem of early lethality. In this system, RAG2-deficient mice fail to rearrange antigen receptor genes, resulting in a complete absence of mature T or B cells. Injection of pluripotent ES cells into RAG2 $^{-/-}$ blastocysts gives rise to chimeric animals in which all lymphocytes are derived from the injected cells. Fruman *et al* isolated ES clones with a homozygous disruption of the $p85\alpha$ gene and injected them into RAG2 $^{-/-}$ blastocysts to show p85 was necessary for B cell development and proliferation (Fruman *et al* 1999). By specifically leaving $p55\alpha$ and $p50\alpha$ intact, and selectively abrogating $p85\alpha$ Suzuki, Terauchi and co-workers were able to link PI 3-kinase to the Btk pathway. The phenotype of these mice was nearly identical to those of X-linked immunodeficiency mice (*Xid* or Btk $^{-/-}$) (Suzuki *et al* 1999).

1.10.3 Bioactive membrane permeant phospholipids and phosphopeptides

Some peptides which mimic the SH2 binding domains of receptors can elicit responses which are downstream of PI 3'-kinase. The advantage of such an approach is that these peptides will activate endogenous p110 via the p85 subunit in a manner analogous to that of activation by RTK's (Derossi *et al* 1998). In addition to their research applications, such peptides may also have potential for development for novel therapeutics.

Biologically active membrane permeable forms of PI (3,4,5)P3 have also recently been developed (Jiang *et al* 1998). Stereospecific ester derivatives of PI (3,4,5)P3

have now been synthesized, and whilst only a little data is available so far, initial studies with these compounds are likely to be revealing. Jiang *et al* have shown that these uncharged lipophilic derivatives are able to deliver phosphatidylinositol 3,4,5-trisphosphate across cell membranes of 3T3-L1 adipocytes and T84 colon carcinoma monolayers. Though PI (3,4,5)P₃ alone was not sufficient to stimulate glucose transport in basal 3T3-L1 adipocytes, the phospholipid was capable of partially overcoming the inhibitory effect of wortmannin on insulin-stimulated glucose transport in these cells. The authors therefore propose a 'bifurcation of the insulin induced signal', one involving PI (3,4,5)P₃, the other independent of this product. Another possibility is that PI (3,4,5)P₃ requires more specific targetting, perhaps to a specific subcellular location. However, in T84 cells, the membrane permeant esters of PI (3,4,5)P₃ were well able to fully mimic the inhibitory effects of EGF on Cl⁻ secretion and efflux through K⁺ channels.

1.11 Endpoint responses to PI 3-kinase activation

1.11.1 Vesicle traffic and glucose transport

A role for D-3 phosphoinositides in the regulation of membrane traffic was initially proposed on the basis of the homology of mammalian PI 3-kinases with the yeast protein VPS34. Schu *et al* demonstrated that this protein was involved in vesicular transport following the observation that point mutations in the lipid kinase catalytic domain of VPS34 led to severe defects in vacuolar protein sorting (Schu *et al* 1993).

One of the major physiological functions of insulin is to stimulate the uptake of glucose from blood into target tissues of the hormone, such as muscle, fat and liver. There are five described mammalian genes encoding hexose transport proteins, with the insulin-sensitive glucose transporter, GLUT4, uniquely expressed in insulin target tissues. In the basal state, GLUT4 is retained on intracellular membrane structures, and rapidly cycled through the plasma

membrane/ endosomal membrane system in response to insulin. It has been shown that there are at least three intracellular pools of GLUT4; one representing constitutive recycling between the plasma membrane and early endosomes (Aledo et al 1997), one responsive to insulin (Munoz et al 1996) and one pool which is exercise sensitive (Coderre et al 1995). Subsequent studies have identified differences in the molecular makeup of these vesicles (Martin et al 1998). In addition, differential cycling levels of GLUT4 and transferrin following insulin stimulation suggest characteristics more often associated with synaptic vesicles than recycling endosomes (Martin et al 1998; Martin et al 1996b). Further evidence of a common pathway with regulated synaptic exocytosis comes from studies using blocking antibodies and peptide inhibitors of syntaxins and t-SNAREs (Cheatham et al 1996; Rea et al 1998).

D-3 phosphoinositides were implicated when initial studies demonstrated that insulin stimulated glucose transport was inhibited by low doses of wortmannin (Kanai et al 1993). Since then, activated p110 constructs have also been observed to have a stimulatory effect on glucose transport (Martin et al 1996a). Nevertheless, it is clear that stimulation of PI 3-kinase is insufficient to stimulate glucose transport, since neither PDGF (Kotani et al 1995; Nave et al 1996) nor PI 3'-kinase recruitment to IRS in response to interleukins (Isakoff et al 1995), is sufficient to stimulate glucose uptake. However, since PI (3,4,5)P₃ added to permeabilized cells in the presence of insulin and wortmannin rescues glucose uptake (Jiang et al 1998), it appears multiple steps under the control of insulin, including activation of PI 3'-kinase, are necessary to regulate glucose homeostasis. The current hypothesis is shown schematically in 1.3.

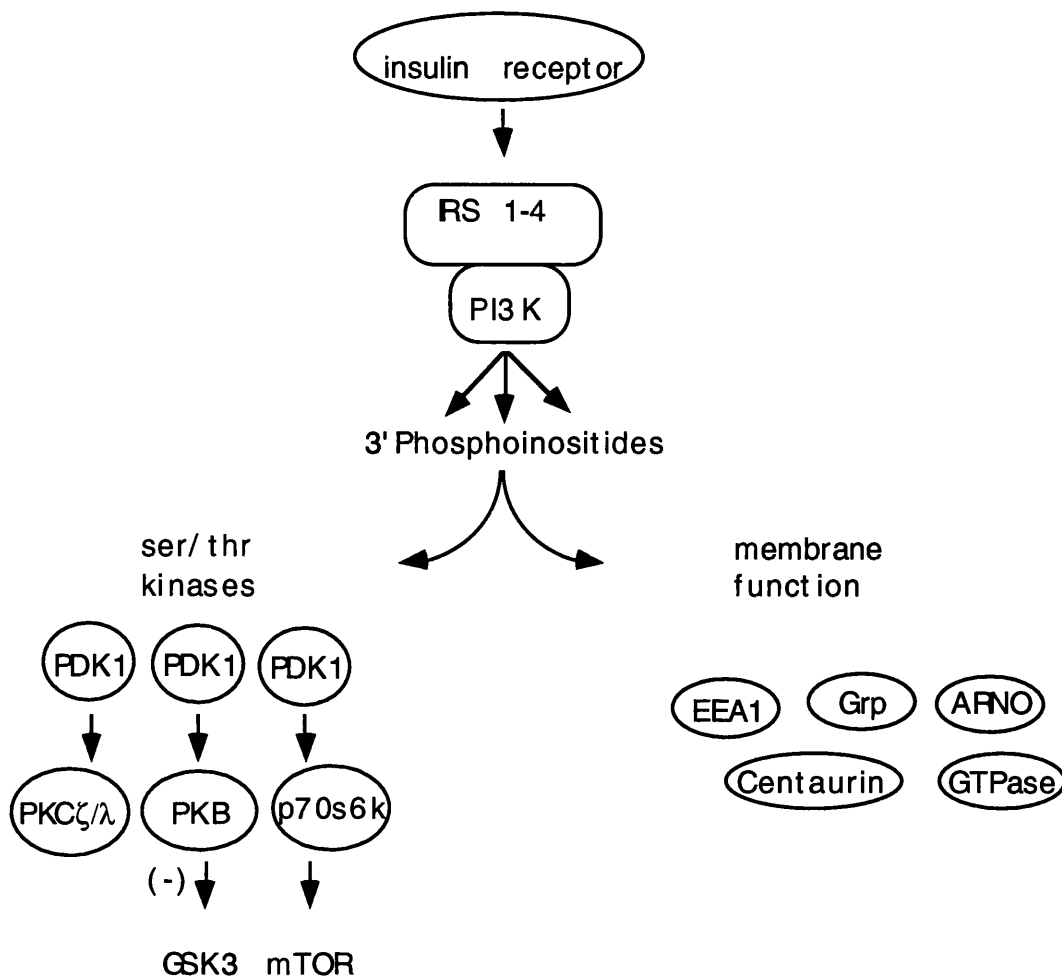


Figure 1.3 Signalling elements downstream of insulin activated PI 3'-kinase

The abbreviations used are: PI3K (PI 3'-kinase), PDK1 (phosphoinositide dependent kinase 1), PKC (Protein kinase C), PKB (protein kinase B), p70s6k (p70 S6 kinase), GSK3 (glycogen synthase kinase 3), mTOR (mammalian target of rapamycin). All molecules shown are discussed in detail in the text.

As discussed previously, one target for PI (3,4,5)P3 is PKB. Kohn et al have shown that activated PKB is sufficient to stimulate GLUT4 translocation using a PKB variant lacking its PH domain made constitutively active by membrane targeting via a Src myristoylation signal (Kohn et al 1996a). When expressed in 3T3-L1 fibroblasts, this activated PKB induced spontaneous differentiation into adipocytes as a result of constitutive lipogenesis. This was shown to be caused by elevated glucose uptake, accounted for by increased GLUT1 expression, as well as GLUT4 translocation elevated to near insulin stimulated levels. Other studies have also suggested activation of PI 3-kinase stimulates GLUT1 expression, and

wortmannin treatment causes intracellular accumulation of this hexose transporter, but this effect is independent of insulin (Kaliman et al 1995). It is noteworthy that the activated PKB construct does not stimulate glycogen synthesis, and in fact expression of activated PKB in adipocytes inhibits insulin stimulated glycogen synthesis by 70-80%.

Another likely component of the GLUT4 translocation machinery is Rab4. The Rab proteins are known to be involved in vesicle fusion and may interact with tether proteins such as EEA1. Rab4 colocalizes with GLUT4 containing cell fractions (Cormont et al 1993), and expression of Rab4 mutants inhibits insulin stimulated GLUT4 translocation (Shibata et al 1997). In addition, insulin has been shown to cause redistribution of this GTP binding protein from intracellular membranes to the cytosol, and unlike VAMP or cellubrevin, insulin stimulates the guanine nucleotide exchange of Rab4 in a PI 3-kinase dependent manner (Shibata et al 1997).

1.11.2 Apoptosis

PI 3-kinase plays a dual role in cell growth and transformation and has been shown to be necessary for both cell division and cell survival (Isakoff et al 1995; Roche et al 1994b). Following the initial discovery of a PI 3-kinase activity associated with oncoproteins, extensive research has implicated this enzyme family in a number of mechanisms of cell survival. The first clear evidence came from studies of PC-12 cells, which Yao and Cooper used to demonstrate that apoptosis induced by serum withdrawal was prevented by wortmannin and LY294002 (Yao & Cooper 1995). It has also been shown that the certain anti-tumour ether lipids inhibit PI 3-kinase (Berrgren et al 1993).

More recently, it has emerged that some of the anti-apoptotic effects of PI 3-kinase activation are mediated by PKB. In neuronal cells, LY294002 blocks cell

survival, as does expression of catalytically inactive PKB, or the PH domain of PKB. Furthermore, overexpression of wild type PKB enhances survival of cerebellar neurons, an effect which was not inhibited by LY294002 (Philpott et al 1997). The PI 3-kinase/ Akt pathway has also been shown to overcome apoptosis mediated by activation of p38 (Berra et al 1998). Inhibition of Erk (extracellular signal-regulated kinase) by serum withdrawal activates p38 and triggers caspase activation, resulting in programmed cell death; this process is inhibited by expression of dominant negative p85 or PKB. Ras mediated inhibition of anoikis (see section 1.4.1.2), programmed death which prevents cells surviving in inappropriate locations, is also mediated via PI 3-kinase. Khwaja et al demonstrated that activation of PI 3-kinase blocks DNA damage induced apoptosis, and inhibition of PI 3-kinase or PKB reverses protection from anoikis conferred by activated Ras (Roche et al 1994b). The fact that high levels of PKB are frequently present in human tumors may explain the molecular basis of cell survival under conditions in which apoptosis would normally prevent malignant growth.

The Bcl-2 protein protects cells from activation of caspase machinery and subsequent apoptosis only if it exceeds levels of pro-apoptotic factors such as Bcl-X_L and BAD. By dimerizing with Bcl-2, BAD and Bcl-X_L stifle the anti-apoptotic effects of this protein. Evidence is now appearing that PI 3-kinase/ PKB protection from apoptosis is mediated by phosphorylation of BAD (Datta et al 1997). Serine phosphorylation of BAD promotes its sequestration by 14-3-3, which prevents dimerization of this molecule with Bcl-2.

Conversely, it has also been shown that disruption of the full length p85 α gene inhibits apoptosis induced by oxidative stress (Yin et al 1998). Yin et al demonstrated that in MEF cells, p85 is upregulated by p53 and is involved in p53-mediated apoptosis in response to H₂O₂. Apoptosis in response to oxidative stress

is not sensitive to wortmannin, suggesting there exists a role in the control of cell survival for p85 which is distinct and opposite to that of p110.

1.11.4 Glycogen synthesis

Although dominant negative forms of p85 do not block insulin stimulated glycogen synthase in CHO-IR cells (Yamamotohonda et al 1995), there is considerable evidence that glycogen synthesis is downstream of PI 3-kinase. Glycogen synthase activity is regulated at least in part by serine phosphorylation, and insulin stimulates dephosphorylation, and hence activation, of glycogen synthase (Brady et al 1998). Whilst insulin and PDGF caused a similar inhibition of GSK3 in adipocytes, only insulin stimulation resulted in elevated PP1 and glycogen synthase activity. Wortmannin completely blocked the ability of insulin to stimulate PP1 activation. From their studies of 3T3-L1 cells, Brady et al have proposed a model in which different mechanisms of insulin stimulation of glycogen synthase occur depending on cell type, resulting in different activation responses. In fibroblasts, the low level of glycogen synthase activation stimulated by insulin is mediated by GSK3 inactivation, whereas in adipocytes, the large stimulation of synthase activity is mediated by insulin stimulated PP1 activation, and this effect lies downstream of PI 3-kinase.

1.11.5 Lipid metabolism

The effect of insulin on lipid metabolism in adipocytes was first shown to be PI 3-kinase dependent by Okada et al, who demonstrated that wortmannin blocked the antilipolytic action of insulin without altering the basal rate or isoproterenol-stimulated lipolytic rates (Okada et al 1994a). In addition to inhibiting lipolysis, insulin can also stimulate lipid synthesis.

Fatty acid synthesis from glucose requires glucose uptake, and activation of pyruvate dehydrogenase (PDH) and acetyl-CoA carboxylase (ACC). PDH has been shown to be activated by dephosphorylation, but this element of the pathway is insensitive to wortmannin. On the other hand, ACC activity is stimulated by insulin in a PI 3-kinase dependent manner (Moule et al 1995).

The ability of insulin to antagonize hormone-activated lipolysis in adipose tissue is in part due to insulin mediated activation of some members of the PDE 3 gene family. The subsequent inhibition of protein kinase A lowers phosphorylation and thereby activity of hormone sensitive lipase (HSL) (Rahn et al 1996). Wortmannin has been shown to block phosphorylation of PDE, implicating PI 3-kinase in inactivation of HSL (Wijkander et al 1998).

In liver, PI 3-kinase may mediate VLDL production via its action on apolipoprotein B (apoB). ApoB is a major structural component of low density lipoproteins and is required for the secretion of triglyceride-rich lipoproteins, and the synthesis and secretion of apoB are insulin regulated (Sparks & Sparks 1994). The downregulation of apoB by insulin has been shown to result from increased degradation of newly synthesized apoB protein and this process is blocked by wortmannin (Sparks et al 1996). Phung and co-workers have shown by subfractionation of LDM on sucrose density gradients that PI 3-kinase and apoB both colocalize to rough ER following insulin stimulation, which correlates with the site of lipoprotein assembly. Insulin induced degradation of apoB in rat hepatocytes occurs in a post-ER compartment, and the colocalization of PI 3-kinase at RER, with its well established role in vesicular traffic, may provide a mechanism for transporting apoB to be degraded.

1.11.6 Cytoskeletal rearrangement

PI 3-kinases have been implicated in the regulation of the actin cytoskeleton since microinjection of p85 lacking a p110 binding site was shown to inhibit insulin stimulated membrane ruffling in KB cells (Kotani et al 1994). Furthermore, microinjection of activated p110 α is sufficient to induce membrane ruffling or stress fiber breakdown in 3T3-L1 adipocytes (Martin et al 1996c). More recently, studies using partial loss of function Ras mutants demonstrated that actin rearrangement was dependent upon the ability of Ras to stimulate PI 3-kinase (Rodriguez-Viciana et al 1997). In addition, it has been shown that PI 3-kinase associates with α -actinin via the SH3 domain of p85 (Shibasaki et al 1994). α -actinin, as well as other actin binding proteins such as gelsolin and profilin contain PI (4,5)P₂ as bound lipid, and these proteins have been shown to stimulate PI 3-kinase activity (Singh et al 1996).

Rac1 has been shown to mediate growth factor induced membrane ruffling via actin reorganization at the plasma membrane (Ridley et al 1992) and in vivo association of Rac and PI 3-kinase stimulated by PDGF (Tolias et al 1995) suggesting that PI 3-kinase mediates membrane ruffling in a mechanism involving GTPases. Reif et al subsequently demonstrated that constitutively activated PI 3-kinase induces a selective subset of Rac dependent pathways, including cytoskeletal rearrangement, but does not induce Rac or Rho dependent gene transcription (Reif et al 1996). In yeast, the PI 3-kinase homologue Tor2 controls cell cycle dependent organization of the actin cytoskeleton via the GTPase Rho1 (Helliwell et al 1998). Tor2 activates Rho via a GDP/ GTP exchange factor, and Tor2 mutants have defective actin organisation (Schmidt et al 1996). Some, but not all Rho1 mutants have severe effects on the yeast cytoskeleton, and these defects can be suppressed by overexpression of PKC1 (Helliwell et al 1998). Helliwell et al also demonstrated that overexpression of the PKC controlled MAP kinase could suppress the actin defect of the Tor2 and Rho1 mutants. This suggests

that in yeast, actin is regulated by Tor2 activating Rho subsets which stimulate the PKC regulated MAP kinase. It remains to be determined if a similar mechanism operates in mammalian cells, or whether a shorter linear pathway controls actin organization in these cells.

The role of PI 3-kinase association with microtubules (Kapeller et al 1993) is less well established. Nevertheless, it has been shown that insulin stimulated phosphorylation of tubulin occurs (Matten et al 1990) and microtubule dependent processes are known to occur in response to growth factor activation. γ -tubulin is the proposed link between the microtubule organizing centre and microtubule polymers, since it can nucleate microtubule formation and bind the centrosome. It has been shown by Kappeller et al that p85/ p110 associates constitutively with α/β -tubulin, and with the γ -isoform following insulin stimulation. This occurs to a lesser extent following cell stimulation with PDGF, but not at all with EGF or NGF, suggesting PI 3-kinase could mediate microtubule responses to insulin. Although γ -tubulin is phosphorylated in vivo in response to insulin, recombinant p85 was able to associate with γ -tubulin from quiescent cells, suggesting that the insulin induced association is dependent in a conformational change in PI 3-kinase, not tubulin. The interaction with α/β -tubulin was shown to be mediated by the inter-SH2 domain of p85, though this domain alone cannot bind γ -tubulin (Kapeller et al 1995).

Preliminary studies by Inukai et al suggest the 55 kDa adapter associated PI 3-kinases may be acutely regulated by insulin with respect to cytoskeletal interactions (Inukai et al 1998). Using GST fusion proteins of the 34 amino acid extensions of p55 α and p55 γ to screen brain cell extracts, β -tubulin was identified as a target protein. Determination of the association of all five PI 3-kinase adapter isoforms with tubulin coexpressed in insect cells confirmed this finding, and

experiments in CHO-IR cells indicate the association of p55 proteins with β -tubulin is stimulated by insulin.

1.12 PI 3-kinase and disease

Given the diversity of signalling pathways in which there exists a necessary role for PI 3-kinase, it is reasonable to suspect that factors which alter its activity, such as reduced expression, changes in activators of the enzyme and molecular changes in phosphoinositide target molecules have the potential to contribute to the development of disease. Indeed, p85 was first identified as a common element in middle-T antigen transformation and PDGF stimulation of cells, immediately presenting a molecular link between growth factors and oncogenesis (Kaplan et al 1987).

Peripheral insulin resistance is a primary defect in the pathogenesis of type 2 diabetes (Beck-Nielsen & Groop 1994; DeFronzo 1988; Kahn 1998). Given the key role of PI 3-kinase in insulin signalling, intensive study has focused on the search for defects in PI 3-kinase activity and localisation as a causative factor in insulin resistance (Haring & Mehnert 1993). There is significant support for such a theory from studies of animal models. In the *ob/ob* mouse, which is a model of obesity and NIDDM, changes in the expression of the PI 3-kinase regulatory subunit have been reported (Kerouz et al 1997). In the livers, but not the muscle of these mice, expression of p55 α was increased nine-fold compared to control mice, whilst expression of p85 was reduced. The early steps of signalling by insulin are greatly altered in *ob/ob* mice, and insulin binding is reduced due to a decrease in receptor number. The mechanism which perturbs p85 α gene splicing in these animals is unknown, although a similar pattern of upregulation of p85 splice variants is observed in other models of obesity, such as the *db/db* and Agouti mice.

Despite numerous studies, evidence for altered expression of PI 3-kinase isoforms in human subjects has been more elusive. However, it has been shown that decreases in insulin stimulation of PI 3-kinase occur in insulin resistant subjects and diabetics (Goodyear et al 1995; Rondinone et al 1997), and there are also reports of polymorphisms in the regulatory subunits in populations of insulin resistant patients. In p85 α , Met³²⁶Ile (Baier et al 1998; Hansen et al 1997) and Arg⁴⁰⁹Gln (Baynes, K. 1999, in press) have been identified in two populations with insulin resistance.

While impairment of PI 3-kinase activation may or may not cause insulin resistance, there is now strong evidence that unregulated activation of this enzyme has an oncogenic effect. The regulatory subunits of PI 3-kinase are known to associate with a number of proto-oncogenes including c-cbl (p85 β) (Hartley et al 1995a), Ras (Rodriguez-Viciana et al 1997) and middle-T antigen (Whitman et al 1985) and activate others including PKB (Franke et al 1995) (Franke et al 1997). A transforming retrovirus encoded PI 3-kinase has been isolated from chicken haemangiosarcomas (Chang et al 1997), and a number of oncogenes derived from the regulatory subunit of PI 3-kinase have also been identified. Jimenez and co-workers generated murine lymphomas by X-ray irradiation and identified a novel 65 kDa protein which cross reacted with antibodies to p85 α (Jimenez et al 1998). Sequencing revealed this molecule to encode the first 571 residues of p85, and the remaining 24 amino acids were homologous to Cys-rich domains found in members of the eph Tyr kinase receptor family. The PI 3-kinase activity associated with p65 was double that associated with p85/ p110, linking PI 3-kinase to mammalian tumor development. An oncogenic fusion product incorporating p85 β has also been identified. Janssen et al induced tumor formation in mice using peripheral blood cell DNA from a patient with a myeloproliferative disorder (Janssen et al 1998). Tumorigenicity was shown to result from a chimeric protein incorporating the first 582 residues of p85 β , and the

His and Cys domains of HUMORF8, a protein previously isolated from immature myeloid cells which has homology to a human oncogene, *trc*.

Increased levels of PI 3-kinase activity have also been observed in colorectal tumors (Phillips et al 1998). In normal and transformed colonic mucosa, almost four times as much PI 3-kinase activity was immunoprecipitated from tumors. Although slightly larger amounts of p85 were immunoprecipitated, this was not sufficient to account for the increased levels PI 3-kinase activity observed in the transformed cells. Western blotting revealed that p85 isolated from tumors had a slightly faster mobility as judged by SDS-PAGE, which was unaffected by alkaline phosphatase treatment. Since the antibody used in the precipitations recognised both p85 α and p85 β , differential expression of PI 3-kinase regulatory isoforms in the transformed and normal colonic mucosa cannot be ruled out.

Overexpression of p110 has been shown to induce DNA synthesis (Frevort & Kahn 1997; Roche et al 1998b) and p110 α has been shown to be mitogenic (Vanhaesebroeck et al 1999), suggesting activated PI 3'-kinase is likely to contribute to cancer by both pro-mitogenic and anti-apoptotic effects. Protein tyrosine phosphatases could potentially suppress tumor development by antagonizing the effects of growth factor stimulated RTK's. For example, glioblastomas are common and malignant cancers, often characterized by constitutive activation of EGF dependent signalling pathways. Tumor suppressor gene products might normally attenuate these signalling pathways, and it is conceivable that loss of function of genes involved in the downregulation of these pathways enables tumor development. PTEN or MMAC1 is a tumor suppressor with homology to the protein tyrosine phosphatase family, and germ line mutations have been identified in a variety of neoplastic disorders. PI (3,4,5)P3 was recently identified as a PTEN substrate by Maehama and co-workers (Maehama & Dixon 1998), see figure 1.4. Subsequent work shows that PTEN

overexpression in HEK293 cells results in decreases in PI (3,4,5)P3, and expression in glioblastoma cell lines disrupts signalling downstream of PI 3-kinase to PKB and BAD (Myers et al 1998b). Significantly, expression in a prostate cancer cell line abrogates cell survival, in a mechanism which is inhibited by expression of constitutively active PKB. Thus it appears that the PI (3,4,5)P3 phosphatase activity of PTEN is critical to its tumor suppressor function, further implicating PI 3-kinase activation to tumorigenesis (Wu et al 1998).

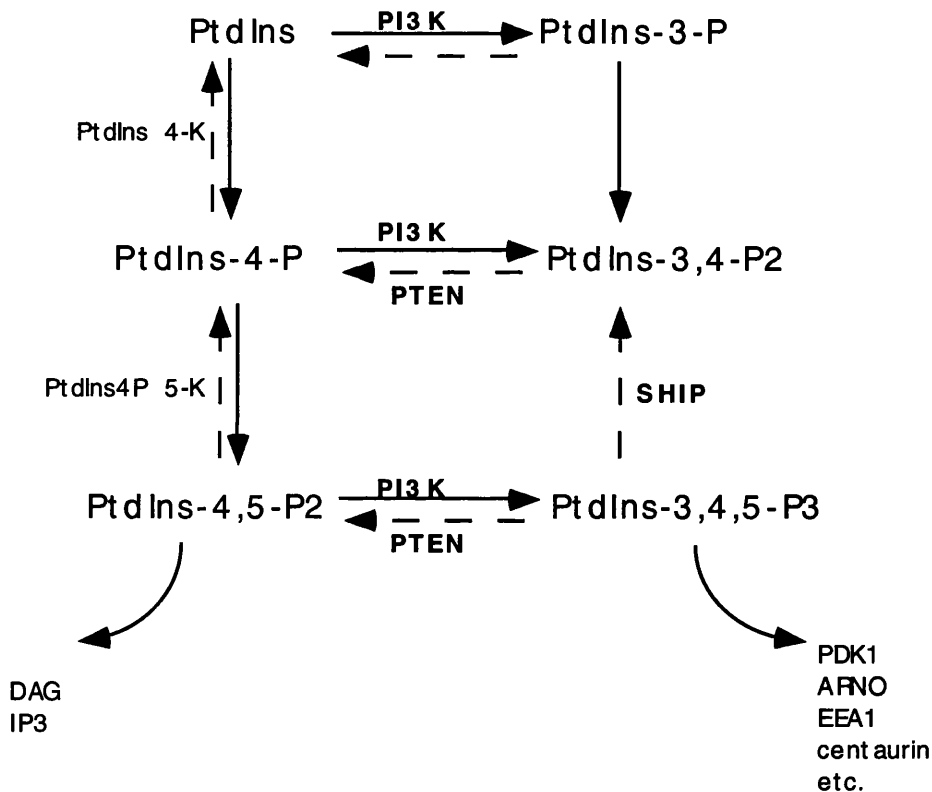


Figure 1.4 PTEN restrains the PI 3'-kinase/ PKB pathway

Class 1 PI 3'-kinases phosphorylate the D-3 position of the inositol ring of phosphatidylinositides to produce PtdIns-3-P, PtdIns-3,4-P2 and PtdIns-3,4,5-P3. PTEN (phosphatase and tensin homolog deleted on chromosome ten) has been shown to dephosphorylate the D-3 of PtdIns-3,4,5-P3 and PtdIns-3,4-P2, thereby reversing the effects of PI 3'-kinase and inhibiting signalling downstream of PI 3'-kinase/ PKB.

Aim of thesis

The studies presented in this thesis were initiated following the publication of two independent papers describing the cloning of two splice variants of the class 1a PI 3'-kinase adapter subunit, p85 α (Antonetti et al 1996; Inukai et al 1997). The aim of the work presented in this thesis was to provide information on the ways in which the different adapter and catalytic subunits of PI 3'-kinase contributed to specificity in signalling pathways. A range of biochemical and computational techniques were employed to facilitate the following studies:

1. Characterization of the role of the BH and SH3 domains of p85 α in PI 3'-kinase catalytic activity.
2. Comparison of the kinase activities of p110 α and p110 β .
3. Determination of the kinetic properties of p110 α and p110 β .

2. METHODS

2. Materials

Tissue culture materials such as medium, PBS, trypsin, and serum, were obtained from Sigma. Chemicals were from Sigma, unless otherwise stated. Monoclonal anti-myc antibody (9E10) was a generous gift from Gerard Evan (ICRF). Polyclonal anti-FLAG antibody was from Santa Cruz (catalogue no. SC-807). Polyclonal anti-p85 antibody, raised against the N-SH2 domain, was provided by Kenneth Siddle (University of Cambridge). Agarose conjugated protein-A (catalogue no. P-1406) and agarose conjugated anti-mouse IgG (catalogue no. A-653) were from Sigma. $\gamma^{32}\text{P}$ -ATP, ^{32}P -orthophosphate, ^{35}S -methionine and ^{125}I -protein A were from Amersham. Phosphoinositides were obtained from Lipid Products, Nutfield Nurseries, Crab Hill Lane, South Nutfield, Redhill, Surrey, RH1 5PG. The source of other reagents and facilities are stated in the text as necessary.

2.1 Nucleic acid manipulation

2.1.1 Preparation of DNA

2.1.1.1 Manipulation of DNA by the Polymerase Chain Reaction

PCR amplifications were performed in 50 μl reaction volumes containing 1 X Thermopol Buffer (20 mM Tris-HCl, (pH 8.8 at 25°C) 10 mM KCl, 10 mM $(\text{NH}_4)_2 \text{SO}_4$, 0.1% Triton X-100), 200 μM each dNTP, 10 ng template DNA, 20 pmol primers and 0.5 units Vent DNA polymerase (all purchased from New England Biolabs). The reaction mixture was overlaid with mineral oil (Sigma Chemical Company). Amplifications were performed in a Techne PROGENE

THERMOCYCLER. Following a 2 minute 'hot start' at 92°C, samples were denatured at 92°C for 30 seconds, and extended at 72°C for 1 minute / KB.

Annealing temperatures were calculated for each primer using the equation

$$T_m (\text{°C}) = (2(A+T) + 4(G+C)) - 1$$

For each pair of primers, the lowest value was used as the annealing temperature.

The completed PCR reaction was removed from below the mineral oil layer, and the extension products were resolved by TAE/ agarose gel electrophoresis. The DNA band was excised from the gel and purified using a QIAX II kit (QIAGEN).

2.1.1.2 DNA digestion with restriction enzymes

All restriction enzymes used were purchased from New England Biolabs. 0.5 µg of DNA were digested in a reaction volume of 20 µl. Restriction enzyme digests were performed in NEB buffers as described in the NEB catalogue Buffer Chart, containing a total of 2µl enzyme. Reactions were incubated at 37°C for one hour.

2.1.1.3 Electrophoresis of DNA fragments

Agarose was dissolved in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) by heating, cooled and ethidium bromide added to a concentration of 1µg/ml before gel casting.

DNA fragments of less than 1Kb in size were resolved on 1.5% (w/v) agarose gels, and 0.7% (w/v) gels were used for resolving fragments of greater than 1 Kb. 6X gel loading buffer (0.25% (w/v) xylene cyanol, 0.25% (w/v) bromophenol blue, 30% glycerol dissolved in water) was added to DNA samples, which were electrophoresed in TAE buffer at 60 mA. One Kb standard (Gibco/ BRL) were

electrophoresed simultaneously. Resolved DNA bands were visualised by illuminating the gel on a UV light box.

2.1.1.4 DNA extraction and purification from agarose gels

DNA fragments of interest were excised from the gel using a clean sharp scalpel. DNA was purified from the gel slice using the QIAEX II gel extraction kit (Qiagen). In brief, the gel slice was solubilised by adding 3 volumes of Buffer QX1, and incubated with 10µl QIAX beads for 10 minutes at 50°C. DNA binds the silica resin beads, and impurities (agarose, protein, ethidium bromide) are washed away. A high salt wash of the bead-DNA pellet in Buffer QX1 removes residual contaminants, and two further washes in an ethanol containing wash remove salt contaminants. The pellet was air dried, and DNA eluted with ddH₂O.

2.1.1.5 DNA isolation and extraction from acrylamide gels

Very small DNA fragments (less than 200 bp) are most easily resolved using a polyacrylamide gel. Polyacrylamide gels for DNA isolation are cast using 6% acrylamide in 1X TBE, and polymerisation is initiated by the addition of 0.1% ammonium persulphate, and 0.01% TEMED. Samples for electrophoresis are prepared in well loading dye as described for agarose electrophoresis (section 2.1.1.4), loaded and electrophoresed in 1 X TBE at 24 mA, until the dye front has migrated through 3/4 of the gel.

The electrophoresed samples were visualised by soaking (without shaking) the gel in 1µg/ml ethidium bromide in water for 20 minutes, rinsing in ddH₂O, and illuminating by UV. Gel slices containing the DNA fragment of interest were cut into small pieces using a sharp scalpel, and DNA was eluted in a microfuge tube by the addition of 2-3 volumes of 0.2% SDS, 0.3M sodium acetate (pH 5.0) and incubating at room temperature for 18 hours. Tubes were then centrifuged at

13000 rpm for 5 minutes in a bench top centrifuge, and eluted DNA (in the supernatant) was transferred to a fresh eppendorf tube. DNA was ethanol precipitated by the addition of 3 volumes 100% ethanol at -20°C, and washed in 70% ethanol before being resuspended in ddH₂O. The oligonucleotide encoding the triple MYC epitope tag was isolated by this procedure.

2.1.2 DNA ligation and transformation

2.1.2.1 Ligation of DNA fragments

Ligation reactions contained a 3-fold molar excess of insert DNA relative to plasmids. Ligations were performed in a 10µl reaction volume containing 1X ligation buffer (120 mM Tris-HCl (pH 7.6), 12 mM MgCl₂, 15 mM DTT and 1.2 mM ATP) and one µl T4 DNA ligase. 100ng total DNA was used, and reactions were incubated at 4°C for 16 hours. A reaction with no insert DNA was performed in parallel as a control.

2.1.2.2 Preparation of competent *E.coli*

Competent *E.coli* JM109 cells for subcloning were prepared by inoculating 20ml TYM (2% Bactotryptone, 0.5% yeast extract (DIFCO Laboratories no. 0123-17-3 and no.0127-17-9), 0.1M NaCl, and 10mM MgSO₄), in a 250 ml flask with a single colony from a freshly streaked agar plate. These were incubated with agitation at 37°C until cells had reached midlog phase (OD₆₀₀~0.2-0.8). The culture was then diluted in 100ml TYM in a 2L flask and grown until cells had reached midlog phase again. Finally the culture was diluted in 500ml TYM and grown to an OD₆₀₀~ 0.6 was reached. Once the culture was cooled rapidly in an iced water bath, the cells were pelleted in a Beckman J6 centrifuge at 4200rpm for 15 minutes. The supernatant was decanted and the cells washed in 100ml buffer TfbI (30mM KC₂H₃O₂, 50mM MnCl₂, 100mM KCl, 10mM CaCl₂, 15%

(w/v)glycerol). After repelleting (4200rpm, 8 minutes), the supernatant was decanted and the cells resuspended in 20ml buffer TfBII (10mM C₇H₁₄NO₄SNa pH 7.0, 75mM CaCl₂, 10mM KCl, 15% (w/v) glycerol). Aliquots were snap frozen in liquid nitrogen, and the competent cells were transferred to a -70°C freezer for storage.

2.1.2.3 Transformation of *E. coli*

Transformation of cells was achieved by heat shocking either 0.5 µg plasmid DNA or 2µl of ligation reaction with 100µl of freshly thawed competent JM109 cells at 42°C for 2 minutes, followed by 10 minutes on ice. Cells were allowed to recover by adding 1ml of Luria Broth (LB) (prepared as described in Manniatis et al) and shaking the cells at 37°C before streaking on agar plates containing 100 µg/ml ampicillin and incubating overnight at 37°C.

2.1.3 Purification of DNA

2.1.3.1 Miniprep plasmid purification

This procedure was used predominantly to screen bacterial colonies for recombinant DNA plasmids.

2ml LB supplemented with ampicillin (100µg/ ml) inoculated with a single colony was incubated with shaking at 37°C overnight and cells harvested by centrifugation at 400rpm for 5 minutes. The cell pellet was resuspended in 100 µl buffer (25 mM Tris-HCl (pH 8), 10 mM EDTA), before 200µl of lysis buffer were added (0.2 M NaOH, 1% SDS). The lysate was mixed by inversion and incubated on ice for 5 minutes, before the reaction was neutralised by the addition of 5 M potassium acetate. Cell debris was precipitated by centrifugation, and the

supernatant containing plasmid DNA was transferred to a new tube. Protein and other debris were removed by extracting with an equal volume of TE saturated phenol/ chloroform, and the aqueous layer was transferred to a clean eppendorf. DNA was precipitated by the addition of 200µl of isopropanol and microfuging for 20 minutes at 14000 rpm. Precipitated DNA was washed with 70% ethanol, before the air dried pellet was resuspended in 20µl ddH₂O.

2.1.3.2 Maxiprep plasmid purification

For large scale preparations of plasmid DNA the Qiagen Maxi-prep kit was used following the manufacturers instructions. Preparations were made from host cells grown in liquid cultures of 100 ml which were pelleted by centrifugation at 6,000g for 15 minutes at 4°C. The plasmid purification protocol is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to Qiagen anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins, dyes and low molecular weight impurities are removed by a medium salt wash. Plasmid DNA is eluted in a high salt buffer and then concentrated and desalted by isopropanol precipitation. DNA is collected by centrifugation at 15,000g for 30 minutes at 4°C, washed with 70% ethanol and air dried. The DNA pellet is resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and the concentration determined. Purified plasmid DNA is suitable for downstream applications, such as DNA sequencing, *in vitro* transcription/translation, or transfection experiments.

2.1.3.4 Quantitation of DNA concentration

DNA concentration was determined by diluting preps 1:100 in ddH₂O and determining OD₂₆₀ values. Concentration was then calculated from the equation:

$$OD_{260} = 1 = 50 \mu\text{g/ml ds DNA}$$

2.1.4 Plasmid manipulation for expression in mammalian cells

2.1.4.1 Construction of myc epitope tagged p85 α , p85 α Δ SH3 and p85 α Δ BCR

All primers used were purchased from Pharmacia and are shown in Table 2.1. A general schematic for generating myc-epitope tagged p85 expression constructs is shown in Figure 2.1. The DNA sequences of the final protein expressed are shown in Appendix 1. The polymerase chain reaction was used to modify wildtype bovine p85 α by inserting a BamHI restriction site prior to the start codon to remove excess 5'-untranslated sequence. The fragment obtained was sequenced and used to replace a 5' untranslated region of cDNA, and the entire coding sequence of p85 α was then subcloned into pBluescript, using the BamHI and EcoRI sites at the 5' and 3' ends respectively.

The polymerase chain reaction was again used to further modify p85 α by replacing the stop codon with an in-frame NcoI site. A fragment was generated from residue 1549 to 2180 using a sense primer which incorporated an Nco I site. This was subcloned into LITMUS 28 using the NsiI and NcoI restriction sites. The 5' region of p85 α (residues 1 to 1573) was isolated from pBluescript (Invitrogen) by XbaI and partial NsiI digestion and subcloned into the LITMUS 28 construct just described at the SpeI and NsiI restriction sites.

In addition, the triple myc epitope (single epitope sequence QKLISEEDLNYDE) was fused to the 3'-end of the construct using in-frame NcoI and XbaI restriction sites. The tagged p85 α (p85 α .M3) was then cloned into pCDNA3 (Invitrogen) using the BamHI and XbaI restriction sites.

Bovine p85 α Δ SH3 (Δ 2–78) was a gift from J. Linacre (LICR) and was myc epitope tagged by subcloning the 5' region into the LITMUS 28 (New England Biolabs) p85 α .M3 construct at the BamHI and HindIII sites. This was subcloned into pCDNA3 at the BamHI and XbaI restriction sites. Bovine p85 α Δ BCR (Δ 119-256), also a gift from J. Linacre, was epitope tagged and subcloned for expression in mammalian cells using the same strategy (see figure 2.1 for schematic). Primers and annealing temperatures used are shown in Table 2.1.

2.1.4.2 Site directed mutagenesis

Point mutations were created in plasmids by site directed mutagenesis using the QUIKCHANGE SITE-DIRECTED MUTAGENESIS in collaboration with K. Baines (University of Cambridge). Primers containing the desired mutation (encoding S608A or S608E) were used in a PCR reaction to generate nicked circular plasmid DNA containing the desired mutation. PCR reaction volumes, dNTPs, primer and template concentrations were as described in section 2.1.1.1, except that a range of plasmid concentrations were used (5, 10, 20, 50 ng of ds DNA template). *PfuTurbo* DNA polymerase from Stratagene was used and 2 min/kb of plasmid length was allowed for the extension time. The enzyme Dpn 1 was used to digest parental methylated DNA, by adding it to the PCR reaction and incubating at 37°C for 1 hour. 1 μ l of Dpn 1 treated PCR reaction was used to transform *E. coli* XL1-Blue Supercompetent Cells. These cells were transformed by incubating on ice with the DNA for 30 minutes, before pulse heating at 42°C for 45 seconds. 0.5

ml of NZY+ broth (prepared as described in (Maniatis et al 1989)) heated to 42°C was then added to the cells, which were then incubated at 37°C with shaking for 1 hour. Pelleted cells were then streaked onto agar plates containing 100 µg/ ml Ampicillin. DNA was purified from colonies and sequenced.

2.1.4.3 Construction of FLAG epitope tagged p110α

Human p110α cDNA was a gift from M.D. Waterfield. The coding region was isolated by restriction digest with BamHI and BglII and subcloned into pCDNA3 using the BamHI site. Orientation of p110α was confirmed by restriction enzyme analysis.

The construct was modified using the polymerase chain to add the FLAG epitope. A fragment was generated from residue 2286 to 3216 using a sense primer which incorporated a quadruple glycine linker and the FLAG epitope. This product was digested to give a 614 bp fragment which was sequenced and used to replace the 3' coding region of p110α in pCDNA3 by subcloning at the ClaI and NotI sites. These manipulations are shown schematically in Figure 2.2.A, and the oligonucleotides are shown in Table 2.1.

2.1.4.4 Construction of FLAG epitope tagged p110β

Human p110β cDNA was a gift from M.D. Waterfield. The coding region was purified and subcloned into pSL301 (Invitrogen) at the HindIII and EcoRI restriction sites. A FLAG epitope with a quadruple glycine linker and stop codon was generated with BglII (5') and EcoRI (3') overhangs by annealing two oligonucleotides (sense, and antisense). The product was inserted into the pSL301.

p110 β construct at the BglII and EcoRI restriction sites. The FLAG epitope tagged p110 β was excised at the HindIII and EcoRI restriction sites and cloned into pCDNA3 for expression. These manipulations are shown schematically in Figure 2.2.B, and the oligonucleotides are shown in Table 2.1.

2.1.4.5 IRS-interSH2 construct

An expression construct of IRS1 containing the p110 binding region of p85 α was a generous gift from Richard Roth (University of San Francisco, California).

Table 2.1 PI 3'-kinase PCR strategies

template	aim of PCR	primer sequence
pGEX2T	sense primer, n1-16 introduces BamHI site	TTTGGATCCATGAGTGCCGAGGGG T
	anti-sense primer, n408-426	TTTCTTTTCAATGGCTT
pBS.p85	sense-primer, n1543-1558	GAAGGCAACGAGACGG
	anti-sense primer, n2165-2172 introduces NcoI site	TTTCCATGGTTCGCCTCTGCTGCG CG
pCDNA3.p85 α .M3	n1812-1842, for point mutation of Ser608 to alanine	GAA GAC CAA TAT <u>GCG</u> CTG GTA GAA GAT GAT
pCDNA3.p85 α .M3	n1812-1842, for point mutation of Ser608 to glutamic acid	GAA GAC CAA TAT <u>GAG</u> CTG GTA GAA GAT GAT
pCDNA3.p110 α	sense primer, n2286-2314	AAATCTCAGGCTTAAAGAGTGTCG AATTA
	antisense primer, n3163-3202 incorporates quadruple glycine linker and FLAG epitope	ACAGTTTAGCGGCCGCTACTTGTC ATCGTCGTCCTTGTAGTCCCCCC CCCCCTCTCAAAGCATGCTGCTT AATTGTTGGAA
pCDNA3.p110 β	used via annealing to create quadruple glycine linker with 5'BglII and 3'EcoRI termini	GATCTGGAGGAGGTGACTACAAGG ACGACGATGACAAGTAAG
	used via annealing to create quadruple glycine linker with 5'BglII and 3'EcoRI termini	AATTC TACTTGTATCATCGTCGT CCTTGTAGTACCTCCACCTCCA

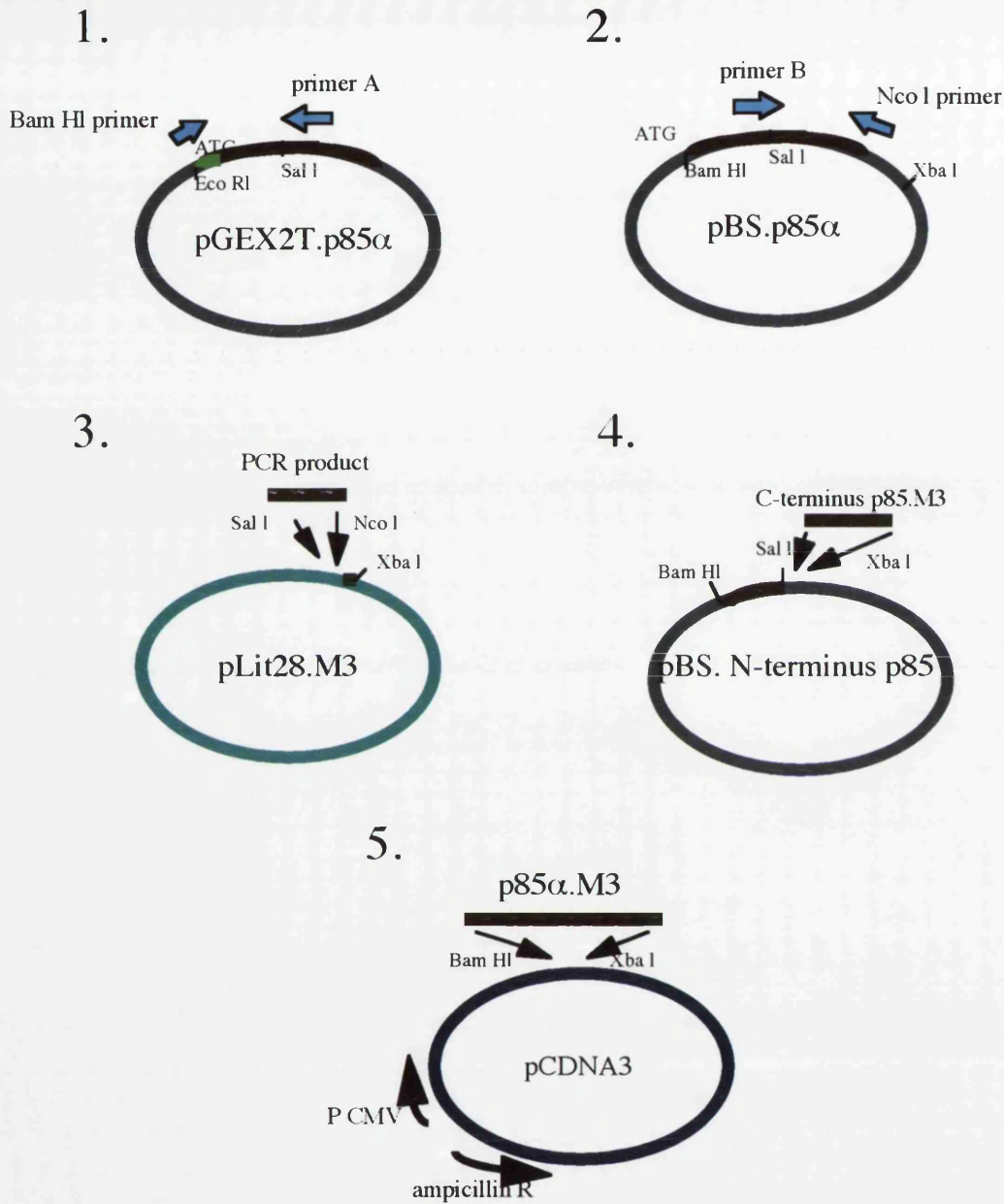


Figure 2.1 Schematic illustration of the strategy used to MYC epitope tag p85 constructs used in this study. A Bam HI restriction site was introduced into pGEX2T.p85α just before the start codon by PCR to remove a region of 5'untranslated nucleotides (1.). p85α was then subcloned into pBluescript using the Bam HI and Xba I restriction sites. This construct (2.) was used as a template for PCR, in which an Nco I restriction site was introduced at the 3' end of p85. The resulting PCR product was restriction digested with Sal I and Nco I, and ligated into Litmus 28 vector containing the triple myc epitope (3.). The myc epitope tagged 3'sequence of p85 was then religated into pBluescript containing the 5' nucleotide sequence of p85 (4.) at the Sal I and Xba I restriction sites. The tagged p85 sequence was then ligated into pCDNA3 at the Bam HI and Xba I sites for expression in mammalian cells. See text for further details.

primer 1
AAATCTCAGGCTTAAAGAGTGTGCGAATTATG

primer 2
ACAGTTTAGCGGC CGCTACTTGTCATCGTCGTCCTTGTAGTCCC
CCCCCCCCCTCTCAAAGCATGCTGCTTAATTGTGTGGAA

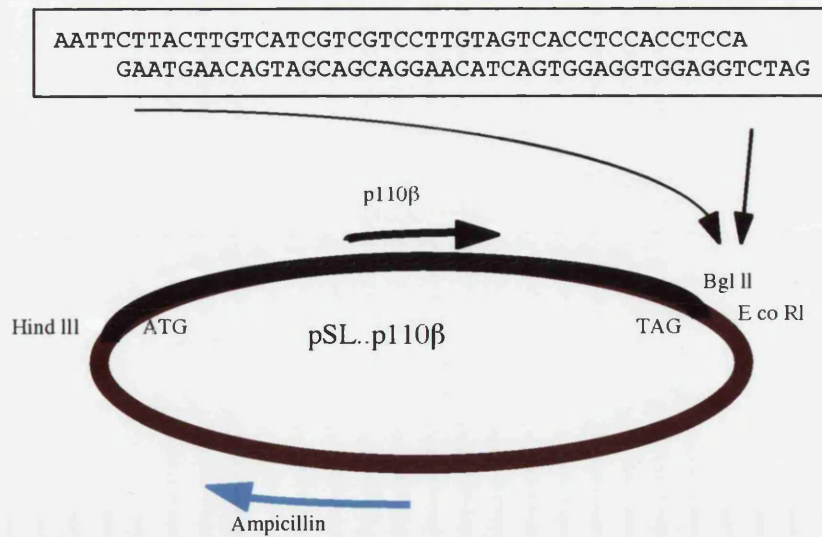
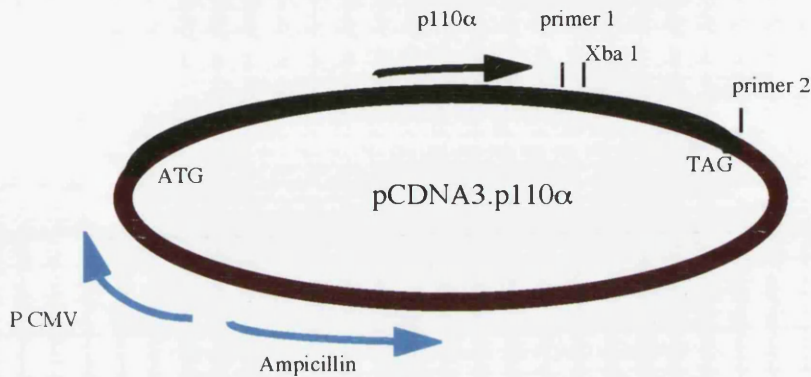


Figure 2.2 Schematic illustration of the strategies used to generate FLAG tagged p110 α and p110 β constructs in mammalian expression vectors. p110 α in pCDNA3 was FLAG eptope tagged using a PCR strategy. p110 β was FLAG epitope tagged in pSL by annealing two oligonucleotides and ligating them into the construct at the Bgl II and Eco RI restriction sites. FLAG tagged p110 β was then cloned into pCDNA3 using the Hind III and Eco RI restriction sites. For further details, see text.

2.2 Mammalian cell culture methodology

2.2.1 General cell culture methodology

2.2.1.1 Thawing HEK 293 cells

All procedures were carried out in a laminar flow hood in a sterile environment. Cells were stored in cryoviles in liquid nitrogen. Cells were removed from storage and thawed quickly at 37°C in a water bath before transfer to a tissue culture flask containing prewarmed medium (DMEM containing 4.5g/L glucose, 10% fetal calf serum, penicillin and glutamine supplements). Following overnight incubation at 37°C in a humidified incubator maintained with 5%CO₂, the medium was changed to remove traces of the freezing medium.

2.2.1.2 Trypsinisation and maintenance of HEK 293 cells

Reagents were prewarmed to 37°C. Medium was removed from confluent cells which were then washed with PBS (without calcium or magnesium). Trypsin/EDTA was added to just cover the cells which were then incubated at 37°C for two minutes. The culture vessel was tapped lightly to dislodge cells, which were then resuspended in medium and plated at a density of approximately 60% of confluence.

2.2.1.3 Freezing of HEK 293 cells

Cells were trypsinized as described and 10ml of medium was added. The resulting cell suspension was transferred to a sterile 15 ml Universal tube and pelleted at 150xg in a precooled Hereaus 12R centrifuge for 5 minutes. The supernatant was poured away and the cell pellet resuspended in ice cold freezing medium (DMEM containing 4.5g/L glucose, glutamine, 20% fetal calf serum and 10% DMSO) to give a final cell density of 1x10⁸cells/ ml. Aliquots were transferred to sterile cryoviles, which were frozen slowly in a polystyrene box in a -70°C freezer

overnight. The following day, cells were transferred to liquid nitrogen for long term storage.

2.2.1.4 Transfection of HEK293 cells

DNA for transfection was prepared with the QIAGEN Plasmid Maxi kit (Qiagen, no. 12163) using the standard protocol. Cells were transfected with an optimised calcium phosphate method. Medium was changed 1 hour prior to transfection to remove traces of trypsin. Up to 20µg of plasmid DNA was used per 10cm tissue culture dish. 2X HBS (280mM NaCl, 50mM HEPES, 750mM NaH₂PO₄, 750mM Na₂HPO₄, pH 7.00) was added to a preparation containing 20µg DNA, 200mM CaCl₂, in 500µl ddH₂O. This was then added dropwise to the cells.

Following incubation at 37°C, in an incubator maintained at 5% CO₂ for 18hours, the cells were washed twice in phosphate buffered saline without calcium or magnesium and fresh medium added. Protein expression of the transfected DNA was found to be optimal if cells were allowed to recover in this medium for 24 hours.

2.2.3 Culturing CHO-IR cells

2.3.3.1 Maintenance of CHO-IR cells

CHO-IR cells were maintained at 37°C in a humidified incubator. Cells were grown in HAM's-F12 nutrient mix (supplemented with 10% fetal calf serum and penicillin). Monolayers were thawed, trypsinised and frozen as described in section 2.2.1.2-3.

2.2.3.2 Transfection of CHO-IR cells

Confluent CHO-IR cells were replated at a dilution of 1:10 in 10 cm plates and grown overnight for transfection using LIPOFECTAMINE reagent (Gibco/ BRL). A total of 25 µg DNA was transfected per dish, and this was suspended in 800 µl serum free, penicillin free, HAM's-F12 nutrient mix. 48 µl of Lipofectamine reagent was diluted in 800 µl HAM's-F12. The medium containing DNA and lipofectamine were mixed together, and following a 45 minute incubation at room temperature 6.4 ml of medium was added. CHO-IR cells were pre-rinsed in PBS before the transfection mixture was added to them.

Cells were incubated with transfection mixture for 5 hours, before the medium was replaced with HAM's-F12 nutrient mix containing 10% FCS and penicillin. Protein expression from the transfected plasmids was found to be optimal 36 hours post transfection.

2.3 Protein isolation and analysis

2.3.1 Immunoprecipitation from cultured cells

Confluent cells were placed on ice and washed twice in phosphate buffered saline (PBS). Cells were then scraped into lysis buffer (1 ml per 10 cm dish) (137mM NaCl, 2.7mM KCl, 1mM MgCl₂, 1% w/v NP40, 10% w/v glycerol, 20mM Tris pH 8.0, 0.5mM sodium orthovanadate, 0.2mM PMSF, and 10µg/ ml leupeptin and aprotinin), and transferred to an eppendorf tube. Nuclei and unbroken cells were removed by centrifugation at 10,000rpm for 10 minutes, and the resulting lysate was then pre-cleared with anti-mouse IgG conjugated agarose by end-over-end mixing for 20 minutes.

To immunoprecipitate the protein of interest, a defined volume (usually 400 µl/ 10 cm dish) was transferred to a fresh eppendorf. Supernatants were transferred to a

fresh eppendorf tube containing appropriate antibody and Anti-Mouse IgG-Agarose (20µl of 1:2 slurry) (Sigma, A-6531) for monoclonal antibodies, or protein A-agarose for polyclonal antibodies. Following end-over-end mixing for 2 hours at 4°C, immune complexes were collected by centrifugation in a microfuge (maximum speed, 1 minute), and washed 3 times in lysis buffer for SDS-PAGE analysis, or as described in specific assay protocols.

2.3.2 Tris-glycine SDS-polyacrylamide gel electrophoresis

Hofer gel apparatus was used throughout. SDS-polyacrylamide gels were prepared as in Molecular Cloning, Maniatis *et al.*, (pp18.47-18.54) 8% gels were used unless otherwise stated. Samples were loaded onto the gel in well loading dye (5x stock containing 50% glycerol, 10% SDS, 500mM DTT, 500mM Tris, pH 6.8) and electrophoresed at 12 mA per gel overnight. Gels were then either prepared for Western blotting, stained with coomassie blue or silver stain, or fixed and dried.

2.3.3 Tris-tricine SDS-polyacrylamide gel electrophoresis

These gels were used for the resolution of peptides. Biorad mini gel apparatus was used throughout. Gels were prepared similarly to those optimised by Schagger and von Jagow (178) for the separation of small proteins in the range from 5 to 20 kDa. Briefly, this was achieved by swapping tricine for glycine in the electrophoresis buffer, and lowering the gel pH.

15% resolving gels were made up containing 12% glycerol, 15% acrylamide, 0.3% SDS, 0.01% ammonium persulphate, 0.003% TEMED in 0.125 M Tris-HCl (pH 8.45). Stacking gels were made to a final composition of 4% acrylamide in 0.1M Tris-HCl (pH 8.45), 0.1% ammonium persulphate, 0.05% TEMED

2.3.4 Transfer of proteins to membranes.

Proteins separated by electrophoresis, as described in section 2.3.2, were transferred onto Immobilon-P polyvinylidene fluoride (PVDF) transfer membrane (Millipore) using a Bio-Rad trans-blot cell. The PVDF membrane, cut to the size of the gel, was pre-wetted in methanol for 15 seconds and then rinsed in water for 2 minutes. Both gel and membrane were equilibrated in transfer buffer (25 mM Tris-base pH, 192 mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol) for 5 minutes. The transfer stack was assembled immersed in transfer buffer. To assemble the transfer stack a foam pad was placed on one side of the transfer cassette and 3 sheets of 3MM filter paper were placed on top. The gel was then placed on top of the filter paper and the membrane on top of the gel. Three sheets of 3MM filter paper were then placed on top of the membrane and a foam pad was put on top of the filter paper to complete the stack. To ensure an even transfer, air bubbles were removed by carefully rolling a pipette over the surface of each layer in the stack. The transfer cassette was closed and placed in the transfer tank such that the gel is facing the cathode (-). The cassette was covered with transfer buffer and the system was run at 35 V for 2 hours, then at 70 V for a further 2 hours.

2.3.5 Western blotting.

Following transfer to PVDF membranes proteins were detected using specific anti-sera and either ¹²⁵I-conjugated protein A or secondary antibody conjugated to horse radish peroxidase followed by enhanced chemi-luminescence (ECL). The membrane was first washed in blocking buffer (5% (w/v) dried skimmed milk, 0.1% (v/v) Tween 20 in phosphate buffered saline (PBS)) for 1-2 hours at room temperature to block non-specific binding sites on the membrane. The primary anti-sera was then diluted appropriately in 10 ml of 1% (w/v) dried milk powder in PBS containing 0.1% Tween-20 and incubated with the membrane on a rocking platform at room temperature for 1 hour or at 4°C overnight. The membrane was then washed three times in 1% milk in PBS containing 0.1% Tween-20 for 10

minutes each. The secondary antibody, conjugated to horseradish peroxidase or ^{125}I -conjugated protein A, was diluted in 10 ml of 1% milk in PBS containing 0.1% Tween-20 and incubated with the membrane for 1 hour at room temperature. ^{125}I -conjugated protein A was used at 1:1000, HRP-conjugated goat-anti-mouse antibody was used at 1:1000, and HRP-conjugated goat-anti-rabbit antibody was used at 1:2000. The membrane was then washed three times in PBS + 0.1% (v/v) Tween 20 for 5 minutes each, and three times in PBS for 5 minutes each. Where HRP conjugated secondary antibodies were used, proteins were detected by incubating the membrane in 10 ml of mixed ECL reagents for 1 minute before wrapping in Saranwrap and visualising using the FUJIFILM LAS-1000 system. Alternatively, if the secondary antibody was conjugated to ^{125}I , the membrane was dried and exposed on cassette for visualisation by phosphorimager (Fuji FLA 2000).

2.3.6 Fixing proteins in acrylamide gels

Following electrophoresis, the stacking gel was carefully cut away and the gel was placed in a clean plastic container. To fix proteins, the gel was submerged in fixing solution (20% (v/v) ethanol, 10% (v/v) glacial acetic acid in ddH₂O) for 1 hour. Gels were then dried *in vacuo* and exposed to phosphorimager cassette.

2.3.7 Coomassie blue staining of proteins in acrylamide gels.

Following electrophoresis, the stacking gel was carefully cut away and the gel was placed in a clean plastic container. For staining, the gel was submerged in coomassie blue stain solution (0.2% (w/v) coomassie brilliant blue R (Sigma), 45% (v/v) methanol, 10% (v/v) glacial acetic acid and 45% (v/v) pure water) for 18 hours. Protein bands were then visualised by washing the gel in a de-staining solution (20% (v/v) methanol, 7% (v/v) glacial acetic acid in ddH₂O).

2.3.7 Preparation of samples for phosphoamino acid analysis

³²P labelled samples were separated by SDS-PAGE using an 8% resolving gel, which was exposed to X-ray film. The band corresponding in size to the protein of interest was excised using a cleaned, sharp scalpel and placed in an eppendorf. The protein in the gel slice was then subjected to acid hydrolysis in 6M HCl at 110°C for 75 minutes. The hydrolysate was dried overnight *in vacuo*, and the sample resuspended in 10µl of electrophoresis buffer containing 10 mM each *O*-phosphoserine, *O*-phosphothreonine and *O*-phosphotyrosine standards. The sample was then spotted onto a Kodak cellulose chromatogram sheet which was sprayed evenly with electrophoresis buffer (pyridine-acetic acid-H₂O 10:100:1890). Electrophoresis was performed at 1000 V, 50 mA for 45 minutes with cooling, after which the plate was dried, stained with ninhydrin, and developed at 110°C for 1 minute. The chromatogram was exposed to X-ray film and the positions of ³²P labelled phosphoamino acids correlated with the ninhydrin stained standards.

2.3.8 Preparation of samples for mass spectrometry

Samples for mass spectrometric analysis separated by SDS-PAGE were excised using a cleaned, sharp scalpel and placed in an eppendorf. Proteins were eluted from the gel slice by extraction with 50% acetonitrile: 5% TFA, and then dried *in vacuo*.

2.4 Preparation of GST fusion proteins

2.4.1 Purification of GST fusion proteins

pGEX2T plasmids containing sequences for V12Rac1 and V14RhoA were gifts from A. Ridley (LICR). These were transformed into E.coli XL1 Blue cells, and a single colony was used to inoculate 50ml of LB containing 100µg/ ml ampicillin.

This was grown overnight with shaking at 37°C. The culture was diluted into 500ml LB supplemented with ampicillin, and grown for 1 hour at 37°C with shaking. Expression of the plasmids was induced by the addition of IPTG to a final concentration of 100µM, and following a further incubation for 3 hours, cells were pelleted at 10,000rpm, 4°C, for 10 minutes. Cells, maintained at 4°C were resuspended in 10 volumes (ml/ g cell pellet) lysis buffer (50mM Tris-HCl, pH 7.5, 50mM NaCl, 1mM DTT, 5% glycerol and 1mM PMSF). The suspension was sonicated using 10 second pulses 6 times, with a 10 second rest on ice between pulses. The lysate was centrifuged at 10,000rpm for 15 minutes to remove cell debris. Protein was extracted from the lysate by mixing end-over-end with Glutathione Sepharose 4B (Pharmacia) in a 15 ml Falcon tube at 4°C. Sepharose beads with bound GST fusion proteins were washed 3 times in buffer, and incubated with thrombin overnight at 4°C. Following centrifugation at 4000rpm for 1 minute, the supernatant was transferred to a clean tube, and incubated with agarose conjugated p-aminobenzamidine for 30 minute. This was centrifuged at 4000rpm for 5 minutes, and the supernatant was dialysed for 18 hours at 4°C. The dialysis buffer contained 50mM Tris-HCl, pH 7.5, 50mM NaCl, 1mM DTT, 20mM CaCl₂. Proteins were concentrated using centricon-10 tubes, and stored in aliquots in liquid nitrogen.

2.4.2 GTP loading of fusion proteins

2µg of Rac1 or RhoA were mixed on ice with 0.4mM GTP, 5mM EDTA, 20mM Tris-HCl, pH 7.5, 5mM NaCl, and 0.1mM DTT. Following a 10 minute incubation at 30°C, the GTP loaded proteins were ready to use.

2.5 Assays

2.5.1.1 *In vitro* PI3'-kinase lipid kinase assay

In vitro PI3'-kinase activity was measured in immunoprecipitates throughout. Immune complexes were washed 3x in lysis buffer (137mM NaCl, 2.7mM KCl, 1mM MgCl₂, 1% w/v NP40, 10% w/v glycerol, 20mM Tris, pH 8.0, 0.5mM sodium orthovanadate, 0.2mM PMSF and 10µg/ml leupeptin and aprotinin). These were followed by 2 washes in buffer 2 (0.5M LiCl, 0.1M Tris, pH 8.0), 1 wash in buffer3 (0.15M NaCl, 10mM Tris, 1mM EDTA, pH 7.6) and finally 1 wash in buffer 4 (20mM HEPES, 1mM DTT, 5mM MgCl₂, pH 7.6). All washes were performed at 4°C. Traces of wash solution were removed with a 29 gauge needle.

The immune complex pellets were resuspended in 40µl of kinase assay buffer (20mM β-glycerophosphate, 5mM sodium pyrophosphate, 30mM NaCl, 1mM DTT, pH 7.2 at 4°C). The immunoprecipitated proteins were then mixed with 20µl of the substrate, phosphatidylinositol (3mg/ ml in kinase assay buffer sonicated with 1% sodium cholate). Following incubation at 37°C for 5 minutes, 40µl of ATP solution containing 8mM MgCl₂, and 30µM ATP containing 5µCi γ-(³²P)-ATP (Amersham, AA0068) and the mixture incubated for a further 10 minutes. (12µM final ATP concentration). The reaction was stopped with 0.45ml chloroform: methanol (1:2 v/v)

2.5.1.2 Lipid extraction

Lipids were extracted using acidified chloroform. Briefly, 150µl chloroform and 150µl 0.1M HCl were added to each tube, which were vortexed and microfuged (5000rpm, 10 minutes, Denver Instrument Microfuge). The organic layer was then transferred to a clean microfuge tube for re-extraction, using 300µl chloroform and 300µl 0.1M HCl. The organic layer was then dried *in vacuo*.

2.5.1.3 Separation of phospholipids by thin layer chromatography

Dried lipids were resuspended in 25µl chloroform: methanol: 0.1M HCl (200:100:1) by vortexing. These samples were spotted onto TLC plates (MERCK '5748', silica gel). The TLC plates had been pretreated with (1% potassium oxalate, 2mM EDTA): methanol (1:1 v/v) and baked at 150°C for 2 hours.

The TLC plates were developed in a tank pre-equilibrated with methanol: chloroform: ammonia (conc.): H₂O (300:210:45:75). Phospholipids were visualised using a Fuji phosphorimager.

2.5.2 *In vitro* PI3'-kinase protein kinase assay

In vitro phosphorylation of the adapter subunit of PI3'-kinase by the catalytic subunit was measured in immune complexes. Immunoprecipitates from transfected or control cells were washed three times in lysis buffer, twice in ' high salt' buffer (0.5M LiCl, 0.1M Tris, pH 8.0), and once in 1% Tween 20 in phosphate buffered saline. Traces of wash solution were removed with a 29 gauge

needle, and the pellet resuspended in 40µl assay buffer (5mM MgCl₂, 50mM NaCl, 5mM MnCl₂, 20mM Tris pH7.4, 0.1mM sodium orthovanadate).

5µl of assay buffer containing 12 µM ATP with 5µCi γ-(³²P)-ATP was added to each tube and incubated at room temperature for 15 minutes. The reaction was stopped by the addition of SDS-polyacrylamide gel loading dye.

The samples were electrophoresed on SDS-polyacrylamide gels, which were fixed, dried *in vacuo*, and the extent of phosphorylation quantitated by phosphorimager.

2.5.3 Peptide phosphorylation assay

A peptide based on bovine p85α incorporating the p85αSer608 site was purchased from Alta Bioscience.

The amino acid sequence is: RRLGNENTEDQYSLVEDDEDL

In addition to the residues from the original protein sequence, two arginine molecules were added at the amino terminus aimed at stimulating binding to phosphocellulose paper. The peptide was solubilised in a 5% aqueous solution of DMSO to a final concentration of 2.4 mM. Serial dilutions were performed in ddH₂O, and added to immunoprecipitated PI 3'-kinase complexes or immunoprecipitated phosphotyrosine complexes (see section 2.3.1). Reactions were started by the addition of ATP to a final concentration of 12µM, and containing 5µCi γ-(³²P)-ATP. Following a 10 minute incubation at 25°C, the reaction was stopped by the addition of polyacrylamide gel loading dye (5X stock containing 50% glycerol, 10% SDS, 500mM DTT, 500mM Tris, pH 6.8).

Samples were electrophoresed on Tris-Tricine SDS-polyacrylamide gels (see section 2.3.3), which were fixed and dried *in vacuo*. Phosphorylation was quantitated by phosphorimager.

2.5.4 Orthophosphate labelling of HEK293 cells

Wildtype or transfected confluent HEK293 cells were washed twice in phosphate free DMEM (Sigma, cat. no. D-3656, made up as in the instructions). Cells were then incubated for 2 hours at 37°C in phosphate free medium supplemented with 50µCi/ml ³²Pi-orthophosphate. The ³²Pi-orthophosphate labelled cells were processed as usual.

2.5.5 Methionine labelling of HEK293 cells

Confluent HEK293 cells were incubated in serum free medium for 12 hours, then in methionine and cysteine free medium for three hours (Sigma, cat. no. D-0422). Cells were then maintained in methionine/ cysteine free media supplemented trans³⁵S-label methionine (100µCi/ ml) as usual. Labelled cells were processed as usual.

3. THE ROLE OF THE ADAPTER SUBUNIT IN THE LOCALISATION AND ACTIVATION OF P110

3.1 Summary

Western blotting of the class 1a PI 3'-kinase adapter proteins present in human skeletal muscle and two cell culture muscle models (rat and mouse) revealed the presence of several variants. This highlighted the need to understand the role of each of the splice variants. A transfection procedure was established whereby specific PI 3'-kinase heterodimers of epitope tagged adapter and catalytic subunit could be expressed. Using this system, p110 α or p110 β were expressed dimerised with either p85 α , p85 $\alpha\Delta$ SH3 or p85 $\alpha\Delta$ BCR, and the characteristics of these heterodimers were studied.

Deletion of the BH and SH3 domains of the adapter subunit was shown to have no effect on the lipid kinase activity of p110 α or p110 β *per se*. However the small GTPase Rac was shown to activate lipid kinase activity of the PI 3'-kinase complex (1.7-fold), and this is demonstrated to occur via a mechanism involving the BH domain of the adapter subunit. The recruitment of PI 3'-kinase to phosphotyrosine complexes following insulin stimulation was affected by deletion of either the SH3 or BH domain of p85 α . Further, p110 β /p85 α heterodimers were better recruited to phosphotyrosine based signalling complexes than p110 α /p85 α indicating the nature of the catalytic subunit can affect the function of the heterodimer.

3.2 Introduction

As described in Chapter 1, five variants of the adapter subunit have been identified. The roles of these isoforms are as yet undefined, although the differences in their modular structures suggests these proteins are unlikely to exhibit functional redundancy. Clearly, the truncated isoforms will be unable to interact with proline-rich motif or SH3 domain containing proteins, and some isoforms, especially the truncated isoforms p55 α and p50 α , have been shown to have restricted tissue distributions (Inukai et al 1997) compared to p85 α . Furthermore, although p85 α and p85 β share the same modular domains, there is variation at the amino acid level, particularly within the amino termini of these proteins; the SH3 domains are 75% identical, and the BH domains 57% identical.

To determine how the adapter isoforms contribute to signalling specificity, studies were undertaken of the functions of the domains of p85 α . Significant progress has already been made in studies of SH2 domains in general, and there is considerable literature regarding the structure and function of these domains in p85 (Panayotou et al 1992; Pawson & Schlessinger 1993; Walksman et al 1993). Similarly, the inter-SH2 domain is well documented as containing the sequence for interaction with the catalytic subunit, as well as an autoregulatory site, which is discussed in Chapter 4, although the structure of this domain is as yet undetermined. This chapter therefore presents studies undertaken to elucidate the roles and functions of the SH3 and BH domains of p85 α .

3.3 Results

3.3.1, Determination of p85 isoforms in skeletal muscle.

We first aimed to determine if the different variants of p85 were detectable in insulin sensitive cell types. Western blots were used to determine the p85 isoforms present in skeletal muscle cell culture models. Two cell culture models were used, C30 (derived from mouse cells and containing a temperature sensitive immortalisation element) and L6E9 (derived from rat cells), and compared with human skeletal muscle. By varying culturing and differentiation conditions, it was possible to optimise expression of the shorter isoforms of the p85 proteins. Specifically, coating culture dishes with matrix (gelatin) promoted expression of the shorter adapter isoforms (Kaliman et al 1998). The proteins in whole lysates from human skeletal muscle, L6E9 myoblasts and myotubes, and C30 myoblasts and myotubes were separated by SDS-PAGE using an 8% resolving gel and transferred to PVDF. (80µg of protein per lane was loaded). The polyclonal antibody raised against the N-SH2 domain of p85 α consistently revealed two proteins at around 85 kDa in human skeletal muscle, but only one in the muscle cell culture models. Immunoblotting using isoform specific monoclonal antibodies showed the lower protein was p85 α while the higher protein was p85 β (Figure 3.1.A.).

The polyclonal antibody also consistently revealed proteins with a molecular weight of 55 kDa and 45 kDa (Figure 3.1.B.) although levels were only substantial in samples of human skeletal muscle and the differentiated C30 myotubes. These bands were also recognised using another antibody to the same domain, but do not appear in blots using an antibody to the SH3 domain of p85 α . These bands have previously been shown to be p55 α and p50 α , splice variants of the p85 α gene.

3.3.2 Structural features of p85 mutant proteins used in these studies

It has previously been shown that the class 1a adapter subunit variants are differentially regulated by insulin in human skeletal muscle (Shepherd et al

1997b). Having established the PI 3'-kinase adapter subunit variants were present only in muscle, it was decided to use a cell culture based expression approach to determine the way in which the NH₂ terminal domains of the adapter subunit affect the response of PI 3'-kinase to insulin. The role of the NH₂-terminus of p85 α in activation and localisation of the p110 proteins was studied using the constructs described in the preceding section and illustrated schematically in Figure 3.2. Full length p85 α and the splice variant of the same gene, p55 α , together with p85 with the BH or SH3 domains deleted, were used to investigate the relevance of the diversity in the NH₂-terminus of the p85 proteins.

3.3.3 The turnover of p85 protein in cells.

It was initially assumed that transfected p85 would associate with endogenous p110, but preliminary experiments in which p85 was immunoprecipitated from transfected HEK293 cells via the myc epitope tag had extremely low lipid kinase activities associated, as shown in Figure 3.3.A. This prompted the experiment to determine the rate of cellular synthesis of PI 3'-kinase protein to determine whether catalytic subunit was being synthesized. Briefly HEK293 cells were labelled with ³⁵S-methionine and lysed at 12 hour time points. Lysates were immunoprecipitated with a polyclonal antibody raised against the N-SH2 domain of p85 and immunoprecipitated proteins were separated by SDS-PAGE using an 8% resolving gel. Proteins in the gel were fixed in 10% glacial acetic acid: 20% ethanol and the gel was then dried *in vacuo*. The autoradiograph is shown in Figure 3.3.B. The band at 85 kDa was only weakly visible at 60 hours, and did not become strong until cells had been labelled for 72 hours. Furthermore, levels of ³⁵S labelled p110 were extremely low, suggesting that in quiescent cells, turnover of endogenous PI 3'-kinase is slow, explaining the poor association of transfected adapter subunit with endogenous p110. However, this provided us with the opportunity to transfect in specific combinations of p85 and p110 and be confident that most of the catalytic activity in resultant p85/ p110 heterodimers

immunoprecipitated via the myc tag was due to the catalytic subunit we had transfected.

3.3.4 Synthesis of specific PI 3'-kinase complexes in HEK 293 cells.

HEK 293 cells at approximately 60% confluence were cotransfected with an adapter and catalytic subunit, using the calcium phosphate transfection protocol described in Methods. 12 μ g of adapter construct DNA and 10 μ g of catalytic construct DNA were used per 10cm dish. This ratio of adapter: catalytic subunit DNA was used as it was found to minimize free p85 or p110. This was important as free p85 might act as a dominant negative inhibitor and expression of p110 only was shown to be cytotoxic. Both p110 α and p110 β are FLAG tagged, and all adapter constructs were tagged with the MYC epitope. Transfected cells were lysed and PI 3'-kinase complexes were immunoprecipitated with a monoclonal α -MYC antibody (9E10). Immunoprecipitated proteins were separated by SDS-PAGE using an 8% resolving gel, and transferred to PVDF. The transfer membrane was cut in two, and the upper section was probed with α -FLAG polyclonal antibody. Visualisation by ECL revealed all lanes expressed the FLAG tagged p110 constructs, except the first lane, in which immunoprecipitates were from cells transfected with p85 only (Figure 3.4.A). This section of the blot was then reprobed with a polyclonal antibody to p110 β (S19, Santa Cruz) and visualised with protein A I¹²⁵ (Figure 3.4.B). These results show that almost no endogenous p110 β is associating with adapter constructs cotransfected with p110 α . Similar experiments could not be conducted on p110 α due to a lack of good p110 α antibodies.

The lower section of transfer membrane was probed with a polyclonal antibody raised against the N-SH2 domain of p85 and visualised by ECL (Figure 3.4.C). The adapter constructs all express well, whether alone (p85 α , first lane) or when cotransfected with either p110 α or p110 β . In the first three lanes of Figure 3.4.C,

the very strong band is recognized by the monoclonal antibody raised against MYC, whereas the weaker band below it is not. This band represents endogenous p85, which co-immunoprecipitates with the MYC tagged adapter constructs, presumably by an interaction mediated by the SH3 or BH domains. (This band is absent from the MYC immunoprecipitates of cells transfected with p55 α). It is important to note that the myc tag affects the migration of p85 during SDS-PAGE such that p85 α Δ SH3 comigrates with wild type p85 α . These data indicate that specific heterodimers of PI 3'-kinase can be generated, with relatively little contamination from endogenous subunits.

3.3.5 The role of the NH₂-terminus of the adapter subunit in PI 3'-kinase lipid kinase activity.

It is known that conformational changes in p85 can alter p110 activity, and it is possible that the SH3 and BH domain have a role via the SH2 domains, in the conformational state of the catalytic subunit (Backer et al 1992; Chuang et al 1994; Herbst et al 1994; Rordorf-Nikolic et al 1995; Yu et al 1998). The effect of the BH and SH3 domains of p85 α on the lipid kinase activity of p110 was therefore investigated. HEK 293 cells were transfected with p110 α or p110 β , and either MYC tagged full length p85 α , p85 α Δ SH3, p85 α Δ BH, or p55 α . 9E10 (anti myc monoclonal) immunoprecipitates were washed extensively and lipid kinase activity was measured as described in Methods. Activity was normalised for protein expression as previously described.

The results (Figure 3.5) show that in quiescent cells, the absence of the BH or SH3 of the adapter subunit does not have a significant effect on the lipid kinase activity of the PI 3'-kinase heterodimer containing either p110 α or p110 β .

3.3.6 Affinity purification of two small GTPases, Rho A and Rac 1

V12Rac1 and V14RhoA, mutants which are unable to hydrolyse GTP, were expressed from pGEX2T expression vectors in *E.coli* and purified as described in

Methods. The purification was monitored by subjecting aliquots from each stage of the purification to SDS-PAGE, and visualizing with Coomassie staining (Figure 3.6). The purified proteins were stored in cryovials in liquid nitrogen.

3.3.7 Stimulation of PI 3'-kinase activity by Rac.

The ability of the small GTP binding protein Rac to stimulate the lipid kinase activity of PI 3'-kinase was investigated. Rho was used as a protein control. Cells were cotransfected with p85 α and p110 α . To ensure results were directly comparable, cell lysates were split into three portions; one aliquot for control PI 3'-kinase assay, one for PI 3'-kinase assay in the presence of Rac and one for PI 3'-kinase assay in the presence of Rho. PI 3'-kinase complexes were immunoprecipitated with 9E10 (monoclonal α -myc antibody). After extensive washing, 2 μ g of GTP loaded Rac or Rho were added to the immunoprecipitates. Because of the divergence in amino acid sequence of p85 α and p85 β at the NH₂ terminus, these were also studied, by immunoprecipitating PI 3'-kinase from wild type cells using polyclonal antibodies raised against either p85 α or p85 β . Immunoprecipitates of endogenous PI 3'-kinase were then treated in the same way as transfected cells. PI 3'-kinase activity was measured as described in Methods. Rac and Rho were GTP loaded as described in Methods. The results show that activated Rho does not significantly stimulate the lipid kinase activity of PI 3'-kinase. Activated Rac stimulates PI 3'-kinase activity 1.7-fold (Figure 3.7).

3.3.8 Rac stimulates PI 3'-kinase activity via the BH domain of p85 α .

The mechanism by which Rac stimulates PI 3'-kinase activity was investigated by measuring the fold stimulation induced in PI 3'-kinase complexes in which the adapter subunit was truncated. Cells were cotransfected with p110 α and either p85 α , p85 $\alpha\Delta$ SH3 or p85 $\alpha\Delta$ BH and each lysate split in two. GTP loaded Rac was added (or not) to 9E10 immunoprecipitates, and lipid kinase activity measured as described in Methods. The results are shown in Figure 3.8.

GTP loaded Rac stimulates equally well the lipid kinase activity of p110 α when dimerised with either full length p85 α , or with p85 $\alpha\Delta$ SH3 (1.7-fold increase over control). However, when p110 α is complexed with p85 $\alpha\Delta$ BH, the stimulatory effect of Rac on the lipid kinase activity of p110 α is absent. As it was previously established that deletion of the BH or SH3 domain does not affect basal activity, this indicates that activated Rac stimulates PI 3'-kinase activity via the BH domain of p85 α .

3.3.9 The role of the SH3 and BH domains of p85 α in recruitment to phosphotyrosine complexes before and after insulin stimulation.

The presence of adapter isoforms of PI 3'-kinase in which the BH and SH3 domains are replaced with a unique stretch of aminoacids prompted the experiment to determine whether any role existed for these domains in localizing full length p85 in cell signalling complexes. HEK293 cells were cotransfected with p110 α or p110 β and either p85 α , p85 $\alpha\Delta$ SH3 or p85 $\alpha\Delta$ BH. Cells were serum starved overnight and stimulated with 100nM insulin or vehicle. Lysates were immunoprecipitated with antiphosphotyrosine antibody (PY99, Santa Cruz), and the proteins separated by SDS-PAGE using an 8% resolving gel. Proteins were transferred to PVDF and probed with a polyclonal antibody to p85 (raised against the N-SH2 domain) and visualised by ECL. The results of 8 separate experiments are shown graphically in Figure 3.9.A. These data show that whilst full length p85 α is recruited to phosphotyrosine complexes 7-fold following insulin stimulation, both p85 $\alpha\Delta$ SH3 and p85 $\alpha\Delta$ BH are recruited only 3-fold. However, as shown in the representative blot in Figure 3.9.B., there is significantly more adapter protein present in phosphotyrosine complexes in the basal state where either the BH or SH3 domain is deleted from p85 α .

The increased levels of the truncated adapter subunits in phosphotyrosine immunoprecipitates is not due to tyrosine phosphorylation of the adapter subunits

themselves as reprobing the blot with antiphosphotyrosine reveals no bands in the size range of the adapter protein constructs, as shown in Figure 3.10. However, a band corresponding in size to IRS4 is clearly visible, and the Western blot indicates that even under basal conditions, there is some tyrosine phosphorylation of the 160 kDa protein.

3.4 Discussion

So far three genes have been identified which encode adapter proteins for class 1a PI 3-kinases. The proteins these produce, including splice variants, vary in size from 45-85 kDa. These share substantial homology at the carboxyl terminus, which contains two SH2 domains, the inter-SH2 region and the second proline rich domain (P2), but diverge dramatically at the amino terminus. In addition, these genes have been shown to be differentially expressed; of particular relevance to insulin signalling is the finding that two splice variants of the p85 α gene are expressed in muscle which is a major *in vivo* target tissue for insulin. The functional roles of each of these adapter molecules have not been defined, but given the requirement of PI 3-kinase in an array of signalling cascades, the differences in the structure of the adapter subunit variants could well provide a mechanism for differential regulation of the enzyme in response to a range of signalling inputs. A few recent studies have provided evidence to support such a hypothesis. Inukai *et al* have shown that PI 3'-kinase associated with different forms of the adapter subunit are activated to greatly differing extents by insulin (Inukai *et al* 1997). Further, in mice with targeted gene disruption of only full length p85 α , upregulation of the p50 α not only compensates for the loss, but appears to confer hypersensitivity to insulin (Terauchi *et al* 1999).

To date, recruitment of adapter subunits via the SH2 domains to tyrosine phosphorylated motifs is the defined mechanism by which growth factors stimulate PI 3-kinase. However, reports of differential recruitment and activation

of p85 proteins following growth factor stimulation in various cell types indicate that modules other than the SH2 domains in the PI 3-kinase dimer influence recruitment to phosphotyrosine complexes and activation of the catalytic subunit. The adapter subunits diverge most at the amino terminus. Whilst the amino termini of p85 α and p85 β both contain a proline rich motif, and SH3 and a BH domains, these are the most unconserved portions of the proteins (75% identity in the SH3 domain, 57% identity in the BH domain). The two splice variants of p85 α lack this proline rich motif, and the SH3 and BH domain, containing instead novel sequences of 33 amino acids (p55 α) or 6 amino acids (p50 α). In general, three roles for these variable regions could be envisaged in terms of activation of PI 3-kinase by growth factors. Firstly, they could modulate the intrinsic activity of the catalytic subunit, for example by inducing allosteric changes in the p110 binding region by a mechanism in which the SH3 and BH domains inter-associate between molecules (Harpur et al 1999). Secondly, they could influence the recruitment of PI 3-kinase to signaling complexes, by differentially 'tethering' the enzyme to subcellular structures. Finally, they could provide a mechanism for the input of signals from effector molecules which could interact with these domains. The studies described here have investigated these possibilities using epitope tagged p85 α , p85 $\alpha\Delta$ SH3 or p85 $\alpha\Delta$ BH constructs coexpressed in HEK293 cells with either p110 α or p110 β .

The effect of SH3 and BH domain deletions on catalytic activity of class 1a PI 3-kinase heterodimers was assessed by immunoprecipitating these complexes from transfected cells, and normalising PI 3-kinase activity to adapter subunit expressed as determined by Western blotting. The results of these studies show that, in unstimulated cells, neither the BH nor SH3 domain affects the intrinsic activity of PI 3-kinase. Nevertheless, it remains possible that in stimulated cells these domains modulate activity of p110. Layton, Harpur and coworkers have shown that growth factor activated PI 3-kinase molecules can dimerize under certain

conditions (Layton et al 1998), enhancing activity, and postulate that this process is likely to involve the SH3 and BH domains since the phenomenon does not occur with the truncated adapter isoforms. Technical difficulties undermined studies of PI 3'-kinase activity in stimulated cells using the deletion adapter constructs, since endogenous PI 3'-kinase would co-precipitate in phosphotyrosine signalling complexes. The development of p85 knockout cell lines would enable determination of the effect of the BH and SH3 domains on PI 3-kinase in stimulated cells.

The potential role of the SH3 and BH domains as targets of effector molecules stimulating PI 3-kinase activity was also addressed in this study. As shown in previous studies and this one, GTP bound Rac stimulates PI 3-kinase activity (Bokoch et al 1996; Toliás et al 1995; Zheng et al 1994) and Rac can bind p85 independently of p110. Both 85 kDa isoforms of adapter subunit contain a region which though lacking any detectable catalytic activity has significant rho-GAP homology, providing a likely site of interaction with Rho family members, though this has never been demonstrated. The studies presented here have used the deletion adapter constructs to address this issue. Initially, it is shown that we can replicate the 2-fold activation of PI 3-kinase by Rac both in immunoprecipitates of endogenous p85 α and β , and also in anti-myc epitope immunoprecipitates of transfected full length p85 α /p110 (p110 α or p110 β). This stimulation was also observed in the p85 α Δ SH3/p110 heterodimers, although Rac had no effect on PI 3-kinase activity in p85 α Δ BH/p110 heterodimers. These data definitively establish the BH domain of p85 α as the target module for Rac stimulation of PI 3-kinase activity. Since Rac is a membrane associated protein, the Rac-BH interaction provides a further mechanism for recruiting PI 3-kinase to the plasma membrane, distinct from growth factor mediated recruitment to phosphotyrosine motifs. This interaction may be particularly significant in membrane ruffling, a PI 3'-kinase

dependent process, and studies using these adapter deletion constructs in p85 knockout cells would provide invaluable insight into this process.

As recruitment to YXXM motifs via the SH2 domains of PI 3-kinase is the major accepted mechanism for activation of the enzyme by growth factors, the differential recruitment of the three p85 α gene products following insulin stimulation (Antonetti et al 1996; Inukai et al 1997; Shepherd et al 1997b) is particularly interesting since these molecules contain identical SH2 domains. This suggests the amino terminal domains of p85 proteins exert an effect on the ability of PI 3'-kinase to interact with IRS and other phosphotyrosine based signalling complexes, and that these are also subject to modulation in a growth factor dependent manner. Regulatory effects of the SH3 and BH domains of p85 on PI 3-kinase recruitment into signalling complexes in response to insulin were studied using the deletion mutants expressed in HEK293 cells. Since previous experiments had shown that only a small proportion of transfected p85 interacted with endogenous p110 (section 3.3.3), and that p85 alone is only poorly recruited (see section 4.3.1), the p85 constructs were cotransfected with catalytic subunit. Transfected wild type PI 3-kinase was recruited equally well into phosphotyrosine based signalling complexes as endogenous enzyme.

It is common for growth factor stimulation of PI 3-kinase to be expressed as a fold increase over basal in the level of protein in phosphotyrosine complexes, since the ability to upregulate responses is relative to basal. By this measure, the fold recruitment of p85 α Δ SH3 and p85 α Δ BH to signalling complexes was greatly reduced following insulin stimulation compared to full length p85 α . Two mechanisms could be envisaged to explain this. Firstly, in light of the results of Rac stimulation of PI 3-kinase, it is conceivable that in addition to binding of SH2 domains to phosphotyrosine motifs, growth factors stimulate PI 3-kinase recruitment by a binding event involving the SH3 or BH domain. Secondly, it is possible that in the basal state, the SH3 and BH domain serve to sequester PI 3-

kinase to specific locations away from signalling complexes. The action of growth factors could then function both to stimulate phosphorylation of YXXM motifs and thereby provide a binding site for SH2 domains, and simultaneously to induce an un-binding of the SH3 or BH domains which anchor the enzyme to a specific subcellular location.

In these studies, the amount of p85 α Δ BH and p85 α Δ SH3 in phosphotyrosine complexes immunoprecipitated from basal cells was substantially greater than the amount of full length p85 α . However, the amount of p85 protein at phosphotyrosine complexes immunoprecipitated from stimulated cells was only marginally reduced in the case of the deletion mutants. This suggests that the function of the BH and SH3 domains of p85 is to sequester PI 3-kinase away from basal state phosphotyrosine complexes which are formed as a result of factors other than insulin. It is interesting to note that basal phosphorylation of IRS4, the predominant protein in anti antiphosphotyrosine immunoprecipitates from HEK293 cells, is higher than would be expected for IRS1. Since p50 α is recruited to phosphotyrosine complexes most effectively following insulin stimulation (Shepherd et al 1997b), it is possible that the 33 amino acid sequence at the amino terminus of p55 α , and the SH3 and BH domains of full length p85 α may attenuate the ability of insulin to induce recruitment of these isoforms, or stimulate their association with signalling complexes in the basal state. These data also indicate that the absence of either the p85 α SH3 and BH domains in p50 α is not sufficient to greatly enhance recruitment to signalling complexes following insulin stimulation.

Overall, these studies identify two broad roles for the amino terminal regions of the adapter subunit. First, they serve as sites for input signals from molecules which can modulate PI 3-kinase activity, as is shown by the interaction of Rac with the BH domain of p85 α which stimulates lipid kinase activity. It will

therefore be important to determine molecules which target the unique sequences of p55 α and p50 α , since these will provide insights into specific downstream pathways from each isoform. In addition, the SH3 and BH domains may cause p85 to be subject to sequestration in subcellular pools which are independent of phosphotyrosine containing complexes, and it will be interesting to determine by what mechanism insulin acts to 'un-tether' the adapter subunits from these subcellular pools to enable them to translocate to phosphorylated YXXM motifs at the plasma membrane.

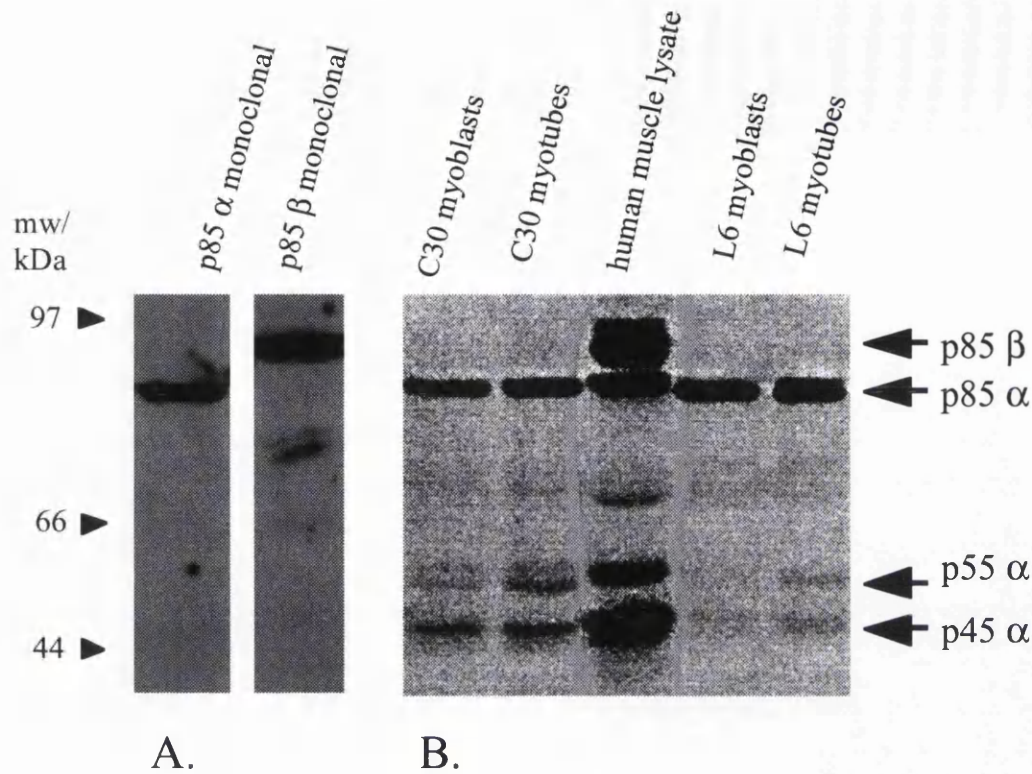


Figure 3.1 The p85 isoforms in human muscle and cell culture muscle models.

A. Control showing human skeletal muscle lysate probed with p85 α or p85 β monoclonal antisera and visualised by ECL.

B. Whole cell lysates from human skeletal muscle, and mouse (C30) and rat (L6) muscle cell lines optimised for differentiation, were analysed by Western blot. p85 isoforms were detected using a polyclonal antisera raised against the N-terminal SH2 domain and visualised using ^{125}I -labelled protein A. The isoforms are indicated.

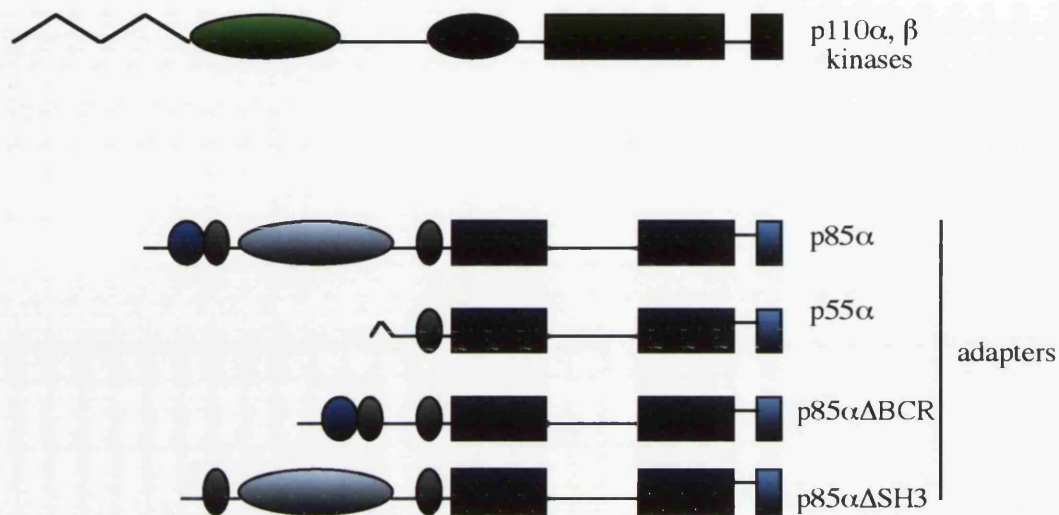


Figure 3.2 Structural features of PI 3'-kinase constructs used in these studies

The constructs shown were subcloned into pCDNA3 for expression in mammalian cells. The protein domains are as follows: p85-interaction region (*large double sawtoothed line*), ras binding domain (*light green oval*), PIK domain (*dark green oval*), catalytic domain (*green rectangle*), FLAG epitope tag (*green square*), SH3 domain (*blue circle*), unique 34 aminoacid NH₂-terminus in p55α (*small sawtoothed line*), proline-rich motif (*grey circle*), BH domain (*light blue oval*), SH2 domain (*dark blue rectangle*), triple MYC epitope (*blue square*).

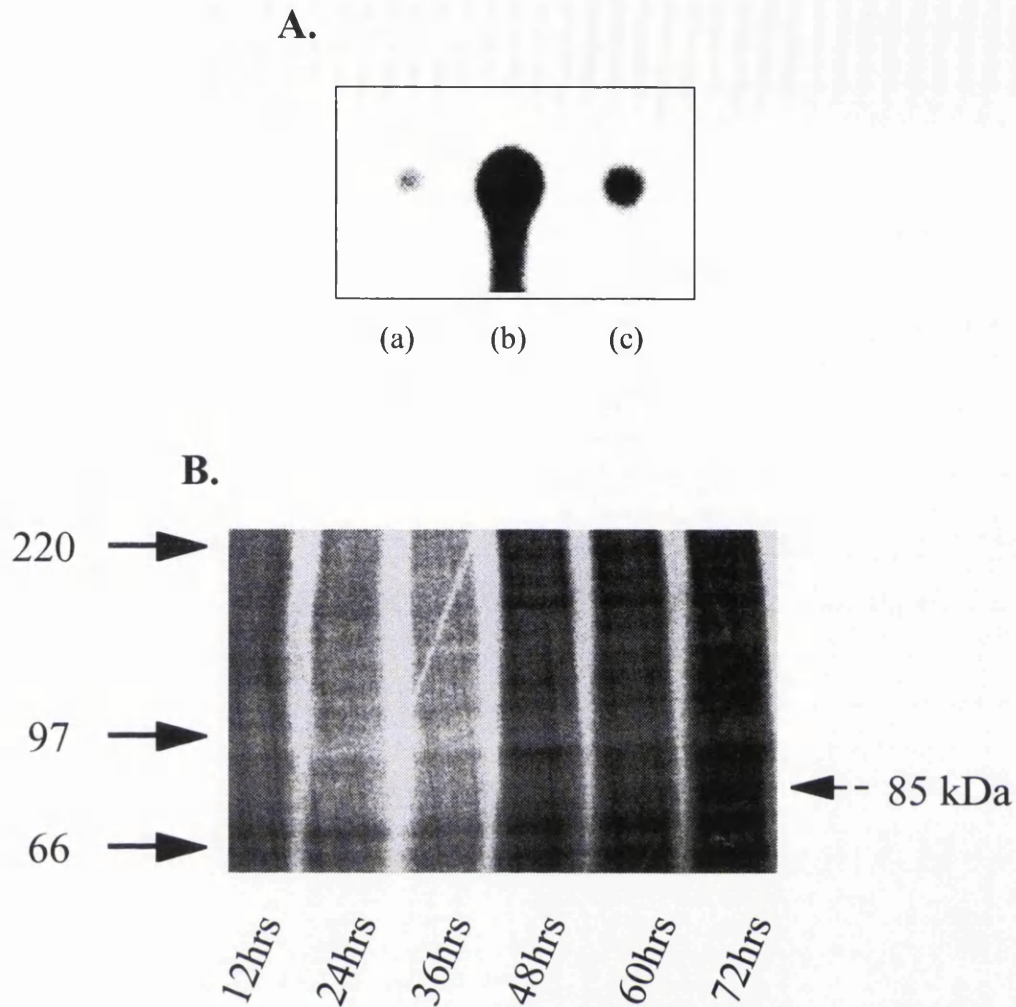


Figure 3.3 Poor association of endogenous p110 with transfected p85 in HEK293 cells.

A. HEK293 cells were transfected with either p85 α alone (a) or with p85 α and p110 α (b) or p110 β (c). Transfected p85 was immunoprecipitated from cells via the myc epitope tag and lipid kinase assays were performed as described in Methods. Protein expression was normalized by western blotting as described previously. A typical TLC plate result is shown. B. Cellular proteins were labelled as described in Methods with Trans ^{35}S -label for the times indicated and then subjected to immunoprecipitation with a polyclonal antibody raised against the SH2 domain of p85 α . Proteins in the immunoprecipitates were separated by SDS-PAGE using a 8% resolving gel, which was fixed and dried before autoradiography.

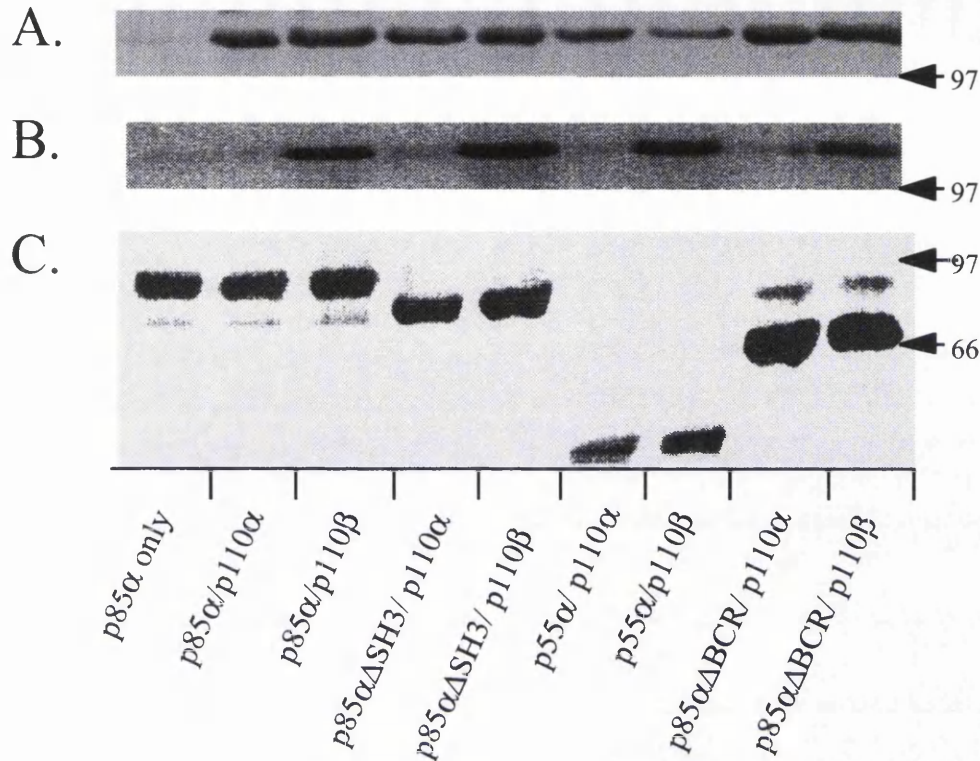


Figure 3.4 Expression of specific phosphoinositide 3'-kinase heterodimers in HEK 293 cells. Cells were cotransfected with either FLAG epitope tagged p110 α or FLAG epitope tagged p110 β , and a MYC epitope tagged adapter construct: p85 α , p85 α Δ SH3, p55 α or p85 α Δ BH. Cells were lysed and complexes were immunoprecipitated with 9E10 (α -myc) antibody. Proteins in the immunoprecipitates were separated by SDS-PAGE using an 8% resolving gel and transferred to PVDF. The transfer membrane was cut and the lower section (0 - 97 kDa) was blotted with a polyclonal antibody raised against the SH2 domain of p85 α and visualised by ECL (C.). The upper section of the transfer membrane was probed first with α -FLAG polyclonal antibody (Sigma), and visualised by ECL (A.), then stripped and reprobed with S19 (α -p110 β antibody, Santa Cruz Biotechnology) and visualised with protein A I¹²⁵ (B.).

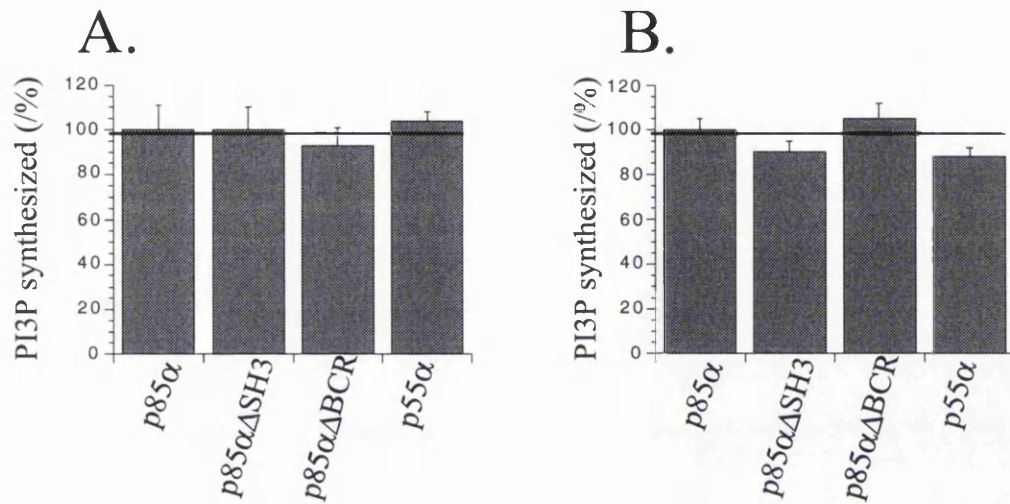


Figure 3.5 The NH₂-terminus of the adapter subunit does not effect the lipid kinase activity of PI 3'-kinase complex.

p110α (A.) or p110β (B.) were immunoprecipitated via the myc tagged adapters indicated and lipid kinase activity was assayed as described in Methods. Protein expression was normalised by Western blotting (n=4 +/- SEM.)

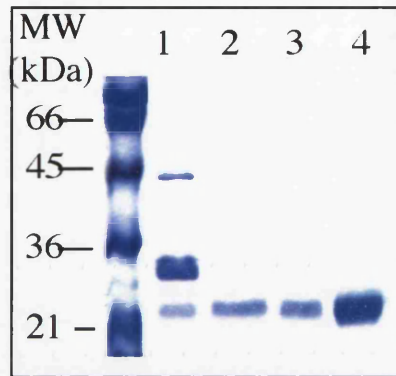


Figure 3.6 SDS-PAGE analysis of GST protein purification.

E. coli transformed with pGEX2T V14RhoA and V12Rac1 were grown at 37°C to an O.D.₅₉₅ 0.6 before induction with IPTG. Cells were lysed and purification of the RhoA and Rac1 proteins was monitored by SDS-PAGE on a 15% resolving gel. Proteins were visualised by staining with Coomassie blue. Lanes: 1, Thrombin cleaved Rac1 from Glutathione sepharose purified GST-Rac1; 2, Rac in eluate following Glutathione sepharose bead removal of GST and GST-Rac1; 3, dialysed Rac1; 4, Concentrated Rac1.

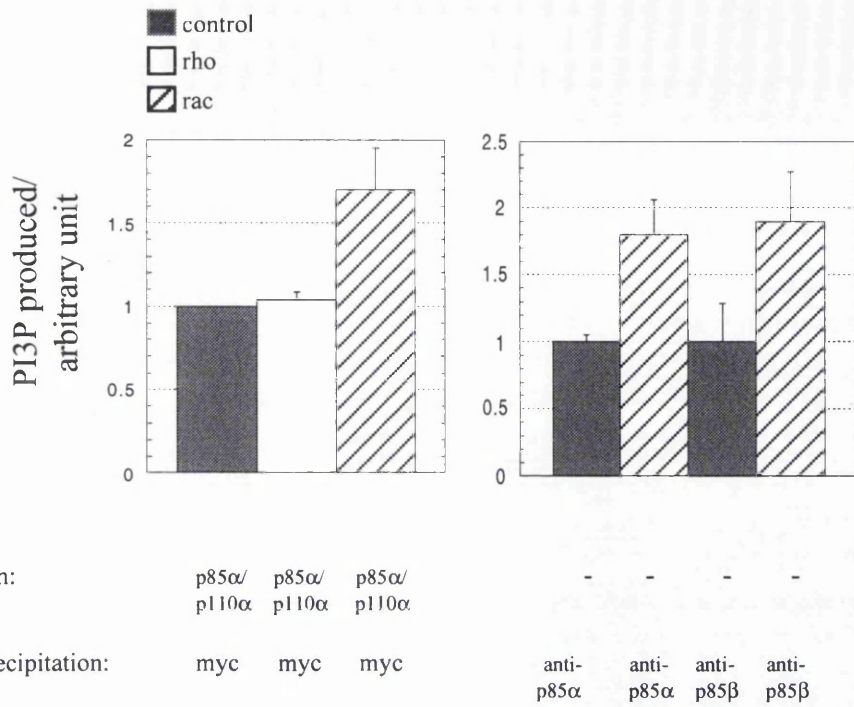


Figure 3.7 Stimulation of phosphoinositide 3'-kinase activity by Rac. Cell lysates were split in three and PI 3'-kinase complexes were immunoprecipitated via the myc epitope if transfected, or using anti-p85 α or anti-p85 β antibodies if cells were wild type. 2 μ g V14RhoA or V12Rac1 were added and lipid kinase activity was measured as described in Methods (n=3 +/- SEM).

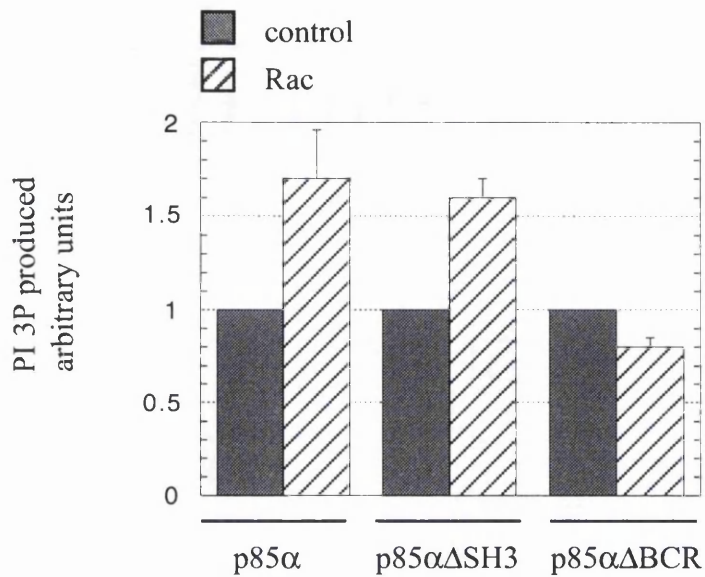


Figure 3.8 Rac stimulates PI 3'-kinase activity via the BH domain of p85α.

Cells were cotransfected with p110α and either p85α, p85αΔSH3 or p85αΔBH. Cell lysates were split in half and PI 3'-kinase complexes were immunoprecipitated via the myc epitope. 2μg of V12Rac1 was added to one half of each lysate. Lipid kinase activity was measured as described in Methods (n=3 +/- SEM).

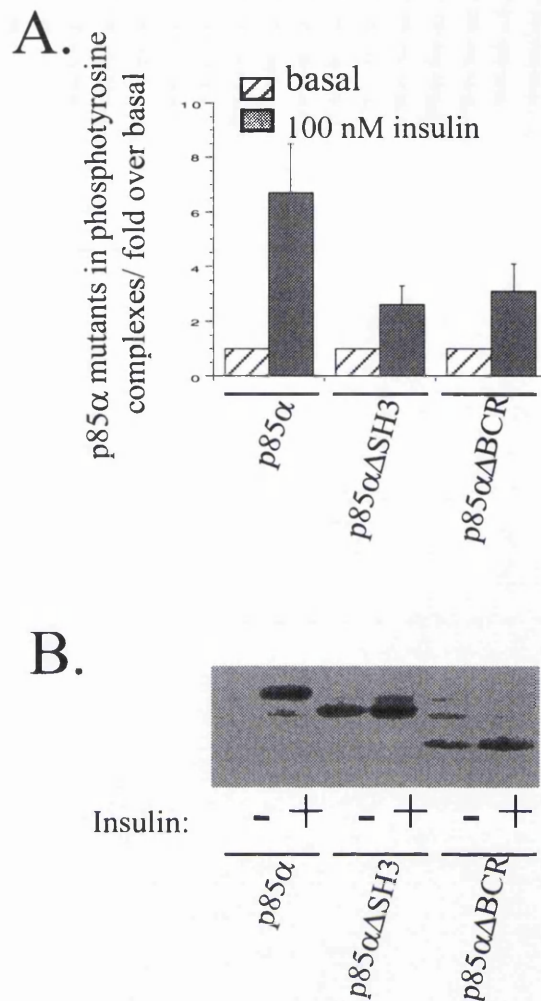


Figure 3.9 The role of the NH₂-terminus of p85α in recruitment to phosphotyrosine complexes following insulin stimulation. Cells were cotransfected with p110 and either wild type p85α, p85αΔSH3 or p85αΔBH. After stimulation with vehicle or 100nM insulin, cells were lysed and subjected to antiphosphotyrosine immunoprecipitation (PY99, Santa Cruz Biotechnology). Proteins in the immunoprecipitates were separated by SDS-PAGE using an 8% resolving gel, transferred, probed with a polyclonal antibody to p85 (raised against the N-SH2 domain) and visualised by ECL. Protein expression was normalised by Western blotting of whole lysates with anti-MYC monoclonal antibody. Figure (A.) shows a graphical representation of four experiments, whilst figure (B.) shows a typical western blot result (n=7 +/- SEM).

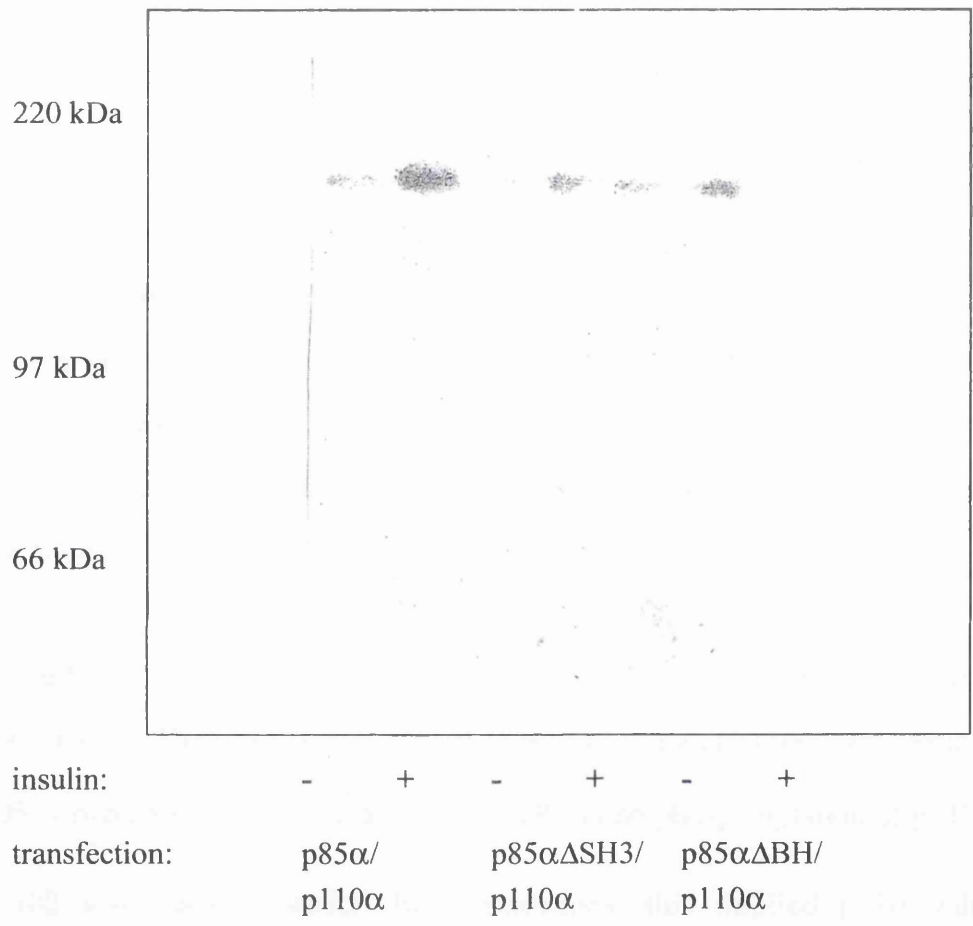


Figure 3.10 Phosphotyrosyl containing proteins in basal and stimulated HEK293 cells. Cells were cotransfected with p110 and either wild type p85α, p85αΔSH3 or p85αΔBH. After stimulation with vehicle or 100nM insulin, cells were lysed and subject to antiphosphotyrosine immunoprecipitation (PY99, Santa Cruz Biotechnology). Proteins in the immunoprecipitates were separated by SDS-PAGE using an 8% resolving gel, transferred, probed with antiphosphotyrosine antibody and visualized with ¹²⁵I-labelled protein A.

4. THE KINASE ACTIVITY OF P110 α AND P110 β

4.1 Summary

The studies presented in this chapter directly compare the catalytic activities of p110 α and p110 β . A number of important functional differences were identified.

The catalytic subunit of PI 3'-kinase is shown to influence recruitment of p85 α protein to phosphotyrosine complexes following cell stimulation with insulin. The lipid kinase activities of p110 α and p110 β dimerised with p85 α were compared, and p110 α was found to phosphorylate approximately 3-fold more PI in a timed period. The protein kinase activity of p110 α towards p85 α was 10-fold higher than the protein kinase activity of p110 β .

The effect of the phosphorylation state of p85 on p110 lipid kinase activity was also studied. The lipid kinase activity of both p110 α and p110 β were reduced by 80% when p85 was preincubated with ATP. As no phosphorylation of p110 α or p110 β was detected under these conditions, this implied p110 induced phosphorylation of p85 was downregulating activity. Ser608 on p85 α was found to be the major, but not only, phosphorylation site. p85 α S608A and p85 α S608E expressed in complex with p110 α were used to determine the level of phosphorylation which occurred at this residue, and to further understand the role of this site in regulating lipid kinase activity. Both the alanine and glutamic acid point mutations at residue 608 of p85 α reduced lipid kinase activity of the PI 3'-kinase heterodimer by approximately 80%.

The ability of PI 3'-kinase to phosphorylate a peptide which mimics the residues incorporating the Ser608 site of p85 α was investigated. Phosphorylation was shown to occur, and this was a Mn²⁺ dependent, wortmannin inhibited event. The peptide was used to develop a novel, SDS-PAGE based assay for cellular PI 3'-kinase activity. Phosphorylation of IRS1 by PI 3'-kinase is studied, and differences in the protein kinase activity of p110 α and p110 β towards this protein are presented.

4.2 Introduction

Three isoforms of the p110 catalytic subunit of PI 3'-kinase have been identified. Two of these, p110 α and p110 β , have a wide tissue distribution, whilst the third, p110 δ is restricted to haemopoietic cells. Recent evidence suggests these have specific functions in the cell (Bi et al 1998; Roche et al 1998b; Siddhanta et al 1998; Vanhaesebroeck et al 1999) however the molecular basis of these differences is unknown. The studies presented in this chapter focus on the intrinsic activity of two catalytic subunits of PI 3'-kinase, p110 α and p110 β , and how these are regulated by the phosphorylation state of p85 α .

Like the p85 adapter proteins, the catalytic subunits which make up the class 1a PI 3'-kinase family are composed of modular domains (see figure 1.2), and share 70% identity at the amino acid level. Initial studies of substrate specificity have been performed, which revealed no obvious structural differences between the p110 proteins. *In vitro*, all the class 1a PI 3'-kinases can phosphorylate PtdIns, PtdIns-4P and PtdIns-4,5,P₂ on the D3 position of the phosphoinositide head group. There have subsequently been very few reports in the literature which address whether the three catalytic isoforms of PI 3'-kinase have redundant functions or provide a mechanism for signalling specificity.

Investigation of p110 has been hindered in the past by an absence of specific antibodies to the different catalytic subunits. In this chapter, potential differences in the function of p110 α and p110 β were studied using transfected p85 α /p110 α and p85 α /p110 β complexes immunoprecipitated from HEK 293 cells. By FLAG epitope tagging p110 constructs, and myc epitope tagging p85, the problem of antibody specificity was avoided. Lipid and protein kinase activities were compared, as was the effect of the catalytic subunit on recruitment of PI 3'-kinase into signalling complexes.

4.3 Results

4.3.1 The effect of the catalytic subunit on recruitment of PI 3'-kinase to phosphotyrosine complexes following insulin stimulation.

To study whether the catalytic subunit has any effect on the recruitment of p85 α to phosphotyrosine complexes, HEK 293 cells were transfected with either p85 α alone, or cotransfected with either p110 α or p110 β . Following overnight serum starvation, cells were stimulated with vehicle or 100nM insulin, lysed and subjected to immunoprecipitation with antiphosphotyrosine antibody (PY99, Santa Cruz).

10% of each lysate was retained prior to immunoprecipitation. Proteins in this lysate were also subjected to SDS-PAGE and transferred to PVDF. Western blotting with anti-myc and anti-FLAG antibodies was performed to normalise for transfected p85 and p110 expression respectively. These Western blots also indicated that p85 α expression was not affected whether p85 α was expressed alone, or in combination with p110 α or p110 β . Figure 4.1.B. shows a typical Western blot result. Immunoprecipitated proteins were separated by SDS-PAGE using an 8% resolving gel, and transferred to PVDF. The transfer membrane was probed with 9E10 (monoclonal α -myc antibody) and visualised by ECL. Figure

4.1.A. shows that when expressed alone, p85 α is recruited to phosphotyrosine complexes less than 2-fold following insulin stimulation. When coexpressed with p110 α , recruitment of p85 α to phosphotyrosine complexes is stimulated 5-fold, whereas coexpression with p110 β results in almost a 10-fold stimulation in recruitment of p85 α protein to phosphotyrosine complexes following insulin stimulation. These data show that the catalytic subunit of PI 3'-kinase has a significant effect on the recruitment of the enzyme heterodimer to phosphotyrosine complexes following insulin stimulation.

4.3.2 Lipid kinase activity of p110 α and p110 β

The lipid kinase activities of p110 α and p110 β were compared using HEK 293 cells cotransfected with p85 α and either p110 α or p110 β (ratio 12:10) as described in Methods. 90% of each sample was immunoprecipitated with 9E10 (monoclonal anti-MYC antibody) and washed extensively, and lipid kinase assays were performed as described in Methods. The remaining 10% of each sample was immunoprecipitated was resolved by SDS-PAGE and Western blotted with antibodies to myc or FLAG tags. This allowed the PI 3'-kinase activity to be normalized for the amount of protein expression of p85 and p110 present in the immunoprecipitates. This method of normalizing activity for protein levels was used throughout the experiments described in this thesis. The results are shown graphically in Figure 4.2. The data shows that when dimerised with p85 α , p110 α is 3-fold more active as a lipid kinase than p110 β using standard assay conditions. For both catalytic isoforms, 100nM wortmannin inhibits lipid kinase activity by 95%.

4.3.3 p85 α is phosphorylated by p110 α and p110 β

The ability of p110 α and p110 β to phosphorylate p85 α was studied. HEK 293 cells were cotransfected with p85 α and either p110 α or p110 β . PI 3'-kinase complexes were immunoprecipitated from lysates with 9E10 (monoclonal α -MYC

antibody). Immunoprecipitated proteins were washed extensively and resuspended in protein kinase assay buffer as described in Methods. Immunoprecipitates were treated with vehicle or 100nM wortmannin. The reaction was started by the addition of ATP to a final concentration of 12 μ M containing 5 μ Ci 32 P- γ -ATP (per immunoprecipitate), and stopped following a 10 minute incubation at 30°C with SDS-PAGE gel loading dye. Proteins in the assay were separated by SDS-PAGE using an 8% resolving gel. Gels were fixed as described in Methods, and dried *in vacuo*. 32 P phosphorylated p85 α was quantified by phosphorimager, and the results are represented graphically in Figure 4.3.A. A typical autoradiograph is shown in Figure 4.3.B.

These results show that both p110 α and p110 β phosphorylate p85 α . However, p110 α shows 10-fold greater protein kinase activity towards p85 than p110 β . As shown, this phosphorylation is inhibited by wortmannin. There was no evidence of p110 phosphorylation.

4.3.4 Phosphorylation of p85 α inhibits PI 3'-kinase lipid kinase activity

Reports in the literature have demonstrated that baculovirus prepared p110 α lipid kinase activity is inhibited by phosphorylation of p85 α although it is not known whether p110 β causes similar effects. Figure 4.4 shows the results of experiments performed with PI 3'-kinase complexes immunoprecipitated from mammalian cells. Briefly, HEK 293 cells were cotransfected with p85 α and either p110 α or p110 β . Cell lysates were split in 2, and proteins immunoprecipitated with 9E10 (monoclonal α -MYC antibody). Immunoprecipitates were resuspended in protein kinase assay buffer and incubated with either vehicle or 10 μ M ATP. Immunoprecipitated proteins were then washed as described for lipid kinase assays, before phosphorylation of PI was determined as usual. The data shows that for both p110 α and p110 β , phosphorylation of p85 α reduces the lipid kinase

activity of PI 3'-kinase by 75% following ten minutes preincubation with ATP in kinase assay buffer.

4.3.5 In vivo phosphorylation of p85 α

There are two possible explanations for the data on the lipid kinase (Figure 4.2) and protein kinase (Figure 4.3) activities of p110 α and p110 β . Either p110 β has significantly reduced kinase activity compared to p110 α , or p110 β is a highly efficient protein kinase which has maximally phosphorylated p85 *in vivo*, thereby switching off the lipid kinase activity accounting for the relatively low level of lipid and protein kinase activity found when the complex is immunoprecipitated.

To determine whether p110 β fully phosphorylates p85 α *in vivo*, HEK 293 cells were cotransfected with p85 α and either p110 α , p110 β or a p110kinase dead construct. The conditions used were known to produce similar amounts of p85 α /p110 α and p85 α /p110 β . Cell proteins were labelled with ^{32}P orthophosphate as described in Methods. Cell lysates were then subjected to immunoprecipitation with 9E10 (monoclonal α -MYC antibody). To analyze the effects on endogenous PI 3'-kinase, untransfected cells were similarly labelled, and immunoprecipitated with an α -p110 β antibody (S19, Santa Cruz). This lysate was then re-immunoprecipitated with an α -p85 antibody. Immunoprecipitated proteins were separated by SDS-PAGE using an 8% resolving gel, which was fixed and dried *in vacuo*. The autoradiograph is shown in Figure 4.5. In these experiments, protein levels were not normalized, but previous experiments indicate expression of p85 in association with either catalytic subunit was usually very similar.

These results indicate that in resting cells, p85 α associated with p110 β is phosphorylated only at low levels. This is true both for endogenous protein (lane (a)), and transfected protein (lanes (e) and (f)). When associated with p110 α , both

endogenous p85 (lane (b)) and transfected p85 α (lanes (c) and (d)) phosphorylation is significant.

Taken together, the lipid kinase data (Figure 4.2), in vitro protein kinase data (Figure 4.3) and in vivo cell labelling data (Figure 4.5) suggest p110 β has less kinase activity than p110 α .

4.3.6 The major target of p110 is Serine 608 on p85 α

Previous study showed by N-terminal sequence and mass analyses that Ser608 is a major phosphorylation site on p85 α . In this study, a mutagenesis approach was used to determine the significance of this site on overall phosphorylation of p85 α , and also on lipid kinase activity. MYC epitope tagged constructs were made in which Ser608 was mutated to either alanine, which cannot be phosphorylated, or glutamic acid, which has been used in similar studies to mimic phosphoserine. A schematic representation of the mutagenesis strategy is shown in Figure 4.6. The primers used are shown in Methods, Table 2.1.

4.3.7 Expression of p85 point mutated proteins and interaction with the catalytic subunit

HEK 293 cells at 60% confluence were cotransfected with either wildtype p85 α , p85 α S608A or p85 α S608E and p110 α . Cells were lysed and proteins were immunoprecipitated with 9E10 (monoclonal α MYC antibody). Western blotting experiments (see figure 4.7) showed that neither expression of the p85 α S608 point mutants in HEK 293 cells, nor their interaction with p110 α , was affected by the point mutation.

4.3.8 Phosphorylation of p85 α S608A mutants by p110 α

HEK 293 cells were cotransfected with p110 α and either wild type p85 α , or p85 α S608A or p85 α S608E. Cell lysates were subjected to immunoprecipitation with 9E10 (monoclonal α -MYC antibody), and immunoprecipitated proteins were

washed extensively as described in Methods. *In vitro* protein kinase assays were then performed as described, and a typical autoradiograph is shown in Figure 4.8.A. The results of 4 separate experiments are shown graphically in Figure 4.8.B. These data show that mutation of Ser608 on p85 α to either alanine or glutamic acid reduces p85 α phosphorylation by p110 α by 80%.

4.3.9 The role of phosphorylation of p85 α S608 in lipid kinase activity of p110

The influence of Ser608 of p85 α on the lipid kinase activity of PI 3'-kinase was further investigated by studying the lipid kinase activities of p110 α dimerised with wild type p85 α , p85 α S608A and p85 α S608E. HEK 293 cells were cotransfected with p110 α and either wild type or point mutated p85 α , and cell lysates were subjected to immunoprecipitation with 9E10 (monoclonal α -MYC antibody). Immunoprecipitated proteins were washed extensively, and lipid kinase assays were performed as described in Methods. Again, a portion of lysate was retained for normalizing expression levels by Western blotting as previously described. A typical TLC plate is shown in Figure 4.9.A., and the results of 4 separate experiments are shown graphically in Figure 4.9.B. For both the alanine and the glutamic acid mutation at Ser608 on p85 α , the lipid kinase activity of p110 α is reduced by approximately 75%. The same reduction was observed if p110 β was cotransfected in place of p110 α and these results are shown in Figure 4.3.9.C.

4.3.10 Development of a novel assay for PI 3'-kinase

Detailed comparison of the protein kinase activities of p110 α and p110 β are difficult due to the fact that the substrate, p85 α , is in a 1:1 complex with the catalytic subunit. The fact that p110 isoforms phosphorylate Ser608 of p85 α was used as the basis for developing a novel peptide assay for PI 3'-kinase. A peptide,

from residues 596 to 616 of bovine p85 α , was purchased from Alta Bioscience, which incorporates the Ser608 site, as shown:

R-R-L-G-N-E-N-T-E-D-Q-Y-S-L-V-E-D-D-E-D-L

Protein kinase assays based on the phosphorylation of peptides frequently use phosphocellulose cation-exchange (P81) paper. The assay technique used is dependent on the substrate. In the simplest case, a peptide which contains lots of basic residues can be designed, and at low pH this will stick to phosphocellulose paper due to the positive charges on the basic residues, whereas the ^{32}P - γ -ATP will not and can be washed away. However, the amino acid sequence surrounding the Ser608 residue on p85 α contains a high proportion of acidic residues. Although two arginine residues were added to the NH₂-terminus of the peptide as shown, preliminary studies found that the peptide did not stick to p81 paper. Instead, another assay technique was optimised, utilising the Tricine SDS-PAGE system. These gels were optimised by Shagger and von Jagow (Shagger & von Jagow 1987) for the resolution of small peptides.

PI 3'-kinase dependent phosphorylation of the peptide, wortmannin sensitivity and ion dependence were studied. PI 3'-kinase was immunoprecipitated from transfected HEK 293 cells via the MYC epitope tag and washed as described in Methods as for PI 3'-kinase lipid kinase assays. Immunoprecipitated proteins were resuspended in protein kinase assay buffer as described in Methods.

2.4mM peptide in water was added to each immunoprecipitate, and the reaction was started by the addition of ATP to a final concentration of 10 μM (containing 5 μCi ^{32}P - γ -ATP). The reaction was stopped by the addition of SDS-PAGE gel loading dye, and the reaction products were separated by tricine SDS-PAGE using a 12.5% resolving gel. Gels were fixed with 50% methanol: 10% acetic acid and dried *in vacuo*. A typical autoradiograph is shown in figure 4.10.A. Lanes (b) and

(c) show in duplicate the phosphorylation of the p85 α S608 peptide by immunoprecipitated PI 3'-kinase from transfected HEK293 cells. The same conditions were used for the reaction in lane (a), which shows that phosphorylation of the peptide is inhibited by 100nM wortmannin. MYC antibody immunoprecipitation from nontransfected cells does not immunoprecipitate any protein which phosphorylates the peptide (lane (d)), whilst the absence of peptide from the reaction results in no band observed corresponding in size to the peptide. The ion dependence of the reaction was also studied, and Figure 4.10.B shows p85 α peptide phosphorylation by PI 3'-kinase is manganese dependent.

In summary, these data show that the peptide is only phosphorylated when PI 3'-kinase is present, the phosphorylation is manganese dependent, and this phosphorylation is inhibited by 100nM wortmannin. These findings together indicate the phosphorylation of the peptide is being mediated by the catalytic subunit of PI 3'-kinase.

4.3.11 Confirmation of peptide identity by mass spectrometry

To further confirm the identity of the band at 4.5 kDa, mass spectrometric analysis of the excised band was performed. The gel band was chopped into small pieces, and the peptide extracted in 50% acetonitrile: 5% TFA. The extract was reduced *in vacuo* and the peptide resuspended in 50% acetonitrile for mass spectrometric analysis. Figure 4.11 shows the laser desorption TOF spectrometric profile of the peptide excised from the gel, with the peptide (2510.1 Da) and phosphorylated peptide (2590.1 Da) clearly visible. The peak at 2590.1 was only visible for peptide extracted from gel when pre-reacted with PI 3'-kinase. The other peaks are probably contaminants from the acrylamide gel.

4.3.12 Ser608 peptide as an assay for cellular PI 3'-kinase

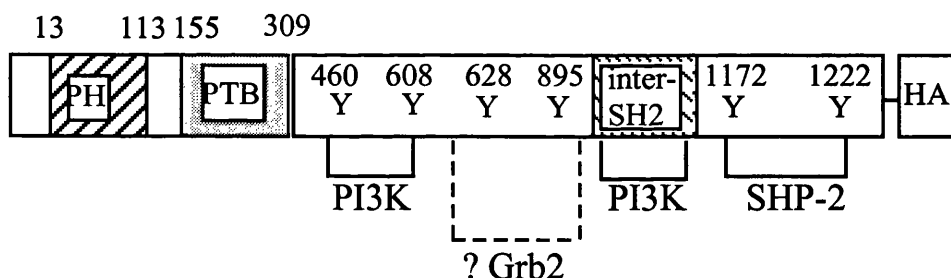
The possibility of using the Ser608 peptide as an assay for cellular PI 3'-kinase was investigated. CHO-IR cells were cotransfected with p85 α and either p110 α or p110 β as described in Methods. Cells were stimulated with vehicle or 100 nM insulin, and cellular proteins were subjected to immunoprecipitation with a monoclonal α -phosphotyrosine antibody (PY99, Santa Cruz). Immunoprecipitated proteins were washed as described in Methods for lipid kinase assay. Lipid kinase assays using phosphatidylinositol as substrate were performed as controls, and a TLC plate separation of extracted lipids is shown in figure 4.12.A. This shows a typical recruitment of 8-10 fold lipid kinase activity in phosphotyrosine immunoprecipitates following insulin stimulation.

Kinetic studies (see Chapter 5) indicated different K_m (peptide) values for p110 α and p110 β , and we had hoped to exploit these to produce an assay which could differentiate between the isoforms of PI 3'-kinase. Therefore, peptide assays were performed at two concentrations, 0.1mM and 2.4mM chosen just above the K_m values for p110 β and p110 α , as shown. The results of the peptide assay are shown in figures 4.12.B and 4.12.C. For wild type and transfected CHO-IR's, an 8-10 fold increase in peptide phosphorylation is seen in phosphotyrosine immunoprecipitates following insulin stimulation at high concentrations of peptide. This is similar to the fold-increase observed when PI 3'-kinase was assayed with PI substrate (Figure 4.12.A). At the low peptide concentration, a reduced but significant stimulation in peptide phosphorylation was observed following insulin stimulation of wild type (3-fold increase in peptide phosphorylation) and p110 β / p85 α transfected CHO-IR cells (5-fold). No increase in peptide phosphorylation was observed following insulin stimulation of p110 α / p85 α transfected cells.

4.3.13 Phosphorylation of IRS1 by p110 α and p110 β

Previous studies have also shown immunoprecipitated PI 3'-kinase, and baculovirus expressed p110 α / p85 α can phosphorylate IRS-1. Identifying the residues on IRS-1 that are phosphorylated by p110 would provide a great deal of new information about the characteristics and substrate specificity of the protein kinase activity of PI 3'-kinase. Therefore, the ability of p110 α and p110 β to phosphorylate IRS1 was compared, and the nature of the phosphorylation event determined.

An IRS1 construct (IRSi) containing the inter-SH2 domain of p85 and an HA epitope tag was a gift from R. Roth (Stanford University School of Medicine, California).



Schematic of IRSi construct

The IRS-1 construct (IRSi), containing the inter-SH2 domain of p85 α used in these experiments was a gift from Richard Roth. The domain structure and the tyrosine residues which are phosphorylated by the insulin receptor to provide docking sites for signalling proteins are shown.

HEK 293 cells were cotransfected with this IRS1 construct and either p110 α or p110 β . Cell lysates were subjected to immunoprecipitation with 9E10 (monoclonal α -MYC antibody), and an *in vitro* protein kinase assay was

performed as described in Methods. A typical autoradiograph is shown in figure 4.13.

4.3.14 Phosphoamino acid analysis of IRS1 phosphorylated by p110

To determine whether the differing levels of phosphorylation of IRS by p110 α and p110 β was due only to the stoichiometry of phosphorylation, or whether there were differences in the actual sites phosphorylated by the two isoforms, phosphorylated IRS1 was subjected to phosphoamino acid analysis. The autoradiograph revealed two bands, both of which had a similar migration to that expected for IRS-1, but only phosphorylation of the upper band was fully inhibited by 100nM wortmannin. The upper band shown in the autoradiograph of figure 4.13 (which completely disappears on addition of wortmannin) was excised with a sharp scalpel and the gel slice subjected to acid hydrolysis in 6M HCl at 110°C for 75 minutes. The hydrolysate was dried *in vacuo* and the phosphoamino acids were prepared for separation by one dimensional thin layer electrophoresis. The samples were resuspended in 10 μ l electrophoresis buffer (pyridine: acetic acid: H₂O 10: 100: 1890) containing 10 mM each O-phosphoserine, O-phosphothreonine and O-phosphotyrosine standards. Samples were spotted on to a Kodak cellulose chromatogram sheet which was then sprayed with electrophoresis buffer. Electrophoresis was performed at 1000 V, 50 mA, for 45 minutes with cooling. The electrophoresed chromatogram was dried, stained with ninhydrin and developed for 1 minute at 110°C. The chromatogram was developed by phosphorimager and the positions of ³²P labelled phosphoamino acids correlated with the ninhydrin stained standards. The results for the two isoforms are shown in figure 4.14, which show that the protein kinase activities of p110 α and p110 β towards IRS1 are different. Whilst IRS-1 associated with p110 α is phosphorylated on serine, threonine and tyrosine residues, IRS-1 coexpressed with p110 β is phosphorylated only on threonine and tyrosine.

4.4 Discussion

The data presented in this chapter have used transfected PI 3'-kinase complexes immunoprecipitated from mammalian cells to demonstrate functional differences between the two isoforms of catalytic subunits studied, p110 α and p110 β . The differences described provide evidence that these isoforms are likely to have distinct roles *in vivo*. By using PI 3'-kinases transcribed from transfected DNA, which had been epitope tagged, it was possible to compare the activities of p110 α and p110 β directly, circumventing difficulties frequently encountered with antibody specificity when studies are undertaken with enzymes isolated from mammalian cells. The constructs were expressed directly in mammalian cells rather than in insect cells. The benefit of this is that post-translational processing of proteins expressed by baculovirus induced expression in insect cells has been shown to differ from that observed in mammalian cells, which display a different array of modifying enzymes (Gout et al 1992; Roche et al 1994a). The PI 3'-kinases used in these studies are therefore more likely to reflect those complexes formed *in vivo* from endogenous genes, and considerable differences in the characteristics of mammalian and insect cell expressed PI 3'-kinase have since been shown (Yu et al 1998). In addition, expression in mammalian cells enables direct comparison of recruitment to signalling complexes within the cell.

Comparison of the sequences of two of the class 1a catalytic isoforms of PI 3'-kinase studied (see Table 1 and Appendix) shows approximately 70% overall identity at the amino acid level. Differences in the activity and functions of p110 α and p110 β determined in these studies are (i) p110 α has a greater lipid kinase activity towards phosphatidylinositol than p110 β , (ii) p110 α has greater protein kinase activity towards p85 α than p110 β , (iii) p110 α demonstrates greater protein kinase activity to two exogenous substrates, a peptide and IRS-1, than p110 β and (iv) the catalytic subunit influences incorporation of p85 α into phosphotyrosine complexes following insulin stimulation.

One of the major mechanisms by which growth factors and other stimuli activate PI 3'-kinase is by recruitment to the membrane localized substrate. This is achieved via binding of the SH2 domains of the adapter subunit to phosphorylated YXXM motifs (Panayotou et al 1992; Pawson & Schlessinger 1993; Walksman et al 1993). To establish whether any role existed for the catalytic subunit in the relocation of PI 3'-kinase to intracellular membranes, experiments were performed to determine whether coexpression of p85 α with either p110 α or p110 β affected recruitment into phosphotyrosine complexes following insulin stimulation. Unexpectedly p85 α expressed alone, which interacts with only negligible amounts of endogenous catalytic subunit (see section 3.3.5), was poorly recruited into phosphotyrosine complexes following insulin stimulation. Since p85 α transfected in the absence of catalytic subunit is expressed equally well as p85 α cotransfected with p110, these results suggest the minimal function of the catalytic subunit in recruitment to signalling complexes is one of stabilizing the function of the adapter subunit. Coexpression of p85 α with p110 α caused 5-fold increase in levels of p85 at phosphotyrosine complexes following insulin stimulation, whilst the increase associated with p110 β coexpression was approximately 10-fold. Probing Western blots of the phosphotyrosine immunoprecipitates with phosphotyrosine antibody did not reveal any band in the expected size range of p85. This indicates that tyrosine phosphorylation of the adapter subunit does not account for the increased levels of p85 α observed in phosphotyrosine immunoprecipitates following insulin stimulation when p110 is coexpressed. It would be interesting to know if the adapter binding regions of the different class 1a catalytic subunits were sufficient to induce differential recruitment of p85 α to phosphotyrosine based signalling complexes following cell stimulation with insulin, since their different interaction with p85 may confer slight structural differences which differentially orientate the SH2 domains. It has also been reported that binding of peptides to the p85 SH2 domains increases the activity of the catalytic subunit of

PI 3'-kinase (Herbst et al 1994; Rordorf-Nikolic et al 1995) and in future studies it will be important to determine whether this is equally true for p110 α and p110 β .

In untransfected cells, the ligand stimulated recruitment of endogenous p85 to phosphotyrosine complexes is reported, as in this thesis, to be 8-10 fold, a figure which is in close agreement to that reported here for insulin stimulated recruitment of p85 α / p110 β . Following completion of these experiments, data was published by the group of Klip which showed p110 β , but not p110 α , was increased at glucose transporter compartments following insulin stimulation of adipocytes (Wang et al 1998). Taken together, it is tempting to surmise that in insulin stimulated cells, p110 β is the major recruited PI 3'-kinase, although no mechanistic explanation can be speculated at the current time.

Perhaps suprisingly, reports in the literature comparing the lipid kinase activities of the catalytic isoforms of class 1a PI 3'-kinases refer only to substrate specificity ((Vanhaesebroeck et al 1997a) and references therein); there are no comparisons of the actual activities of these isoforms. Furthermore, the existance of a protein kinase activity of p110 β was only reported a few weeks prior to completion of this thesis (Roche et al 1998a). In the studies presented in this chapter, it is shown that p110 α and p110 β phosphorylate PI to differing extents under the conditions described, and that both can phosphorylate associated p85 α . The lipid kinase activity of p110 α towards PI was 3-fold greater than that of p110 β , and the protein kinase activity of p110 α towards p85 α was 10-fold greater than that of p110 β . The results of these studies lead us to undertake a detailed comparison of the kinetics of p110 α and p110 β , and these are discussed in Chapter 5.

It has been shown that p85 α is more readily phosphorylated at serine residues, whilst p85 β is predominantly tyrosine phosphorylated in resting T cells, and this is

exacerbated following cellular activation (Reif et al 1993). p85 β possesses a site analogous to the regulatory ser608 on p85 α but it is not yet known if this is phosphorylated by p110 and what the consequences of this might be. The serine 608 site of p85 α has previously been reported to be an autoregulatory site, whereby phosphorylation by p110 α inhibits lipid kinase activity of the heterodimer (Carpenter et al 1993; Dhand et al 1994b). The studies presented here show that phosphorylation of p85 α by p110 β also downregulates the lipid kinase of this catalytic subunit, although to a slightly lesser extent than that observed in the case of p110 α . The p85 α S608A and p85 α S608E constructs used here also provide the first evidence that p110 β targets p85 α at serine residue 608. Orthophosphate labelling of p85 α associated with either of the two isoforms of catalytic subunit showed a similar pattern of phosphorylation occurs in vivo, and this was obliterated by coexpression of p85 with a kinase dead construct of p110 α .

Assays performed with mutant p85 α , in which serine 608 was replaced with either alanine or glutamic acid, indicate this was the major target of both p110 α and p110 β protein kinase activity, confirming the findings of Dhand et al (Dhand et al 1994b). Glutamic acid is commonly used to mimic phosphoserine in studies of activity or structure, and kinase assays of p110 associated with p85 α S608E demonstrated an expected reduction in lipid kinase activity. Unexpectedly, the lipid kinase activity of p110 associated with p85 α S608A showed a parallel decrease. This suggests that it is necessary to have a serine residue at position 608 and that any perturbation at this site causes a downregulation of lipid kinase activity. It is also of interest that there are reports that the tyrosine at 607 can also be phosphorylated in some cell types and given its position it is likely this would have effects on PI 3'-kinase activity. Furthermore, a splice variant of p85 α has been reported in which 8 amino acids are inserted immediately upstream of ser608

(Antonetti et al 1996). This introduces two potential serine phosphorylation sites so it is likely this version of p85 will have a big impact on the activity of p110.

The ability of p110 isoforms to phosphorylate serine 608 within the sequence of p85 was investigated as a possible basis for a novel assay for growth factor activation of PI 3'-kinase. Both p110 α / p85 α and p110 β / p85 α were able to phosphorylate the peptide which comprised 21 amino acids of p85 α centred about the serine608 site. Although Tyr607 has also been shown to be phosphorylated by p110, phosphorylation at this site is not altered by insulin. The level of phosphorylation differed between the two PI 3-kinase isoforms, reflecting the extent of phosphorylation of full length p85 α . In addition, phosphotyrosine immunoprecipitates from insulin treated cells stimulated an increase in phosphorylation of the peptide compared to precipitates from basal cells. The degree of increase in peptide phosphorylation was almost identical to the insulin induced increase in PI 3'-kinase lipid kinase activity in antiphosphotyrosine immunoprecipitates. This establishes that the described peptide provides a suitable basis for a novel assay for cellular PI 3'-kinase activity. Furthermore, whilst PI can be phosphorylated by enzymes other than the class 1a PI 3'-kinases, it is likely that the serine 608 peptide will be a unique substrate for this subfamily of enzymes since the target is unusual in that it is immediately flanked by relatively hydrophobic residues but the regions both upstream and downstream are extremely acidic. A search of the Phosphobase database for recognition sequences within the peptide for other enzymes indicated only a potential affinity by casein kinases. Casein kinase I appears to target serine residues with a glutamate residue on the amino terminal side in the n-2 position (the serine 608 peptide has glutamate at the n-4 position) although this preference has been determined from studies on only a few substrates. However, casein kinase II has been better characterised in terms of substrate specificity, and clearly targets serine residues with a cluster of acidic amino acids carboxyl to the target site. Aspartate residues

are better acidic determinants compared to glutamic acid, and the serine 608 peptide contains the sequence E-D-D-E-D at position n+3 to n+7 from the serine target site (Tuazon & Traugh 1991). Furthermore, insulin and EGF have been shown to increase the activity of casein kinase II under some circumstances (Ackerman et al 1990; Sommercorn et al 1987). Taken together, this suggests casein kinase II could potentially phosphorylate the serine 608 peptide and further investigations are underway to determine whether or not this occurs.

It has been shown that IRS immunoprecipitated from insulin stimulated cells is phosphorylated by a wortmannin sensitive mechanism (Freund et al 1995; Lam et al 1994). There are some speculative suggestions that phosphorylation of IRS1 by PI 3'-kinase reduces p85 phosphorylation presumably at least in part by competitive inhibition, suggesting the serine kinase activity may have a dual function in regulating IRS1 and PI 3'-kinase. In addition to phosphorylating full length p85 α and a peptide of the region incorporating serine 608 of p85 α , it is shown in this chapter that p110 α and p110 β both phosphorylate IRS-1. In the studies presented, interaction between IRS-1 and PI 3'-kinase was rendered insulin independent by use of an IRS-1 construct containing the sequence for the inter-SH2 domain of p85. This was cotransfected with p110, producing complexes either of IRS-1/p110 α , or IRS-1 p110 β . These studies show the IRS construct was phosphorylated in both cases and this was blocked by wortmannin, suggesting it was specifically due to the p110 subunit. Phosphoamino acid analysis of phosphorylated IRS-1 revealed a differential pattern of phosphorylation induced by the two isoforms of catalytic subunit. Only p110 α phosphorylated IRS-1 at serine residues; there was no evidence of IRS-1 phosphorylation on serine residues by p110 β . However, IRS-1 associated with either isoform was phosphorylated on threonine and tyrosine residues, which was inhibited by wortmannin. To date, there are no reports of tyrosine or threonine kinase activity associated with PI 3'-kinase. These data therefore raise the possibility that p110 α

and p110 β have an intrinsic threonine/ tyrosine kinase activity, perhaps acting as 'promiscuous' kinases. Alternatively, it is possible that IRS-1/ PI 3'-kinase complexes associate tightly with protein kinases with these activities, and these were inhibited at the concentrations of wortmannin used. Lam *et al* also presented evidence of tyrosine phosphorylation of IRS proteins bound to PI 3'-kinase, although that study was not performed in parallel with PI 3'-kinase inhibitors.

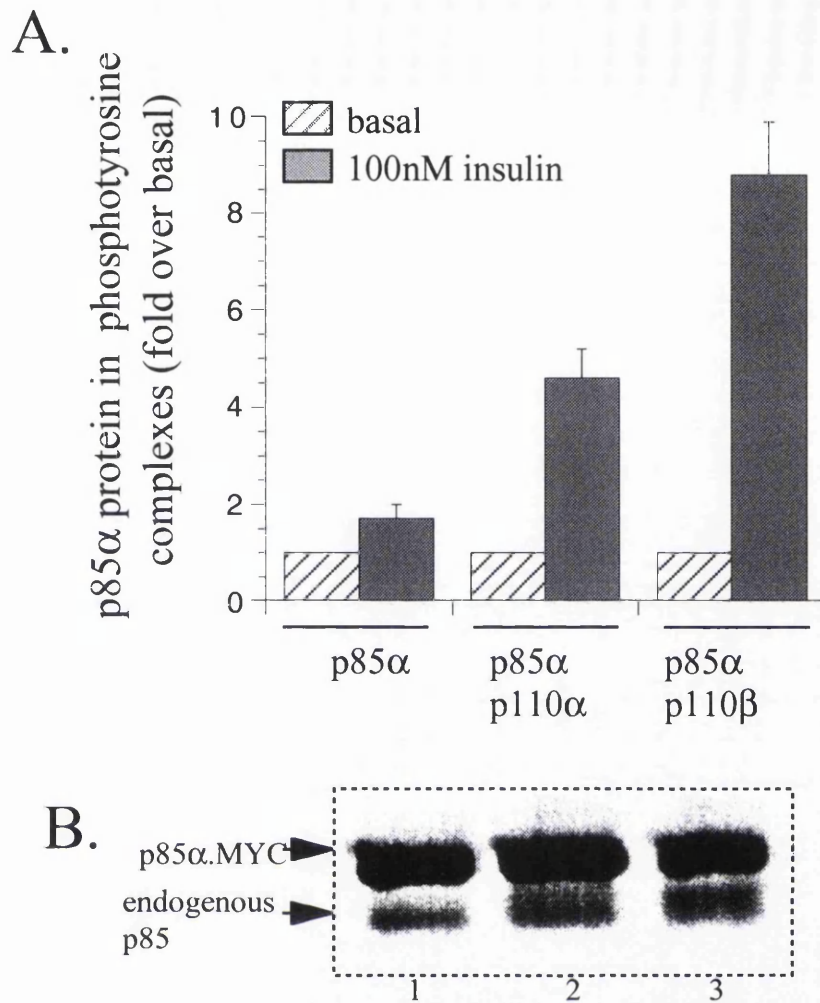


Figure 4.1 The catalytic subunit of PI 3'-kinase affects recruitment of p85α to phosphotyrosine complexes following insulin stimulation.

Cells were stimulated with vehicle or with 100nM insulin and lysed. Immunoprecipitated phosphotyrosine complexes (PY99 monoclonal antibody, Santa Cruz) were resolved by SDS-PAGE and analysed by Western blot, using the 9E10 (myc monoclonal) antibody (A.) (n=5 +/-SEM). Protein expression was normalised by Western blotting with a polyclonal p85 antibody, and a typical normalisation blot is shown in (B). The lanes are 1) p85α expressed alone, 2) p85α coexpressed with p110α and 3) p85α coexpressed with p110β.

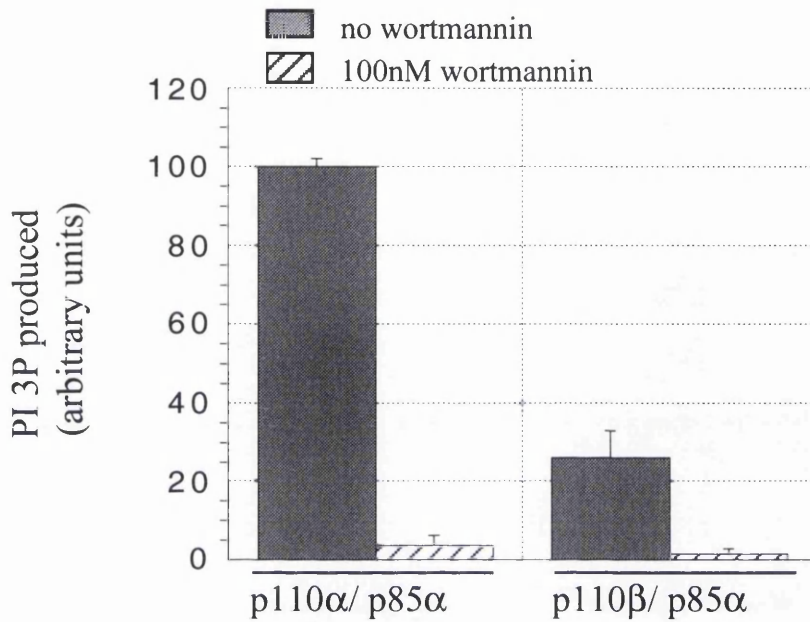


Figure 4.2 Lipid kinase activity of phosphoinositide 3'-kinase

HEK293 cells were cotransfected with p85α and either p110α or p110β. PI 3'-kinase was immunoprecipitated via the myc tag and lipid kinase assays were performed as described in Methods (n=5 +/- SEM). Protein expression was normalized by Western blotting with anti-myc antibody, and myc and FLAG levels were also compared.

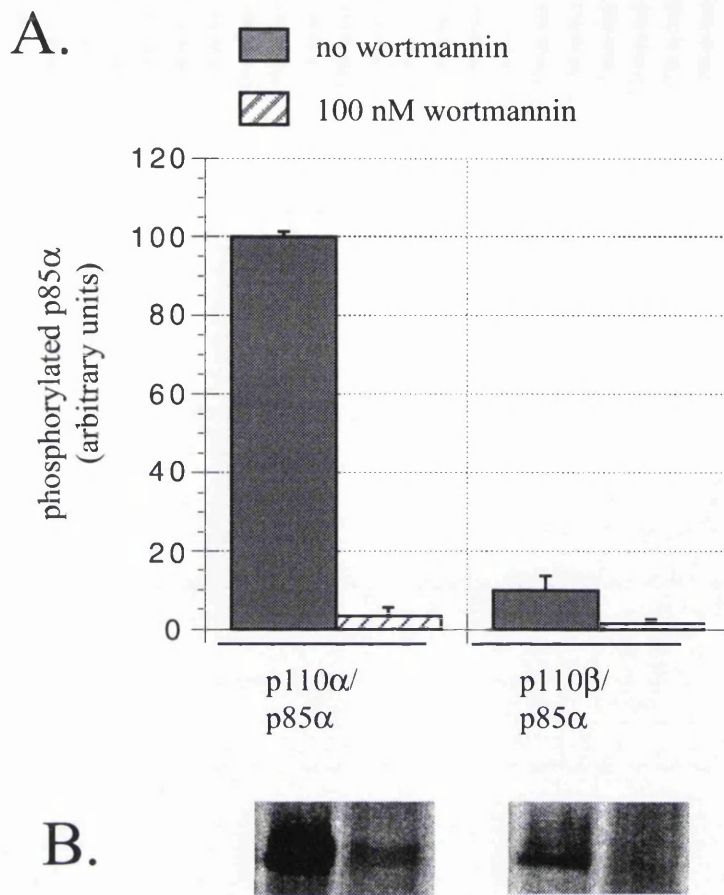
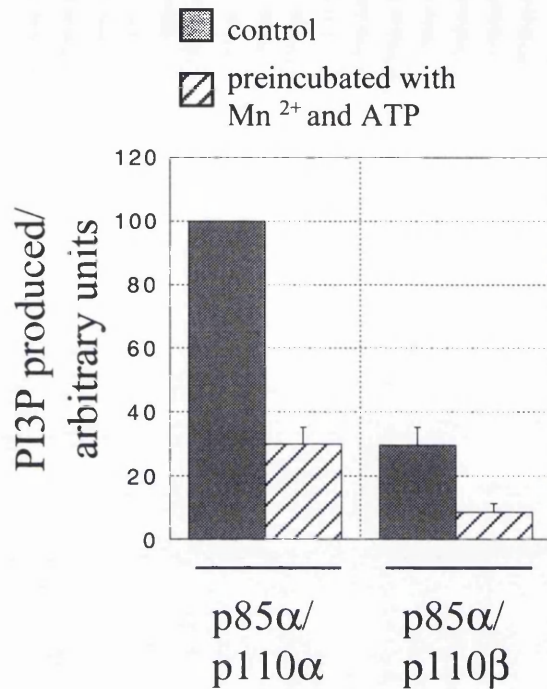


Figure 4.3 Phosphorylation of p85 α by the catalytic isoforms of phosphoinositide 3'-kinase.

HEK293 cells were cotransfected with p85 α and either p110 α or p110 β . PI 3'-kinase was immunoprecipitated via the myc epitope tag on the adapter subunit, protein kinase activity of p110 α and p110 β towards p85 α was measured as described in Methods. Briefly, ^{32}P phosphorylated p85 were resolved by SDS-PAGE and quantitated by phosphorimager. Protein expression was normalised by Western blotting. The results of 4 experiments are shown graphically in A (n=4 \pm SEM), whilst B. shows a typical autoradiograph.

A.



B.

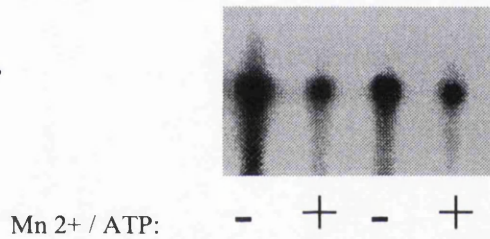


Figure 4.4 Phosphorylation of p85α by p110 inhibits PI 3'-kinase lipid kinase activity. Cells were cotransfected with p85α and either p110α or p110β. Lysates were split in half and PI 3'-kinase complexes were immunoprecipitated via the myc epitope. One immunoprecipitate was pre-treated with Mn²⁺ and ATP to induce phosphorylation of p85α and then washed extensively. Phosphorylation of PI was measured as described in Methods. The results of 3 experiments are shown graphically in A (n=3 +/- SEM), and B. shows a representative autoradiograph.

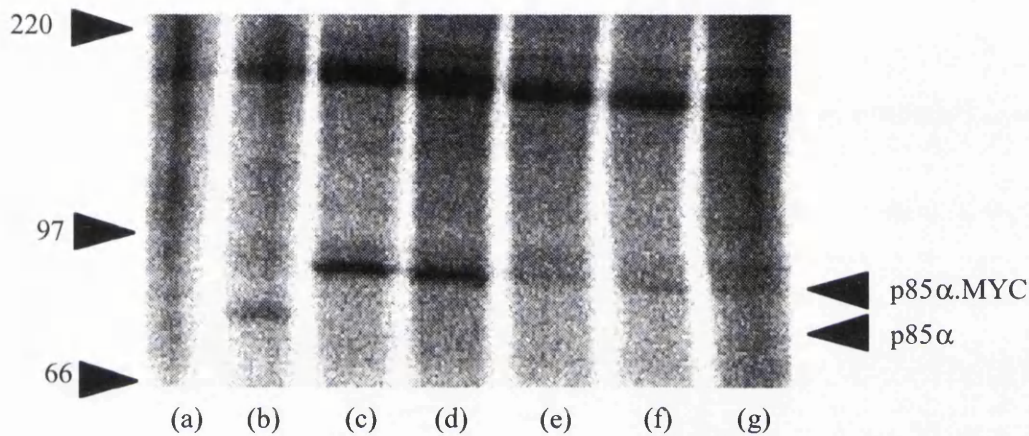


Figure 4.5 Autoradiograph showing *in vivo* phosphorylation of p85 α in HEK293 cells.

Phosphorylation of endogenous or transfected p85 α in quiescent cells was determined by labelling cells with ^{32}P orthophosphate and resolving immunoprecipitated proteins by SDS-PAGE, as described in Methods. The first lane (a) shows a p110 β immunoprecipitation from wild type cells. The next lane (b) is a re-immunoprecipitation of this lysate with an anti-p85 α antibody. Lanes (c) and (d) show in duplicate the *in vivo* phosphorylation of transfected p85 α .MYC by transfected p110 α , and lanes (e) and (f) show *in vivo* phosphorylation of transfected p85 α .MYC by transfected p110 β . The final lane (g) shows *in vivo* phosphorylation of p85 α .MYC by a p110 α kinase dead protein. All transfected proteins were immunoprecipitated with 9E10.

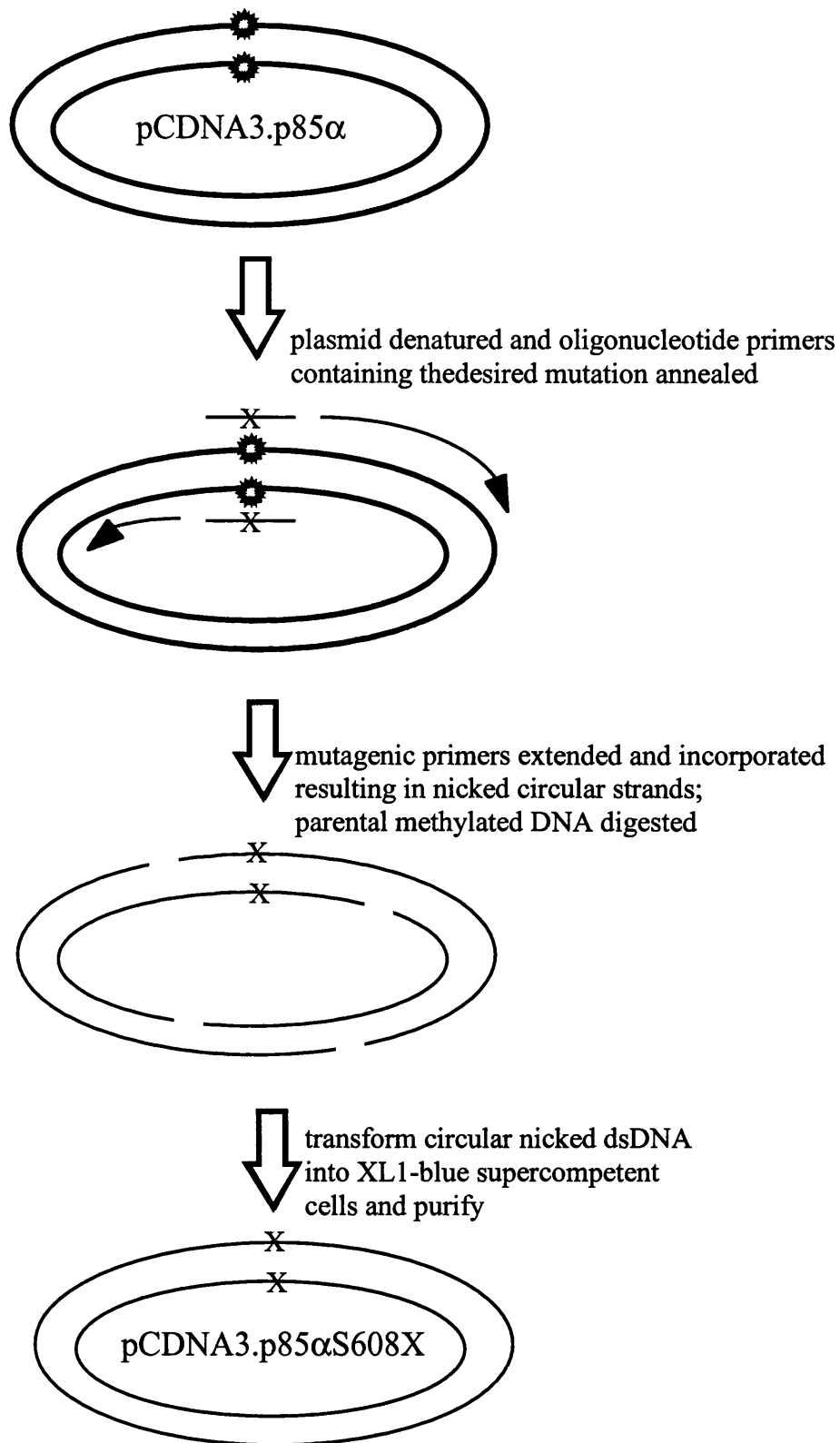


Figure 4.6 Schematic of the site directed mutagenesis method used to generate the p85αS608A and p85αS608E point mutated constructs. The p85 point mutants described in the text were made using the QuikChange mutagenesis kit from Stratagene. Primers used are described in the text.

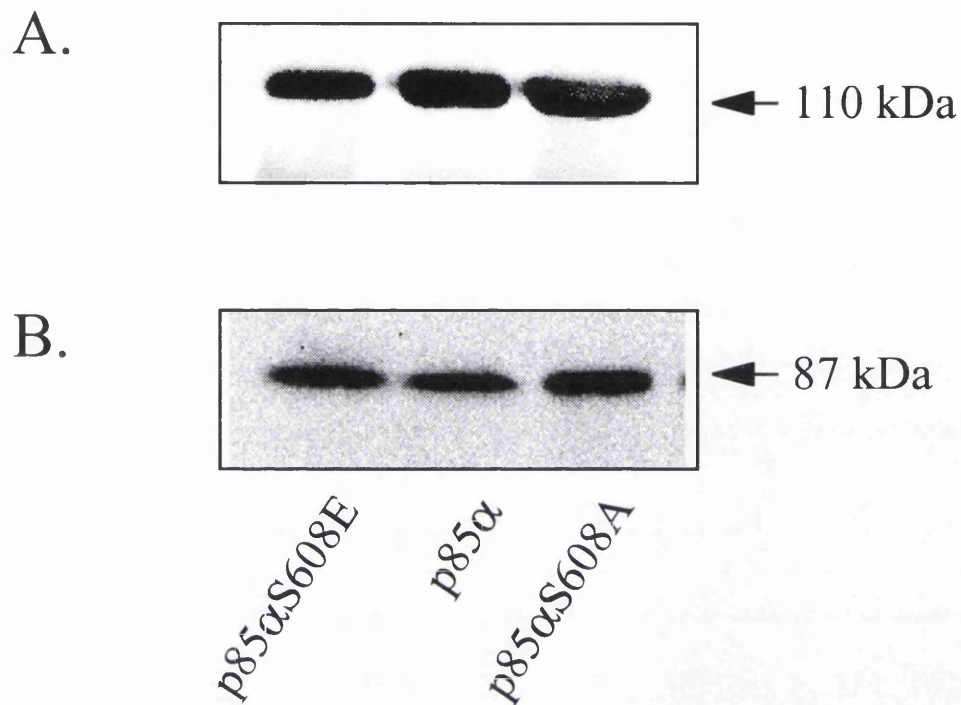


Figure 4.7 Expression of p85αS608 mutants and interaction with p110 subunit.

HEK293 cells were cotransfected with p110α and either p85α, p85αS608E or p85αS608A. Following cell recovery, cell lysates were subjected to SDS-PAGE using a 10% resolving gel. Proteins were transferred to PVDF. Immunoblotting was performed with anti-FLAG antibody, and the reactive protein visualised by ECL via HRP conjugated anti-rabbit antibody (A.). The membrane was then stripped and reprobed with anti-myc antibody, and the reactive proteins were visualised by ECL via HRP-conjugated anti-mouse antibody (B.).

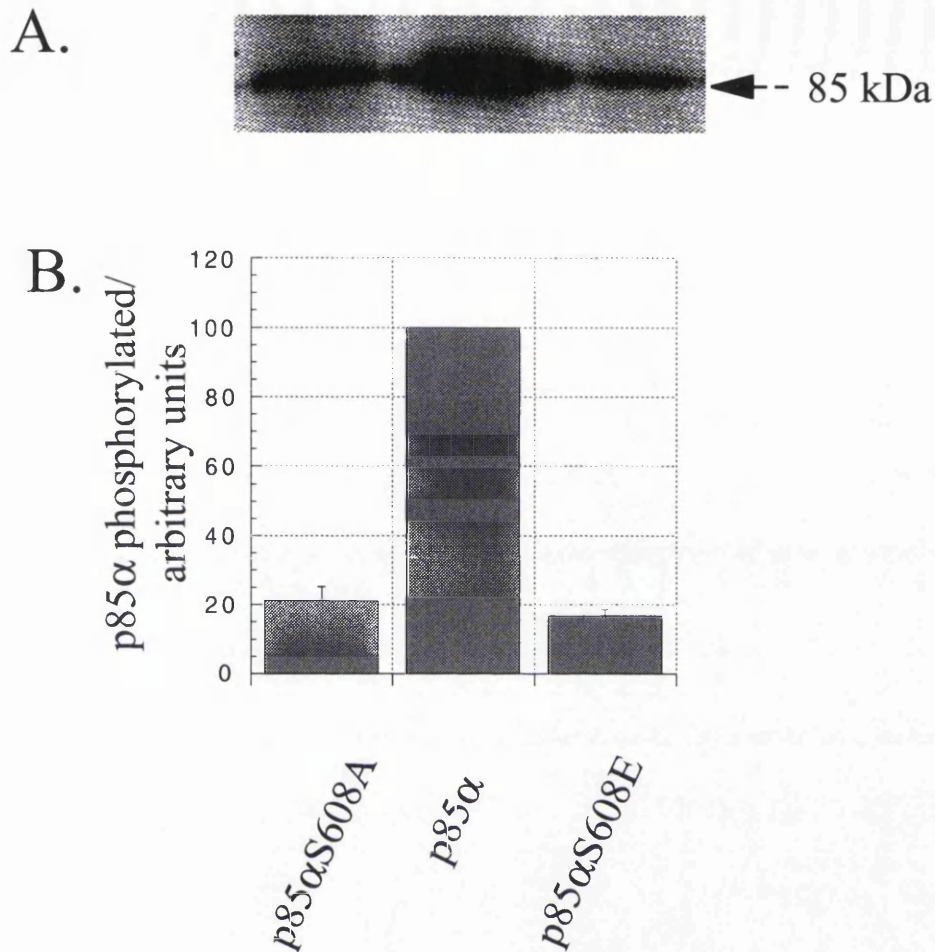


Figure 4.8 Phosphorylation of p85αS608 mutants by p110α. p110α was cotransfected with either wild type p85α, p85αS608A or p85αS608E, and the heterodimers were immunoprecipitated via the myc epitope. Phosphorylation of p85α was measured as described in Methods, and protein expression was normalised by Western blotting (B.) (n=3 +/- SEM). A typical autoradiograph is shown in (A.). Similar results were obtained when the p85 constructs were cotransfected with p110β.

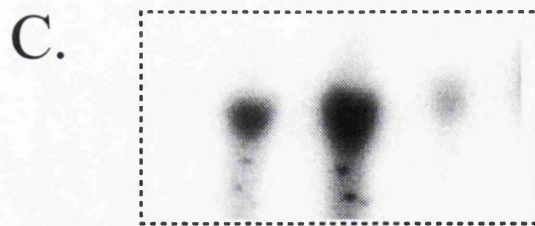
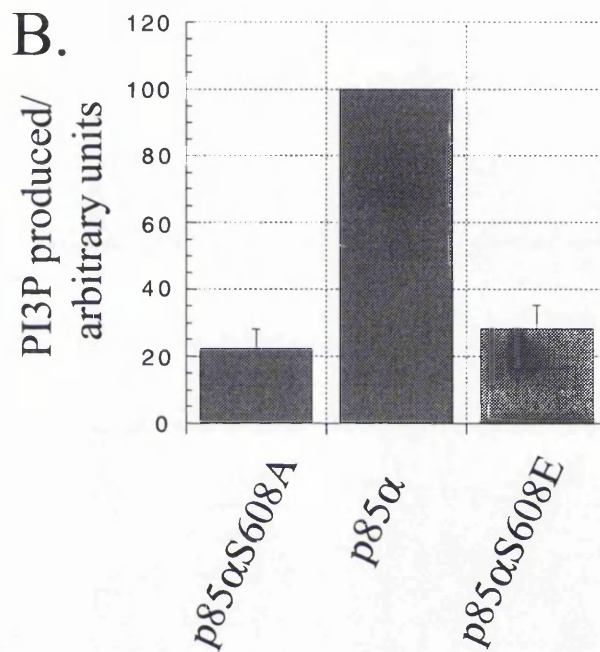
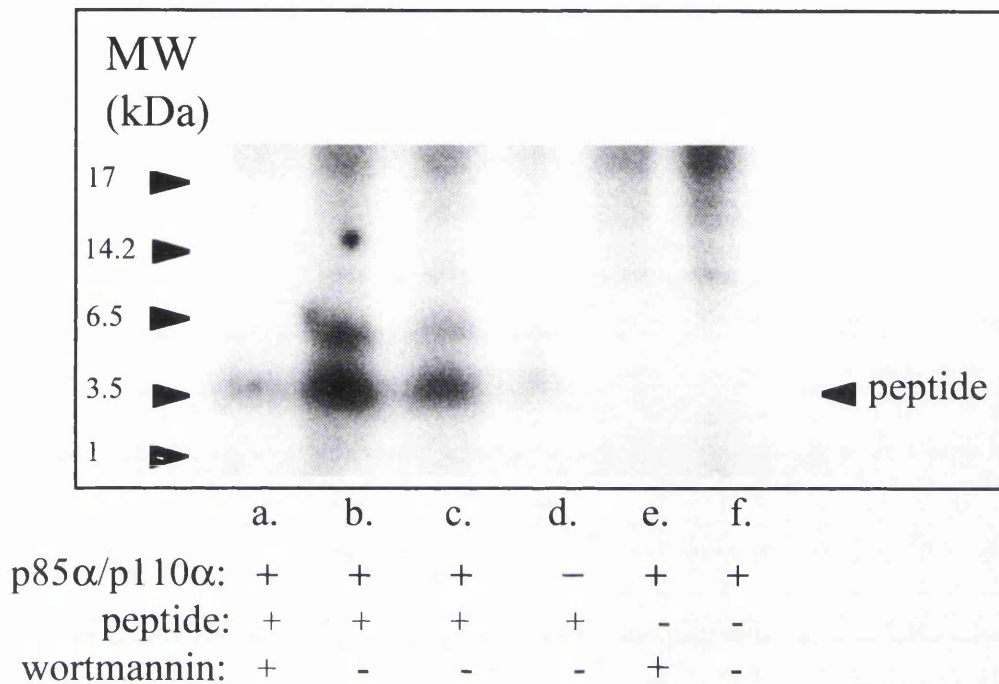


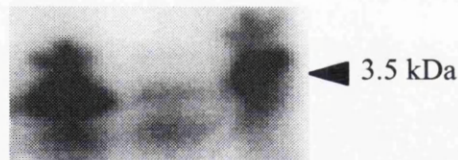
Figure 4.9 The lipid kinase activity of p110 associated with p85 α is dependent on the residue 608 of p85 α . p110 α was cotransfected with either wild type p85 α , p85 α S608A, or p85 α S608E, and the heterodimers were immunoprecipitated via the myc epitope. Phosphorylation of PI was determined as described in Methods, and normalised for protein expression by Western blotting with anti-myc antibody (B.) (n=3 +/-SEM). A typical TLC plate is shown in (A.). Similar results were obtained when the p85 point mutants were cotransfected with p110 β (C).

peptide: R-R-L-G-N-E-N-T-E-D-Q-Y-S-L-V-E-D-D-E-D-L

A.



B.



Mn:	+	-	+
Mg:	+	+	-

Figure 4.10 Optimization of p85αS608 peptide phosphorylation by PI 3'-kinase and resolution of product by Tricine SDS-PAGE. HEK293 cells were cotransfected with p85α/p110α. PI 3'-kinase was immunoprecipitated via the myc epitope on the adapter subunit. Peptide was added to a final concentration of 2.4mM, and protein kinase assays performed as described in Methods. Proteins in the assay were subjected to Tricine SDS-PAGE, using a 12.5% resolving gel. These were fixed and dried and a typical autoradiograph is shown in Figure (A.). The band with an apparent molecular weight of 6.5 kDa is also immunoprecipitated with anti-RAFT antibodies (B.Nave, personal communication). The ion dependence of peptide phosphorylation by PI 3'-kinase was also investigated, and this is shown in Figure (B.).

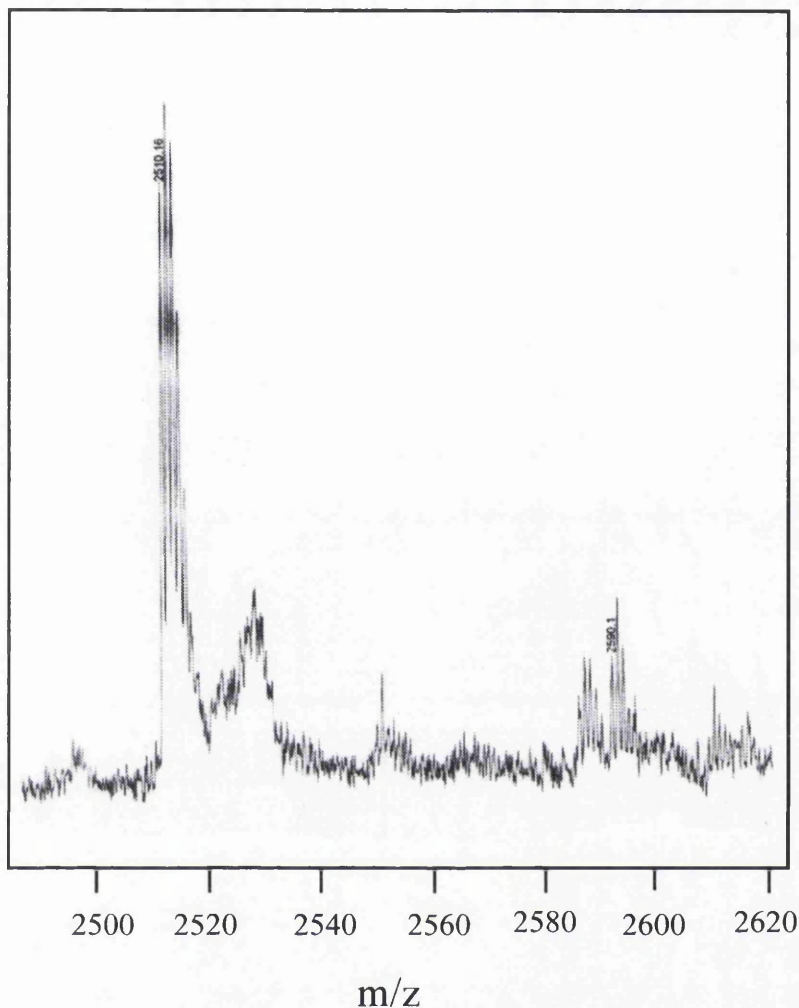


Figure 4.11 Mass spectrometric confirmation of identity of protein which migrates at 4.5 kDa

HEK293 cells were cotransfected with p110 α and p85 α . PI 3'-kinase protein was immunoprecipitated via the myc epitope tagged adapter and resuspended in protein kinase assay buffer. p85 α peptide was added and a protein kinase assay performed as described previously. The reaction products were separated by Tricine SDS-PAGE and the band which comigrated with the 3.5 kDa standard was excised. Peptide was extracted as described in the text, and laser-desorption ionisation mass spectrometry performed. The peak at 2510.1 corresponds to native peptide, the peak at 2590.1 corresponds to phosphorylated peptide. Mass spectrometric analysis of the band comigrating with the 3.5 kDa marker from reactions performed in the absence of PI 3'-kinase showed no peak at m/ z 2590.1.

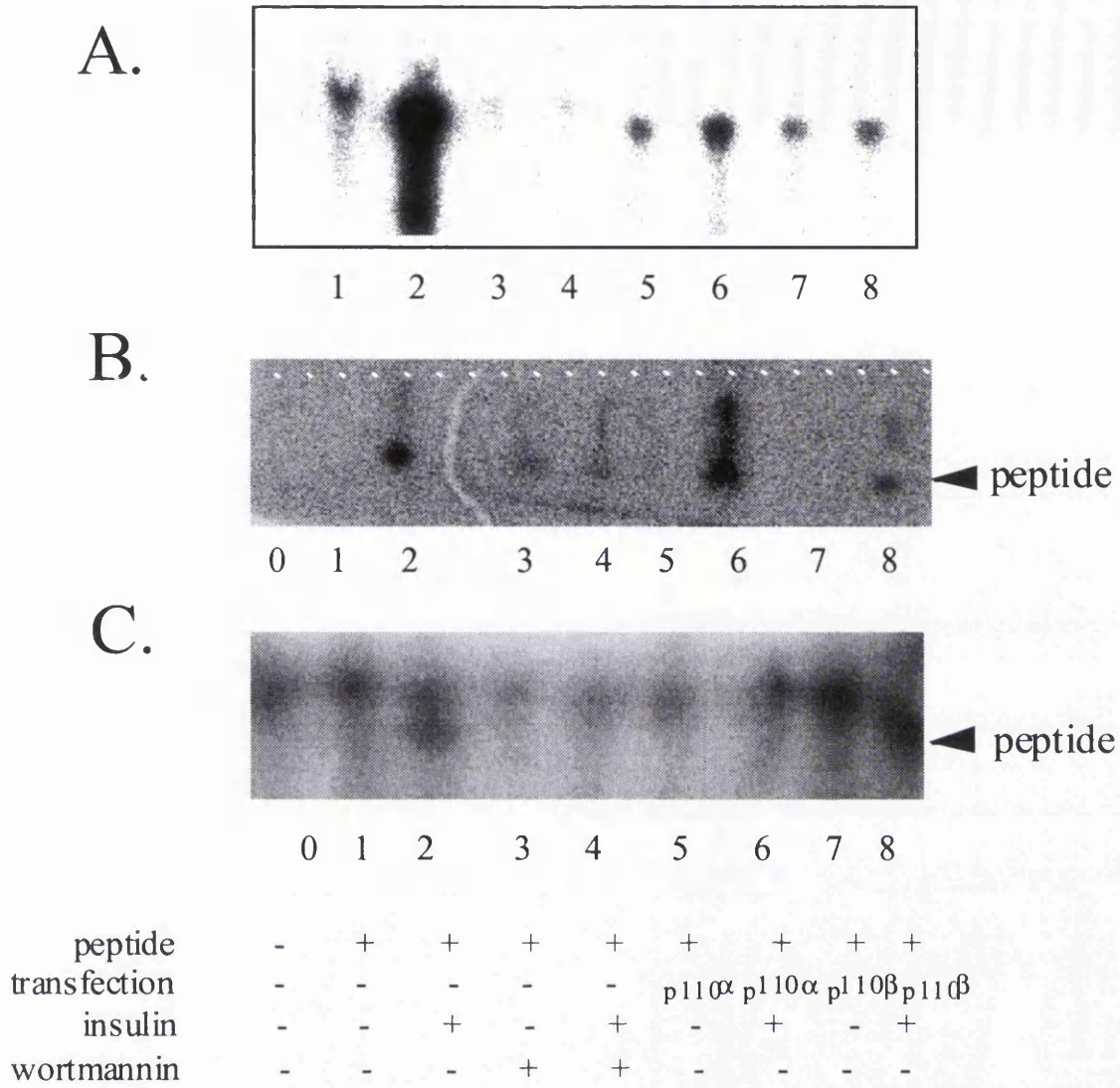


Figure 4.12 p85 α peptide as a novel assay substrate for PI 3'-kinase Wild type and transfected CHO-IR cells were serum starved overnight and stimulated with vehicle or 100nm insulin. Cell lysates were subject to antiphosphotyrosine immunoprecipitations and kinase assays were performed using PI substrate (A.), where transfections, and addition of insulin and/ or wortmannin matched the legend shown for the peptide substrates, (B.) 2.4mM peptide or (C.) 120 μ M peptide. Typical autoradiographs of TLC plates (A.) and gels (B.) and (C.) are shown. For further details, see text.

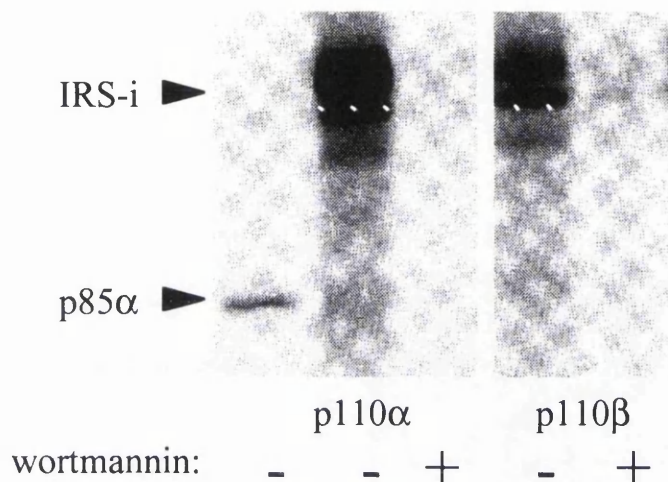


Figure 4.13 Phosphorylation of the insulin receptor substrate by the catalytic subunit of PI 3'-Kinase. Protein kinase activity was measured as described in Methods. Briefly, IRS-i (IRS1 construct containing the p85 inter-SH2 domain and an HA epitope tag) was cotransfected with either p110α (lanes 2 and 3) or p110β (lanes 4 and 5) and immunoprecipitated from cells via the HA tag. Following an *in vitro* protein kinase assay, samples were electrophoresed and the gel exposed to X-ray film. In lane 1, cells were cotransfected with p85α and p110α, immunoprecipitated via the myc epitope and assayed as for the IRSi as a control.

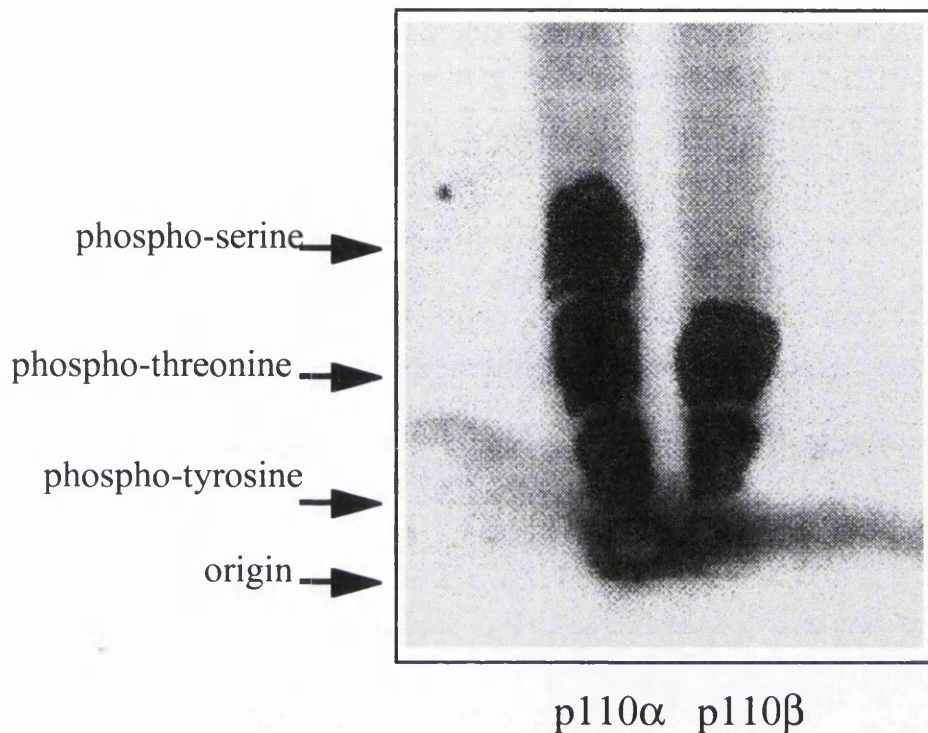


Figure 4.14 Phosphoamino acid analysis of the Insulin Receptor Substrate phosphorylated by p110 α and p110 β . HEK 293 cells were cotransfected with IRS and either p110 α or p110 β . IRS/p110 complexes were immunoprecipitated from cell lysates via the HA epitope on IRS, and a protein kinase assay was performed. Proteins were subjected to SDS-PAGE, and the gel exposed to film. The band corresponding in size to IRS was excised, acid hydrolysed, and prepared for phosphoaminoacid analysis as described in Methods. The product was resuspended in 10 μ l electrophoresis buffer containing *O*-phosphoserine, *O*-phosphothreonine and *O*-phosphotyrosine standards. After spotting onto a Kodak cellulose chromatogram sheet, electrophoresis was performed at 1000 V, 50 mA for 45 minutes with cooling. The plate was dried, stained with ninhydrin and developed. The chromatogram was then exposed to X-ray film and the ^{32}P -labelled phosphoamino acids correlated with the ninhydrin stained standards.

5. KINETIC STUDY OF THE KINASE ACTIVITY OF P110 α AND P110 β

5.1 Summary

The kinetics of phosphate incorporation into lipid and p85 α peptide substrates by p110 α and p110 β were examined. Pre-equilibrium data was analyzed mathematically to identify the steps at which the kinetic constants differ significantly for the two isoforms of catalytic subunit of PI 3'-kinase. The progress curves for incorporation of phosphate into phosphatidylinositol were sigmoidal, with an initial slow start followed by an acceleration before equilibrium was reached. Non-linear least squares analyses of these curves identified a substantial difference (50-fold) in the K_m (lipid) values for p110 α and p110 β . Analysis of the progress of peptide phosphorylation was simpler due to the apparent linearity of the reaction, and again, substantial differences (70-fold) in the K_m (peptide) were identified, as well as a 3-fold difference in the V_{max} of the p110 α and p110 β isoforms.

5.2 Introduction

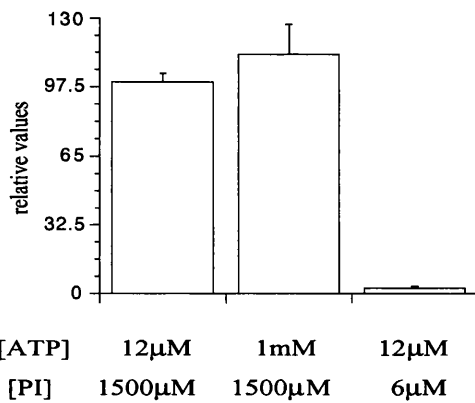
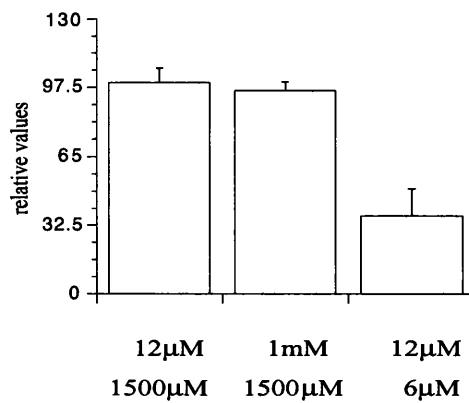
In section 4.3.2, data was presented which showed that over a ten minute period, three fold more phosphate was incorporated into PI and in section 4.3.3 it is shown that ten-fold more p85 α was phosphorylated by p110 α than by p110 β . These differences have not previously been reported. Further studies were undertaken of the pre-equilibrium progress of these reactions at different substrate concentrations, and the data was analyzed mathematically to determine how the kinetic constants vary between the isoforms of PI 3'-kinase studied.

The peptide described in sections 4.3.10 - 4.3.14 was used to study the kinetics of p110 α and p110 β protein kinase activity, and the data observed Michaelis-Menton principles. The phosphorylation of PI by the two isoforms of PI 3'-kinase was sigmoidal, and the resulting progress curves are unsuitable for Michaelis-Menton analysis. These data were analyzed by numerical integration and non-linear least squares optimization (Clare & Chance 1977; Clare & Chance 1978). The in vivo relevance of the different kinetic constants are discussed. The peptide and lipid substrate pre-equilibrium data showed similar trends in kinetic constants for the two isoforms of p110 studied.

5.3 Results

Assay conditions for kinetic studies

PI 3'-kinase assays described in this thesis used conditions commonly described in the literature. The final ATP concentration is 12 μ M. The concentration of ATP within a cell is approximately 1mM. To ensure ATP was not limiting in the studies presented in this thesis, the effect of 1mM ATP on lipid kinase activity was determined compared to 12 μ M ATP. As shown graphically below, ATP at 12 μ M was not limiting, although PI 3'-kinase activity was dependent on the two lipid concentrations used.

A.**B.****Assay conditions for kinetic studies**

Lipid kinase assays of p110 α / p85 α (A.) and p110 β / p85 α (B.) were performed on Myc immunoprecipitates from transfected HEK293 cells as described in Methods but at lipid and ATP concentrations as indicated. Values are relative to 12 μ M ATP/ 1500 μ M PI.

5.3.1 Pre-equilibrium incorporation of phosphate into phosphatidylinositol by PI 3'-kinase

The pre-equilibrium incorporation of phosphate into phosphatidylinositol by p85/p110 α and p85/p110 β complexes is shown in figure 5.1. These experiments were performed with enzyme complexes prepared from the pooled lysates of transfected HEK293 cells as described previously. Briefly, aliquots were subject to immunoprecipitation with anti-myc antibody, and washed as described in Methods in preparation for lipid kinase assay. Phosphatidylinositol was prepared at a range of concentrations by serial dilution in kinase assay buffer of the preparation described in Methods, and added to the immunoprecipitated PI 3'-kinase complexes. The reactions were started by the addition of 12 μ M ATP containing 5 μ Ci 32 P- γ -ATP, and stopped at timed intervals by the addition of chloroform: methanol (1:2 volume) as described in Methods. Extracted lipids were resolved by TLC and quantitated by phosphorimager. The observed progress curves are generally sigmoidal with an initial slow start followed by an

acceleration in phosphorylation of PI by both isoforms of the PI 3'-kinase substrate. The non-linearity of these reactions (which increases with increasing substrate concentration), makes the resulting data unsuitable for Michaelis-Menton analysis.

5.3.2 Analysis of non-linear pre-equilibrium progress curves

A model of the reaction scheme of phosphate incorporation into PI was postulated to enable the mathematical analysis of the experimental results (i.e. the observed increase of PIP3 as a function of time). Non-linear least squares analysis was carried out using ARChek software (ARC Scientific Ltd, 257 Woodstock Road, Oxford). Archek calculates a theoretical progress curve by varying the unknown kinetic parameters to minimize the sum squares of deviation between the calculated curve and the observed values. By varying the unknown initial values, a set of parameters which best fit the data using the postulated reaction mechanism is obtained. Confidence levels of the unknown parameters are also calculated.

The assumed reaction scheme is shown in Figure 5.2.A, and the resulting sequence of mass action law differential equations is shown in Figure 5.2.B. The differential equations are the same for p110 α and p110 β , i.e. it was assumed that both isoforms of the enzyme employ the same mechanism of reaction, but the kinetic parameters are different.

5.3.3 Calculation of kinetic constants from pre-equilibrium data

Since the differential equations are the same for both p110 α and p110 β , the question to be answered is which of the coefficients differ between isoforms to account for the differences observed in the pre-equilibrium reaction progress curves. Digitized data from the phosphoimager was used for the optimization, and the remaining rate constants were calculated by Archek. Validity of these rate

constants was tested by correlation to the observed data points. In Figure 5.3, the points are the experimentally measured incorporation of phosphate into PI, and the lines are the outcome predicted from the calculated rate constants determined from the data and the reaction scheme shown in Figure 5.2.A.

By studying the progress of phosphate incorporation by p110 α and p110 β at each PI concentration, it is clear that in addition to a close correspondence between observed and predicted values, it can be seen that there is likely to be considerable difference in values for either K_m and/ or V_{max} . At low substrate concentrations, the p110 β isoform of PI 3'-kinase is more effective at phosphorylating PI. As substrate concentration increases, the activity of p110 α increases more quickly than the activity of p110 β , until 0.167mM PI, at which concentration both isoforms of the the enzyme produce PIP3 at approximately equal rates. Above 0.167mM, p110 α is more efficient at phosphorylating PI.

5.3.4 Calculation of K_m / V_{max}

The calculated internal constants show close correspondence between observed and predicted values for the incorporation of phosphate into PI by the two isoforms of p110. The equations solved by ARChek are shown below.

$$V_1 = \frac{V_{m1} \times [ATP]}{[ATP] + K_{m1}}$$

$$V_2 = V_m \left\{ \left(\frac{[X]}{[X] + K_x} \right)^n \left(\frac{[L]}{[L] + K_{mL}} \right) \right\}$$

The progress of these reactants are shown in Figure 5.4, and are subject to the following constraints:

- (1.) The reaction has fourth order kinetics i.e. $n=4$
- (2.) At equilibrium, $\frac{\text{PI3P} \times \text{ADP}}{\text{PI} \times \text{ATP}} = 1000$
- (3.) $V_{\max} \text{p110}\alpha = 30 \mu\text{M s}^{-1}$ $V_{\max} \text{p110}\beta = 2.2 \mu\text{M s}^{-1}$
- (4.) $K_m \text{p110}\alpha(\text{PI}) / K_m \text{p110}\beta(\text{PI}) = 1000 \mu\text{M} / 20 \mu\text{M} = 50$
- (5.) $K_{\text{mx}} = Q / (Q-1) K_x$ for $Q = (1/2)^n$
so for $n = 4$, $Q = 0.841$, $K_{\text{mx}} = 5.29 K_x$
- (6.) $K_m \text{p110}\alpha(\text{ATP}) = 2 \mu\text{M}$ $K_m \text{p110}\beta(\text{ATP}) = 6 \mu\text{M}$

5.3.5 Phosphorylation of a p85 α S608 peptide by PI 3'-kinase

The peptide described in sections 4.10-4.12 was used in a study to compare the kinetics of the protein kinase activities of p110 α and p110 β . PI 3'-kinase complexes of p85 α and either p110 α or p110 β were prepared from the pooled lysates of transfected HEK293 cells and immunoprecipitated and washed as described in section 5.3.1.

Peptide was prepared as previously described, and subject to serial dilution in assay buffer. Peptide and immunoprecipitated PI 3'-kinase complexes were mixed and the the kinase reaction started with the addition of 12 μM ATP containing 5 μCi γ -(^{32}P) ATP. The reactions were stopped at timed intervals by the addition of SDS-PAGE gel loading dye, and proteins in the samples were separated by SDS-PAGE using a 12.5% Tricine resolving gel as described in Methods. Gels were

fixed and dried as described previously. Typical autoradiographs are shown in Figure 5.3.5.

5.3.6 Analysis of peptide phosphorylation data

The phosphorylated p85 α phosphopeptide band was quantitated by phosphorimager, and the results are shown graphically in Figure 5.6. The results shown are typical of 3 similar experiments.

5.3.7 Michaelis-Menton analysis of protein kinase progress curves

Michaelis-Menton analysis of the initial rates of incorporation of ^{32}P - γ -ATP in the peptide is shown in Figure 5.7. The initial reaction rates were substrate concentration are shown as double reciprocal plots, and the values of the K_m and V_{max} were calculated from the following equations:

$$\text{at } y = 0, x = -1/ K_m \quad \text{and} \quad \text{at } x = 0, y = 1/ V_{max}$$

5.4 Discussion

In the previous chapter, it was shown that over a ten minute period, using the assay conditions described, p110 α was three fold more effective at phosphorylating p85 than p110 β . Likewise, phosphorylation of p85 within the PI 3-kinase heterodimer was ten-fold greater when catalysed by p110 α than by p110 β . The data presented in this chapter were the result of studies undertaken to determine the steps in the phosphorylation reactions at which the rate constants differed significantly for the two isoforms of catalytic subunit of PI 3'-kinase studied, thereby showing why p110 α appears to phosphorylate its substrates at greater levels than p110 β .

The pre-equilibrium progress curves (Figures 5.1 and 5.6) can be regarded as the integration of the differential equations of the reaction schemes. The peptide data was suitable for analysis by Michaelis-Menten equations, whilst the non-linear progress of phosphatidylinositol phosphorylation was analysed by Arched software. The data for both peptide and lipid phosphorylation by PI 3'-kinase revealed significant differences in the K_m values for the substrates by p110 α /p85 α and p110 β /p85 α . A 50-fold difference in the K_m for phosphatidylinositol was found between p110 α and p110 β . No significant difference was found between the V_{max} values of these isoforms. The effect of the difference in the rate constants determined is clearly observed in Figure 5.4, which demonstrates that at low concentrations of substrate, p110 β phosphorylates PI more readily than p110 α . This is due to the fact that at this concentration of substrate, the β isoform of p110 is working at its K_m and therefore achieves V_{max} , and subsequently equilibrium, more quickly. As the substrate concentration increases, the difference between the apparent activities of p110 α and p110 β diminishes, until conditions are such that p110 α can more rapidly achieve V_{max} , and this isoform phosphorylates PI more rapidly.

The data relating to p85 α peptide phosphorylation by p110 α and p110 β revealed a 70-fold difference in the ratio of $K_{m\alpha}/K_{m\beta}$, and that the V_{max} of peptide phosphorylation by p110 α is 3-fold greater than the equivalent constant for p110 β . Experimental constraints meant that different concentrations of peptide were used to determine kinetic constants for p110 α and p110 β , which prevents direct comparison of the progress of phosphorylation by these two isoforms at a given substrate concentration. Nevertheless, assay of p110 α at the lowest concentration of peptide used (0.15mM) compared to assay of p110 β with 0.1mM peptide, clearly demonstrates that at low levels of substrate, p110 β more readily phosphorylates the peptide (1750 phosphorimager units) than p110 α (1200 phosphorimager units). (Where phosphorimager units are directly proportional to

peptide phosphorylated). The greater efficiency of p110 β then rapidly diminishes as peptide concentration increases, and at 1mM substrate p110 β phosphorylates the equivalent of 2400 phosphorimager units, whilst at 1.2mM substrate, p110 α phosphorylates the equivalent of 4000 phosphorimager units.

The literature indicates that p110 α and p110 β are unstable and rapidly degraded unless coexpressed with an adapter subunit in mammalian cells (Yu et al 1998), and to date there are no reports of the adapter subunit of PI 3'-kinase existing independently of the catalytic subunit although there are two reports of p85 effects mediated independently of p110 activity (Jimenez et al 1998; Yin et al 1998). Therefore the ratio (and subsequently concentration) of adapter to catalytic subunit will be equal in any in vivo situation, and the relevance of the kinetic differences of p85 α peptide phosphorylation by p110 α and p110 β may at first appear questionable. However, there are two issues which merit consideration. Firstly, in immunoprecipitates of PI 3'-kinase phosphorylation of residue 608 results in inhibition of the lipid kinase activity of the heterodimer. The differential kinetics of peptide phosphorylation reflect why p110 α significantly phosphorylates associated p85 α more rapidly than p110 β . The close proximity of the serine 608 site of full length p85 in a heterodimer with p110 means that its effective concentration is relatively high. Under these conditions, p110 α results in greater phosphorylation of p85 in immunoprecipitates than p110 β . Furthermore, the action of phosphatases on phosphorylated p85 α will be more rapidly be reversed by the action of p110 α than p110 β . Secondly, the high localized concentrations of PI3'-kinase at intracellular membranes following cell stimulation could provide a mechanism for intermolecular phosphorylation of p85, particularly by p110 α , thereby providing a mechanism for downregulation of the enzyme once a signalling cascade has been initiated. Furthermore, PI 3'-kinase has been shown to dimerize via full length p85 (Harpur et al 1999) albeit under

very specific conditions. This intermolecular binding may provide a further mechanism for downregulation by blocking the binding of exogenous ligands for the p85SH3 domain, a process which could be further facilitated by the enhancement of p85 phosphorylation.

Previously, studies of the kinetics of PI 3'-kinase action have been published by Woscholski *et al* (Woscholski et al 1994; Woscholski et al 1995) which focused on the substrate specificity of p110 α as modulated by detergents, and on the effect on p110 α activity as regulated by p85 α . These studies produced apparently linear pre-equilibrium progress curves of lipid phosphorylation which were then approximated to Michaelis-Menton kinetics, whilst those presented in this chapter were clearly sigmoid. It is possible that these differences arise from the PI 3'-kinase source. The data reported by Woscholski was obtained using PI 3'-kinase purified from baculovirus infected insect cells, as opposed to the mammalian system used in this thesis.

Of particular relevance to the studies presented in this chapter was the finding that reconstitution of PI 3'-kinase *in vitro* from purified p110 α and p85 α does not result in a heterodimer with the same enzymological properties as purified p110 α /p85 α coexpressed *in vivo* (Woscholski et al 1994). Since reconstituted heterodimers most closely resembled free catalytic subunit activity, the implication is that it is not the physical presence of the adapter subunit itself which affects the enzymology of PI 3'-kinase under these conditions. Rather, it suggests a co-translational process which is dependent on the *in vivo* formation of the complex. The most readily detectable post translational modifications observed *in vivo* are serine and tyrosine phosphorylation of p85. Tyrosine phosphorylation of p85 is not detectable in insect cells unless tyrosine kinases are coexpressed (Gout et al 1992; Roche et al 1994a), but has been proposed to activate the lipid kinase activity of PI 3'-kinase *in vivo*. Serine phosphorylation is

readily observed on p85 purified from baculovirus infected insect cells, and has been shown to downregulate the lipid kinase activity of p110 α (Carpenter et al 1993; Dhand et al 1994b) and p110 β (Section 4.3.4 and (Roche et al 1998a)). Published data (Woscholski et al 1994; Yu et al 1998) indicates that coexpression with p85 α inhibits the lipid kinase activity of p110 α , consistent with post translational serine phosphorylation of p85, although the effect was only partially alleviated by phosphatase treatment.

The sigmoidal pre-equilibrium progress curves of phosphoinositide phosphorylation by PI 3'-kinase presented in Figure 5.1 are consistent with expectations for an activated enzyme. These data were obtained using PI 3'-kinase immunoprecipitated from quiescent cells, in which there is considerable phosphorylation of p85 α , as shown in the previous chapter (Figure 4.7). This phosphorylation is not completely inhibited by wortmannin, suggesting at least some phosphorylation was p110 independent, possibly the result of a tyrosine kinase. Although not specifically investigated as part of this study, data on tyrosine phosphorylation of p85 in quiescent cells has been variable. In addition, it is possible that the immunoprecipitation process itself activates PI 3'-kinase. It is therefore possible that activation of PI 3'-kinase contributes in part to the sigmoidal nature of these curves, i.e. the sigmoidal curve is the result of superimposition of a changing activity state of the PI 3'-kinase catalytic subunit. A substantial attempt was made to fit the experimental data to such a model, but was not successful.

Before discussing the in vivo relevance of the differences of p110 α and p110 β phosphorylation of phosphatidylinositol, it is necessary to iterate some concepts specific to studying lipid kinetics. The hydrophobic nature of any lipid substrate results in the formation aggregated structures in aqueous solutions, be they micelles, vesicles, liposomes or bilayered sheets. The protocol described in

Methods produces detergent-mixed micelles of a relatively uniform size (Lichtenberg et al 1983). Substrate dilution, in this case phosphatidylinositol, can be defined as a function of dilution of the surface concentration of substrate within a vesicle, decrease in vesicle number, or decrease in vesicle size where the ratio of substrate: detergent is maintained. To determine absolute kinetic parameters, both the 'bulk' concentration expressed in terms of molarity, and the surface concentration expressed in terms of mol%, must be varied to produce a matrix of data points. The mole fraction of substrate in the surface of a micelle is varied by the addition of neutral dilutors, which will neither interact with the enzyme or substrate, nor change the physical characteristics of the micelle surface (Carmen et al 1995).

The lipid kinase data in this thesis were obtained by dilution of the bulk concentration, and therefore are a function of vesicle size. Because surface dilution studies were not undertaken, these data cannot be taken as absolute values, but as comparative ones. In the case of 'bulk' concentration changes, such as described here, it is necessary to take into account also the changing levels of detergent in any kinetic analysis. It has been shown that cholic acid concentration, (the detergent used in these experiments) affects neither the K_m values for the lipid substrate nor the ATP (Woscholski et al 1994). Importantly, this is true only when PI is used as substrate, and not when PI (4,5)P₂ is used. As PI was used in the current study, it is assumed that cholic acid concentration had no effect on kinase activity.

The substantial difference in the K_m values for the phosphatidylinositol substrate of p110 α and p110 β described here could have enormous implications if the substrate demonstrated differential concentrations within different domains within membranes. Although once considered to serve mainly a structural function, membrane lipids are now well established as precursors for a number of signalling

molecules. Evidence from pulse-labelling suggests the most significant target of agonist stimulated class 1a PI 3'-kinases are PI(4)P and PI(4,5)P₂, the 3-phospho derivatives of which are barely detectable in quiescent cells, but rise to 1-3% of total phospholipids in stimulated cells (Rameh & Cantley 1999). Furthermore, in some cell types, insulin stimulates an increase in PI (3,4,5)P₃ only, whilst in the same cells PDGF can elevate both PI (3,4,5)P₃ and PI (3,4)P₂ (Gagnon et al 1999). It seems reasonable to assume then that a definite universal target for agonist stimulated class 1a PI 3'-kinases is PI (4,5)P₂.

As would be expected for a signalling molecule precursor, evidence is now available that certain lipids are not randomly distributed throughout membranes, but are compartmentalized (Pike & Casey 1996) (Hope & Pike 1996). Evidence has been put forward for the existence of 'lipid rafts' within membranes, where like molecules self associate, and the subsequent preferential migration of proteins into these lipid patches. Significantly, the concentration of PI(4,5)P₂ is increased in these lipid rafts which are detected as low density detergent resistant pools and membrane invaginations (Liu et al 1998). This means that in addition to variations between cell types, the concentration of a lipid is not uniform within a cell. Therefore the data presented in Figures 5.1-5.4, suggests that p110 β PI 3'-kinase is active at small vesicles, whilst p110 α PI 3'-kinase metabolizes phosphoinositides on larger vesicles, or the plasma membrane, since the in vitro data suggests maximal activity at the higher concentrations of PI used. The methodology employed in these studies means the high concentrations of PI most likely produce large vesicles, which have less curvature than small vesicles, and are therefore more akin to a flat surface such as the plasma membrane. There is evidence to suggest that insulin signalling pathways take advantage of these functional differences in p110 isoforms. For example, Wang et al have shown that p110 β , but not p110 α , is increased at glucose transporter compartments (low density microsomes) following insulin stimulation of adipocytes (Wang et al

1998), and data presented in this thesis, (section 4.3.1) also suggests that p110 β /p85 is more responsive to insulin stimulation than p110 α /p85. Certainly the data shown here suggests the p110 β isoform would be highly active at small vesicles such as those found in endosomes.

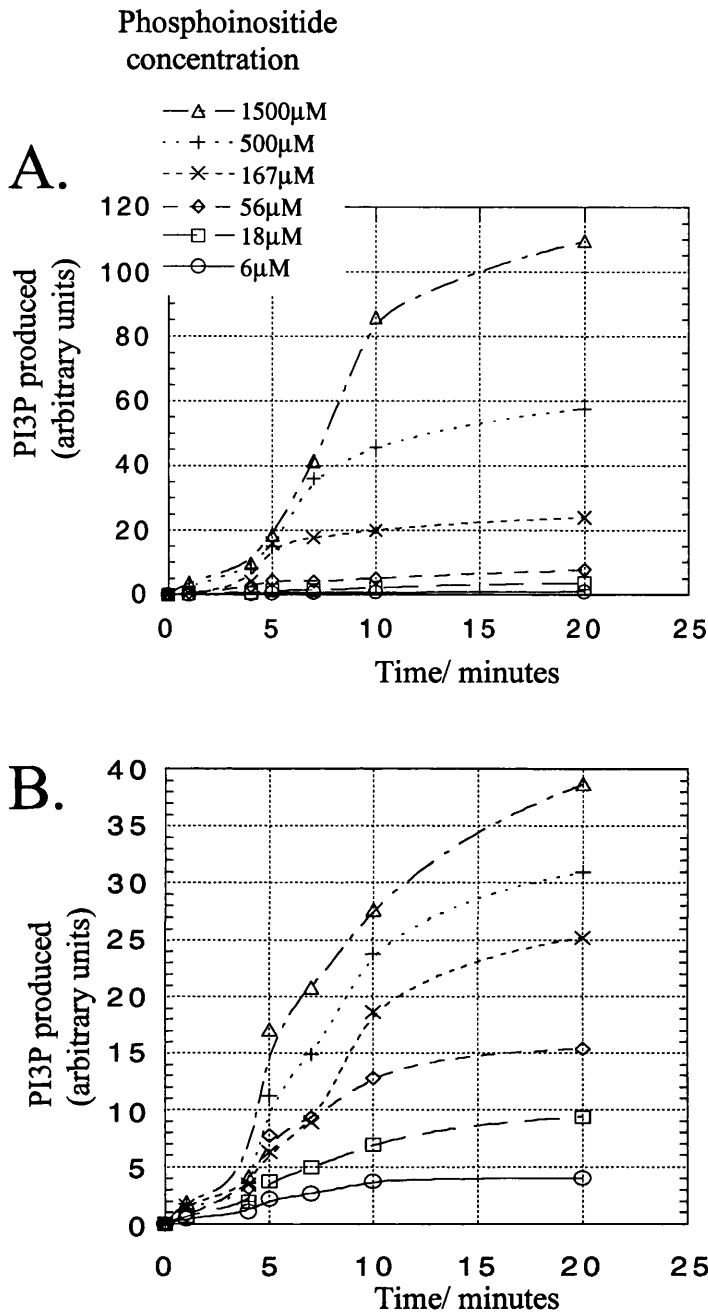
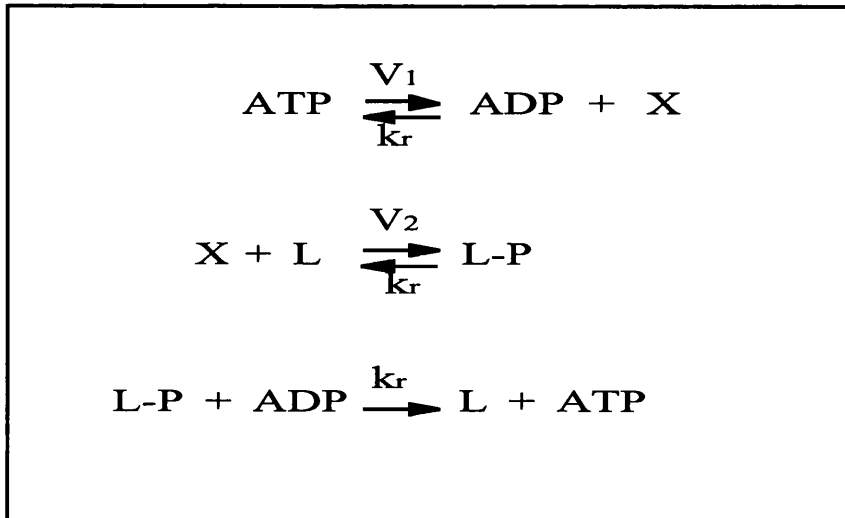


Figure 5.1 Time course of PI3P synthesis from varying concentrations of PI by p110 α and p110 β complexed with p85 α .

HEK293 cells were cotransfected with p85 α and either p110 α (A.) or p110 β (B.). PI 3'-kinase was immunoprecipitated via the myc epitope of the adapter subunit. PI 3'-kinase assays were performed as described, except for dilution of the PI/cholate solution (see text). Reactions were stopped at timed intervals and the extracted lipids resolved by TLC. PI3P was quantitated by phosphorimager. Shown are the values obtained from one of three similar experiments.

A.



B.

$$\frac{d\text{ATP}}{dt} = -V_1 + k_r \cdot \text{ADP} \cdot (\text{X} + \text{L-P})$$

$$\frac{d\text{ADP}}{dt} = - \frac{d\text{ATP}}{dt}$$

$$\frac{d\text{X}}{dt} = V_1 - k_r \cdot \text{ADP} \cdot \text{X} - V_2 + k_r \cdot \text{L-P}$$

$$\frac{d\text{L}}{dt} = -V_2 + k_r \cdot \text{L-P} + k_r \cdot \text{L-P} \cdot \text{ADP}$$

$$\frac{d\text{L-P}}{dt} = V_2 - k_r \cdot \text{L-P} \cdot \text{X}$$

Figure 5.2 Model of phosphatidylinositol phosphorylation by PI 3-kinase

To determine rate constants from the non-linear pre-steady state progress curves of phosphatidylinositol phosphorylation by PI 3'-kinase, experimental data was analysed using the computer program ARCHEK. This relies on a predicted reaction scheme (5.2.A) described by a series of differential equations (5.2.B) which were solved by ARCHEK. The abbreviations used are: X, phosphorylated enzyme intermediate; L, phosphatidylinositol; L-P, phosphatidylinositol 3-phosphate. Values for V1 and V2 are as described in section 5.3.4.

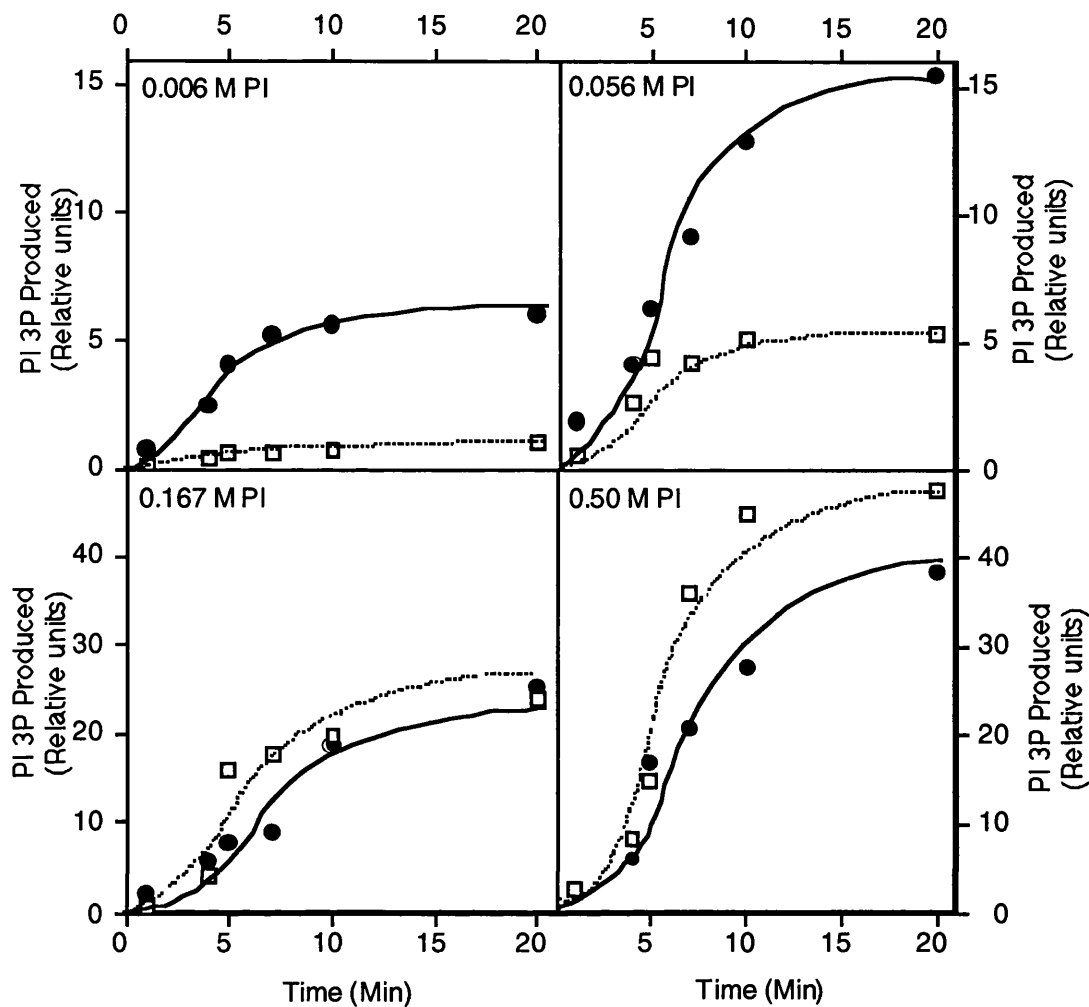


Figure 5.3 Theoretical progress curves of PI phosphorylation by PI 3'-kinase predicted by ARChek

ARChek predicts the progress of a reaction from calculated constants determined by numerical integration of the data and optimization using with the postulated reaction mechanism. The points are the experimental data obtained for PI phosphorylation by p110 α (\square) and p110 β (\bullet) dimerized with p85 α , and the lines are the outcome predicted from the calculated rate constants by ARChek for p110 α (dashed line) and p110 β (solid line). Note the different scales of the y-axis in the upper and lower panels.

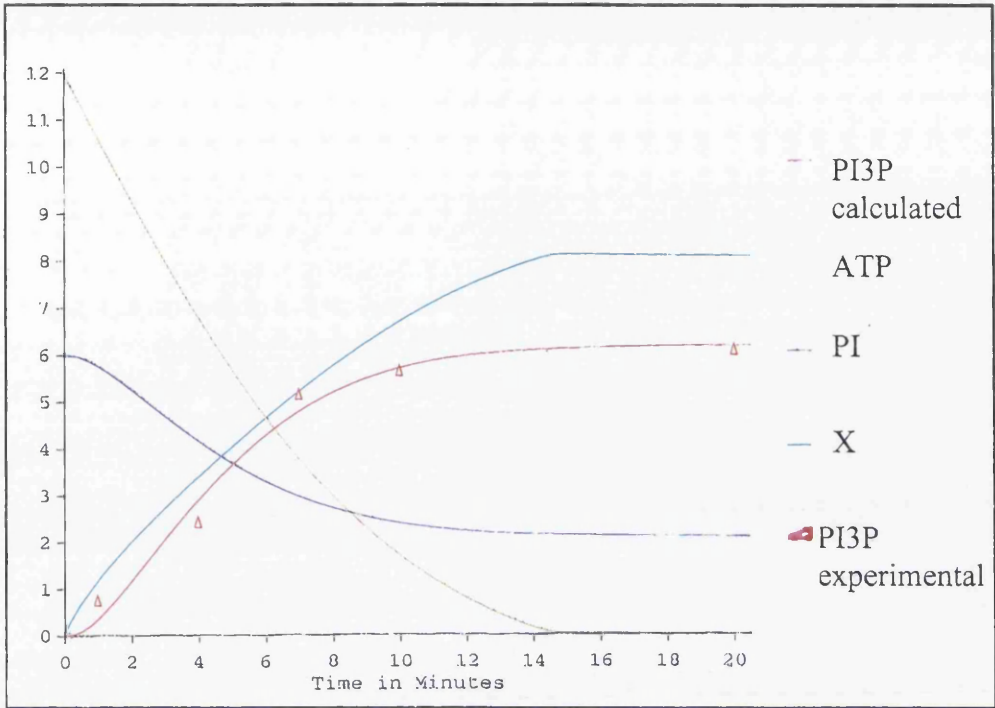


Figure 5.4 Reaction profile of PI phosphorylation by PI 3'-kinase
 The kinetic constants calculated by ARChek were constrained by the equilibrium position, ATP usage and K_m/V_{max} (calculated as described in section 5.3.4) of the reaction. The consumption of ATP, the formation of the intermediate X, and the calculated progress of PI phosphorylation are shown together with the experimental data of PI3P formed by the action of p110 β /p85 α on 6 μ M PI.

peptide: R-R-L-G-N-E-N-T-E-D-Q-Y-S-L-V-E-D-D-E-D-L

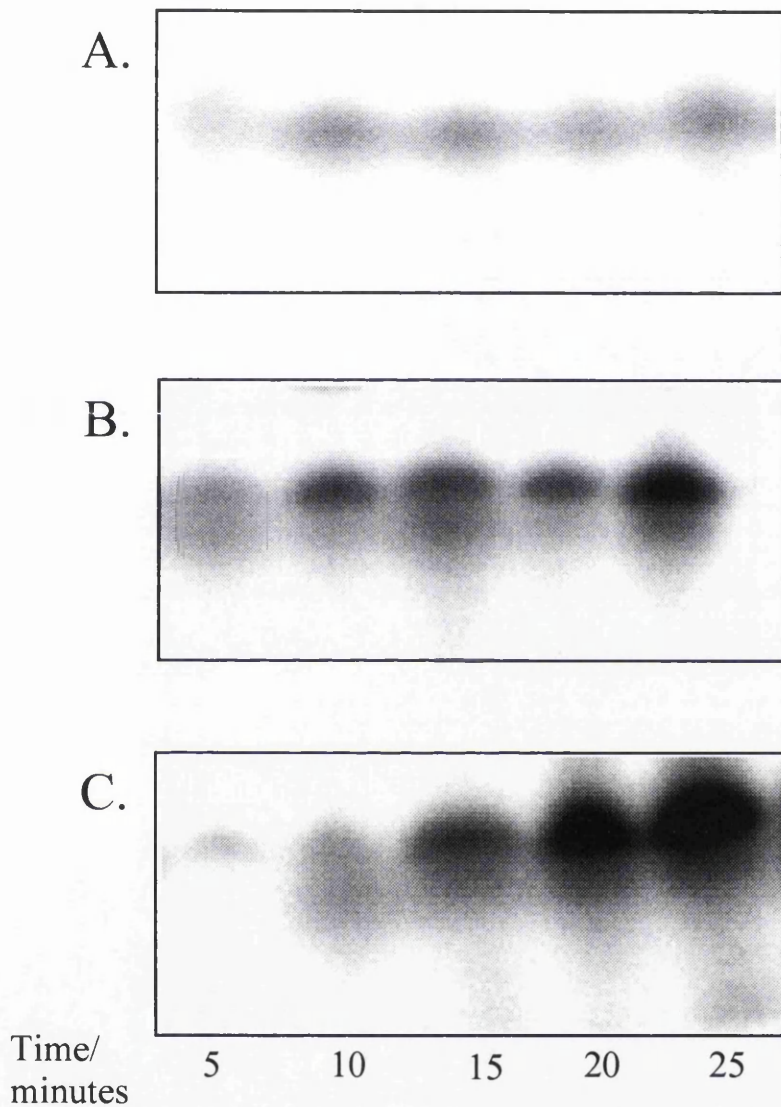
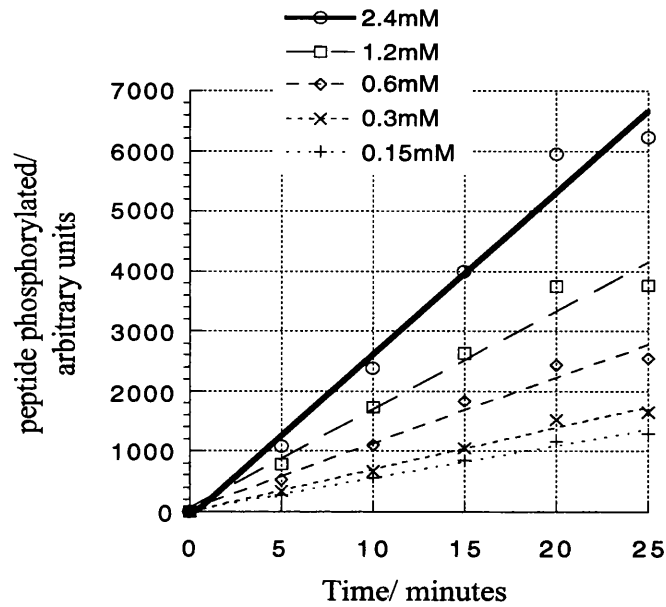


Figure 5.5 Time Course of Phosphorylation of p85 α S608 peptide by PI 3'-kinase. HEK 293 cells were cotransfected with p85 α and either p110 α or p110 β , and cell lysates were subjected to immunoprecipitation with anti-myc antibody. The 21-mer peptide was dissolved in water, and added to the immunoprecipitated protein complexes. 12 μ M ATP with 5 μ CI 32 P- γ -ATP was added in protein kinase assay buffer to start the reaction. Protein kinase reactions were stopped at timed intervals by the addition of loading dye, and proteins were separated by SDS-PAGE using a 12.5% tricine resolving gel (see Methods). Gels were fixed and dried *in vacuo* and phosphorylated peptide was quantified by phosphorimager. Typical results are shown for A. 0.3mM; B. 1.2mM; and C. 2.4mM peptide.

A.



B.

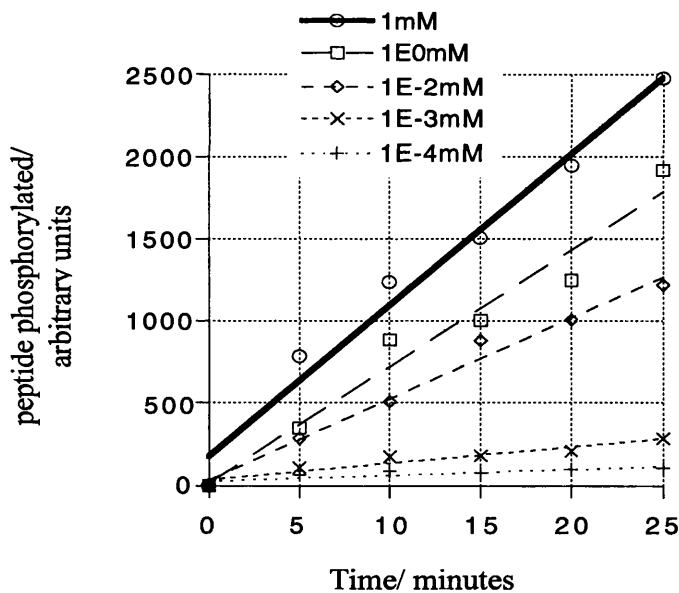
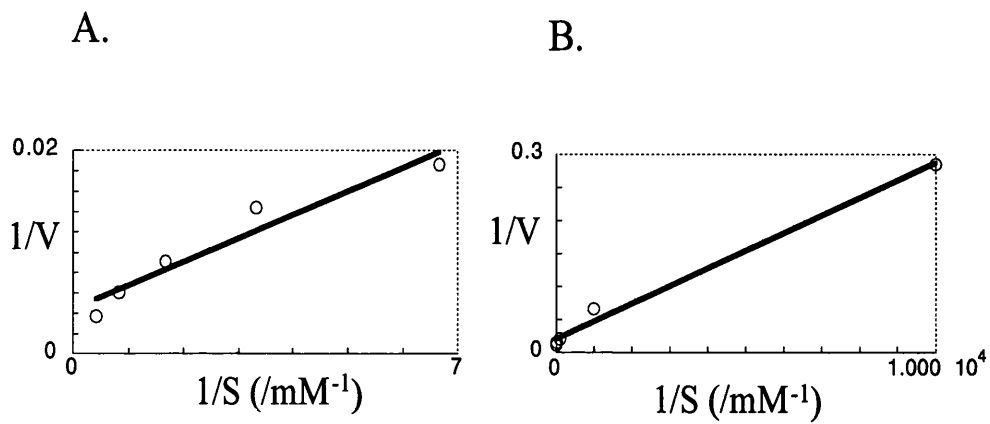


Figure 5.6 Time course of p85 α peptide phosphorylation by PI 3'-kinase.

HEK293 cells were cotransfected with p85 α and either p110 α (A.) or p110 β (B.). PI 3'-kinase was immunoprecipitated via the myc epitope tag on the adapter subunit. Immunoprecipitated protein was suspended in protein kinase assay buffer, and peptide added to the concentrations shown. The reactions were started by the addition of ATP and stopped at timed intervals by the addition of well loading dye. The proteins in the reaction were separated by SDS-PAGE using 12.5% resolving gels, fixed and dried, and the phosphorylated peptide quantitated by phosphorimager.



C.

	p110 α	p110 β
K_m	5.5×10^{-1} mM	8×10^{-3} mM
V_{max}	250	83

Figure 5.7 Kinetic analysis of p85 α S608 peptide phosphorylation by PI 3'-kinase.

The data shown in Figure 5.6 were processed and plotted as double reciprocal plots. The data for peptide phosphorylation by p110 α is shown in A., and the data for peptide phosphorylation by p110 β is shown in B. The V_{max} and K_m values subsequently calculated are shown in (C.).

6. General Discussion

Many of the signalling proteins downstream of the insulin receptor have been identified, although only a fraction of these have well defined roles or mechanisms of regulation. What has definitely emerged is that insulin action at the cellular level is a complex network characterised by both multiple pathways and isoforms of the key proteins involved (see Chapter 1). At the first level are the multiple substrates of the insulin receptor tyrosine kinase. Some of these substrates contain ligands for several SH2 domain containing proteins, and can bind multiple molecules simultaneously. Amongst these SH2 containing proteins are the Class 1a PI 3'-kinases. Alterations in the early steps of insulin signalling have been recognized as components in the pathogenesis of many insulin resistant states. Decreased insulin binding, decreased receptor tyrosine kinase activity, decreased IRS and subsequent decreases in associated PI 3'-kinase activity, have all been demonstrated in animal models of insulin resistance. In parallel, it has also been shown that perturbation at one level is often compensated by changes at another, thereby limiting the impact of alterations made to one signalling molecule. Some perturbations can be compensated better than others however, and the picture that is emerging is one of a deluge of molecules with a range of functions, some of which overlap at the periphery. In the IRS1 knockout mouse for example, many pathways are rescued by IRS2 (Araki et al 1994; Patti et al 1995), whilst p110 β cannot rescue the loss of p110 α which results in embryonic lethality (Bi et al 1998).

Seven isoforms and splice variants of adapter subunit for the class 1a PI 3'-kinases have so far been identified, which means that in combination with p110 α , p110 β and p110 δ , some cells could contain up to 21 different combinations of this heterodimeric enzyme. Tissue specific expression of these isoforms, and the differences in their modular structure suggests the different adapter/ catalytic

subunit heterodimers will have identifiable functional differences. However, following the initial effort to identify new members of the PI 3'-kinase families, little progress has been made in identifying differences between them. Similarly, regulation of PI 3'-kinases via their intra-molecular domains has remained poorly understood. The work described in this thesis has addressed some of these issues. The major conclusions which can be drawn from these studies have been discussed within each result chapter. The aim of the following section is to summarize what factors are known to affect PI 3'-kinase activity following activation by an agonist, and to put the findings of this thesis into the context of the current literature.

It is shown that as with p110 α , the lipid kinase activity of p110 β is down regulated by phosphorylation of the serine 608 residue of p85 α , although the uninhibited lipid kinase activities of p110 α and p110 β are not the same (Chapter 4), and the steps at which the kinetic constants differ between these isoforms have been determined (Chapter 5). Whilst most signalling functions of Class 1a PI 3'-kinases are considered due to their ability to phosphorylate phosphoinositides, p110's also have a known serine kinase activity. The specificity and function of this protein kinase activity is largely ill-determined, though as shown here and elsewhere, activation of the serine kinase activity of p110 inhibits the lipid kinase activity of the enzyme. We have shown that a peptide which is composed of the residues surrounding the serine 608 site of p85 α is also a target for the protein kinase activity of PI 3'-kinase. Although this peptide obviously contains the consensus sequence for p110 recognition, it was initially unclear if PI 3'-kinase would be able to bind it. Since it clearly can, there is potential to develop this peptide into a cell permeable, class 1a PI 3'-kinase specific inhibitor, which could prove a powerful tool for delineating specific signalling pathways.

The serine kinase activity of p110 may also serve to regulate insulin mediated signalling by phosphorylating IRS proteins. One potentially very important difference which was identified between the two catalytic isoforms studied was that only the serine kinase activity of p110 α targets IRS-1 (Chapter 4). Under several conditions resulting in cellular insulin resistance, decreased insulin stimulated tyrosine phosphorylation of IRS-1 is observed concomitant with increased serine phosphorylation, inhibiting the binding of PI 3'-kinase. Agents such as phorbol esters and tumor necrosis factor are thought to attenuate the ability of insulin to activate PI 3'-kinase by increasing serine/ threonine phosphorylation of IRS proteins (Hotamisligil et al 1996a). However, it has also been shown that serine phosphorylation of IRS-1 is associated with a decrease in the serine phosphorylation of p85, an event which may contribute to the activation of PI 3'-kinase (Freund et al 1995).

An important known factor determining the activity of PI 3'-kinases is the nature of the substrate. In vivo membrane constituents, such as cholesterol sulphate, can selectively alter the specificity of PI 3'-kinase to enhance its activity towards PI (4,5)P₂, while inhibiting its reaction with PI (Woscholski et al 1995), and the curvature of the surface on which the lipid is presented has also been shown to influence enzymatic activity (Hubner et al 1998). Small spherical structures stimulate activity, but are subject to chemical remodelling by kinases, since phosphorylated derivatives destabilize bilayers more strongly than PI. In addition, changes in local membrane structure are induced by the presence of peptides and proteins, so the effect of receptor activation and clustering serves to activate PI 3-kinase not only by providing a docking substrate for SH2 domains, but by inducing curvature in the surface on which the lipid substrate is presented. The studies presented in this thesis showed that p110 β was a more active kinase at low bulk concentrations of the PI substrate (corresponding to smaller vesicles), and p110 α was more active under conditions where bulk substrate concentration was

high (approximating lipid bilayer sheets). Thus it is that lipid membranes themselves provide an important mechanism to feed forward or back on enzyme activity.

Another aspect of PI 3'-kinases which will necessarily require understanding if an accurate model of non-redundancy of p110 α and p110 β function is to be made is of their tertiary structure. The solution of the crystal structure of PIPK II β (a PI5P 4-kinase) suggests a structural basis for the specificity for membrane embedded phosphoinositides (Roa et al 1998). The structure revealed a large flat area formed by homodimerization, wherein the catalytic sites form the face of this surface. It has been postulated that the cluster of basic residues at the dimer interface would provide electrostatic interactions to enable the kinase to skim along the surface of the membrane bilayer (Carpenter & Cantley 1998). Class 1a PI 3'-kinases have also been shown to dimerize in vivo, via a mechanism involving the SH3 and BH domains of p85 (Harpur et al 1999), and it is possible that this will influence the shape of the 'face' of PI 3'-kinases. The catalytic domains of p110 α and p110 β share approximately 70% identity at the amino acid level, and it will be interesting to see if the sequence differences are sufficient to create differently shaped substrate recognition sites in these two proteins, in which the actual substrate is the same, but with differential presentation at different subcellular compartments providing unique roles for the different PI 3'-kinase isoforms. Taken together with the lipid kinetic studies (Chapter 5), the recruitment to phosphotyrosine based signalling complexes (Chapter 4) and the recruitment to GLUT4 containing vesicles (Wang et al 1998), structural studies could explain why it is emerging that p110 β is the more insulin responsive isoform of PI 3'-kinase.

A consistent feature among signalling molecules is that many appear to be made from a limited repertoire of small modular domains. Moreover, it has been shown

that these domains are key participants in the regulation of signalling cascades. While some domains have been well characterized, the roles of others is less well understood. The different modular make-up of the adapter subunits of PI 3'-kinase suggests these are likely to provide mechanisms for specificity in signalling, and during the preparation of this thesis, some studies have been published which complement the work presented here. It is well established that the inter-SH2 domain of the adapter subunits contain a stretch of residues for interacting with the catalytic subunit, and that p85 α also has a serine residue close to this region which when phosphorylated downregulates p110 α . In Chapter 4, evidence is presented that this site can also regulate the activity of p110 β . Furthermore, we present evidence that this site is highly sensitive, since it is not only phosphorylation which regulates kinase activity of associated p110, but any small perturbation at this residue. Unexpectedly, an alanine substitution also down regulated PI 3'-kinase activity, and it would be interesting to determine whether a similarly small change to threonine would inhibit activity. The inter-SH2 domain is predicted to be a coiled coil (Dhand et al 1994a), and structural comparisons of native and phosphorylated p85 α , and of p85 α S608A would provide invaluable insights into p110 regulation.

The role of SH2 domains as docking units at phosphorylated tyrosine motifs is well documented, and there has been significant progress in determining the specificity of the targets of the SH2 domains of p85 (see Chapter 1). Distinct roles for each of the two SH2 domains are now being assigned as regulators of p110 activity. The inhibitory effect of p85 on p110 lipid kinase activity observed in vitro (Woscholski et al 1994) has been assigned to the nSH2 domain (Yu et al 1998), and two possible mechanisms have been put forward to explain this. In the first, the presence of an SH2 domain at the N-terminal of the inter-SH2 region would exert a conformational strain on the coiled-coil, thereby promoting a conformational change in p110 α . Alternatively, the nSH2 domain has been shown

to interact directly with p110 (Cooper & Kashishian 1993) and it may be this which inhibits p110 activity. Phosphopeptide binding to the nSH2 domain, when attached to the inter-SH2 domain has been shown to relieve the inhibitory effect of nSH2, although binding to the cSH2 only activates p110 if the cSH2 domain is in the context of a complete molecule (Yu et al 1998). Thus, phosphopeptide modulation of PI 3'-kinase activity appears to occur through distinct mechanisms of the nSH2 and cSH2 domains. Studies have shown the proline rich motifs of p85 are potential ligands for the p85 SH3 domain (Kapeller et al 1994; Rickless et al 1994). This suggests both inter- and intra-molecular interactions of p85 are possible. The latter would form the amino-terminus of p85 into a compact unit adjacent to the nSH2 domain, and phosphopeptide binding here would induce a substantial conformational change in the overall structure of p85, transmittable to p110 via strains exerted on the inter-SH2 coiled coil. By this model, phosphopeptide binding to the cSH2 domain would be expected to exert a lesser effect.

It has been discussed elsewhere in the text that the SH3 and BH domains of p85 α enable PI 3'-kinase to dimerize (Harpur et al 1999). This establishes one function for these domains. In Chapter 3, the role of these domains in activation and localization of PI 3'-kinase was discussed. The major conclusions were that these domains did not affect the kinase activities of associated catalytic subunit, but were potential ligands for activators of this class of enzyme. We have definitively shown that Rac1 stimulation of PI 3'-kinase activity occurs via the BH domain of p85 α . As described in the above section, there is potential for intra-molecular interaction within the p85 molecule, and it is highly conceivable that Rac1 binding the BH domain disrupts the compact unit of the amino-terminus. The effect of disrupting this compact unit would be to exert a conformational strain via the nSH2 domain to the p110 binding region, thereby activating the kinase activity of this enzyme. Taking this model into account, it would be reasonable to assume

that there would be differences in the kinase activities of p85 Δ BH or p85 Δ SH3 at activated signalling complexes compared to wild type PI 3'-kinase. Unfortunately, practical difficulties precluded experiments to determine whether lipid kinase activity at phosphotyrosine signalling complexes was altered in p85 Δ BH or p85 Δ SH3 PI 3'-kinase. The recent development of p85 α knockout cell lines would simplify these experiments immensely, as only the activity of transfected PI 3'-kinase would be present at phosphotyrosine signalling complexes.

It was also shown in Chapter 3 that the fold increase in p85 α recruitment to phosphotyrosine based signalling complexes following insulin stimulation was altered in the absence of either the SH3 or BH domain of p85 α . In similar studies undertaken to compare the splice variants of p85 α , a differential association of these adapters with IRS-1 has been shown following cell stimulation with insulin (Antonetti et al 1996; Inukai et al 1997; Shepherd et al 1997b). The smallest splice variant, p50 α , shows the highest level of recruitment to IRS proteins, and the authors postulate this is due to the amino-terminal domains of p85 α and p55 α forming complexes with other molecules, resulting in an inability to bind IRS as efficiently as p50 α .

Our studies were undertaken using antiphosphotyrosine, rather than anti-IRS, immunoprecipitates, although immunoblotting revealed only one major phosphotyrosine band in these samples, which corresponded in size to IRS-4 (the predominant isoform in HEK293 cells). In contrast to the conclusions of the studies by Inukai et al, we found that deletion of the SH3 or BH domains of p85 caused a decrease in fold recruitment to signalling complexes, which was accounted for primarily by the increased levels of PI 3'-kinase associating in the basal state. The effect was similar whether the BH or SH3 domain was deleted. Though sparse, the current available data suggests the domains in the amino-terminus of p85 α are frequently dependent on each other to function. For

example, dimerization occurs via the SH3 domain of one molecule binding to the first proline rich motif of another p85, yet deletion of the BH domain inhibits dimerization to the same extent as deletion of the SH3 domain (Harpur et al 1999). This suggests that although the boundaries of these modules are physically separated by almost 50 residues, including a 20 residue proline motif, their functional boundaries are blurred. It is likely that the large amount of p85 Δ BH and p85 Δ SH3 observed in phosphotyrosine complexes from basal cells is due to dysregulation and location of PI 3'-kinase in the absence of a functional amino-terminus in the adapter subunit. It is therefore tempting to speculate that the BH and SH3 domains function together as a unit which anchors the enzyme to another subcellular location. In this case, the differential recruitment of p85 splice variants following insulin stimulation would depend both on insulin stimulated formation of phosphotyrosine motifs on IRS, and a simultaneous mechanism to 'un-anchor' the adapter proteins. Although no targets have been identified for the unique regions of p55 α or p50 α , they are unlikely to be the same targets as those of the BH/ SH3 unit. p50 α may be the most recruitable adapter isoform because insulin most stimulates its detachment from whichever molecules it associates with in the basal state.

In conclusion, we find that in addition to the complexities of signalling systems in general, the regulation of p85/ p110 PI 3'-kinase is particularly complicated. Modulation of downstream effects of this class of enzyme are achieved by a combination of different intrinsic kinase activities of the catalytic subunits, differential modular domains of the adapter subunit enabling cross-talk to other signalling systems, tissue specific expression, differential substrate presentation specificities as well as varying recruitment patterns and p110 elevating activities of the adapter subunits. The intracellular pools of substrates and products are only just beginning to be identified and still there is the biology of the lipid phosphatases to be understood. Nevertheless, there has been substantial progress

in recent years, and it cannot be long before it is possible to design compounds which can differentially modulate the activities of these enzymes for research and clinical applications. The issues discussed and the results reported in this thesis have added some useful insights towards this aim.

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Appendix

humanp110a	...MPPRPS	SGELWGIHLM	PPRIL.....	.VECLLPNGM	IVTLECLREA
humanp110b	MCFSFIMPPA	MADILDIWAV	DSQIASDGS	PVDFLLPTGI	YIQLEVPREA
humanp110a	TLVTIKHELF	KEARKYPLHQ	LLQDESSYIF	VSVTQEAERE	EFFDETRRLC
humanp110b	TISYIKQMLW	KQVHNYPMFN	LLMDIDSYMF	ACVNQTAVYE	ELEDETRRLC
humanp110a	DLRLFQPFLLK	VIEPVGNREE	KILNREIGFA	IGMPVCEFD	VKDPEVQDFR
humanp110b	DVRPFLPVLLK	LVTRSCDPGE	K.LDSKIGVL	IGKGLHEFDS	LKDPEVNEFR
humanp110a	RNILNVCKEA	VDLRDLNSPH	SRAMYVYPPH	VESSPELPHK	IYNKLDRGQI
humanp110b	RKMRKFSEEK	ILSLVGLSWM	DWLKQTYPPE	HEPS..IPEN	LEDKLYGGKL
humanp110a	IVVIWVIVSP	NNDKQKYTLK	INHDCVPEQV	IAEAIRKKTR	SMLLSSEQLK
humanp110b	IVAVHF....	ENCQDVFSFQ	VSPNMNPIKV	NELAIQKR..	.LTIHGKED
humanp110a	LCVLEYQGGY	ILKVCGCDEY	FLEKYPLSQY	KYIRSCIMLG	RMPNLKMMAK
humanp110b	VSPYD....Y	VLQVSGRVEY	VFGDHPLIQF	QYIRNCVMNR	ALPHFILVEC
humanp110a	ESLYSQLPMD	CFTMPSYSRR	ISTATPYMNG	ETSTKSLWVI	...NRALRIK
humanp110b	CKIKKMYEQE	MIAIEAAINR	NSSNLPLPLP	PKKTRIISHV	WENNNPFQIV
humanp110a	ILCATYVNLN	IRDIDKIYVR	TGIYHGGEPL	CDNVNTQRPV	CSNPR.WNEW
humanp110b	LVKGN..KLN	TEETVKVHVR	AGLFHGTELL	CKTIVSSEVS	GKNDHIWNEP
humanp110a	LNVDIYIPDL	PRAARLCLSI	CS...VKGR	KGAK.....EE
humanp110b	LEFDINICDL	PRMARLCFAV	YAVLDKVKTK	KSTKTINPSK	YQTIRKAGKV
humanp110a	HCPLAWGNIN	LFDYTDTLVS	GKMALNLW.P	VPHGLEDLLN	PIGVTGSNPN
humanp110b	HYPVAWVNTM	VDFDKGQLRT	GDIILHSWSS	FPDELEEMLN	PMGTVQTNPY
humanp110a	KE.TPCLELE	F.DWFSSVVK	FPDMSVIEEH	ANWSVSREAG	FSYSHAGLSN
humanp110b	TENATALHVK	FPENKKQPY	YPPFDKIIEK	AAEIASSDS.ANVSS
humanp110a	RLARDNELRE	NDKEQLKAIS	TRDPLSEITE	QEKDFLWSHR	HYCVTI.PEI
humanp110b	RGGKKF....	..LPVLKEIL	DRDPLSQLCE	NEMDLIWTLR	QDCREIFPQS
humanp110a	LPKLLLSVKW	NSRDEVAQMY	CLVKDWPPIK	PEQAMELLDC	NYPDPMVRGF
humanp110b	LPKLLLSIKW	NKLEDVAQLQ	ALLQIWPKLP	PREALELDF	NYPDQYVREY
humanp110a	AVRCLEKYLT	DDKLSQYLIQ	LVQVLKYEQY	LDNLLVRFLL	KKALTNQRIG
humanp110b	AVGCLRQ.MS	DEELSQYLLQ	LVQVLKYEPE	LDCALSRFLL	ERALGNRRIG
humanp110a	HFFFVHLKSE	MHNKTVSQRF	GLLLESYCRA	CGMYLKHLNR	QVEAMEKLIN
humanp110b	QFLFWHLRSE	VHIPAVSVQF	GVILEAYCRG	SVGHMKVLSK	QVEALNKLKT
humanp110a	LTDILKQERK	DETQKVQMKF	LVEQMRRPDF	MDALQGLLSP	LNPAHQGLNL
humanp110b	LNSLIKLNVA	KLNRARCKEA	MHTCLKQSAY	REALSDLQSP	LNPCVILSEL
humanp110a	RLKECRIMSS	AKRPLWLNWE	NPDIMSELLF	QNNIEIFKNG	DDLRODMLTL
humanp110b	YVEKCKYMDS	KMKPLWLVYN	NKVFGEDSV.	...GVIFKNG	DDLRODMLTL
humanp110a	QIIRIMENIW	QNQGLDLRML	PYGCLSIGDC	VGLIEVVRNS	HTIMQIQ.CK
humanp110b	QMLRLMDLLW	KEAGLDRML	PYGCLATGDR	SGLIEVVSTS	ETIADIQLNS
humanp110a	GGLKGALQFN	SHTLHQWLKD	KNKGEIYDAA	IDLFTRSCAG	YCVATFILGI
humanp110b	SNVAAAAAFN	KDALLNWLKE	YNSGDDLDR	IEEFTLSCAG	YCVASYVLGI
humanp110a	GDRHNSNIMV	KDDGQLFHID	FGHFLDHKKK	KFGYKRERVP	FVLTQDFLIV
humanp110b	GDRHSDNIMV	KKTGQLFHID	FGHILGNFKS	KFGIKRERVP	FILTYDFIHW
humanp110a	ISKGAQECTK	TREFERFQEM	CYKAYLAIRQ	HANLFINLFS	MMLGSGMPEL
humanp110b	IQG..KTGN	TEKFGFRQC	CEDAYLILRR	HGNLFITLFA	LMLTAGLPEL
humanp110a	QSFDDIAYIR	KTLALDKTEQ	EALEYFMKQM	NDAHGGWTT	KMDWIFHTIK
humanp110b	TSVKDIQYLK	DSLALGKSEE	EALKQFKQKF	DEALRESWTT	KVNWMAHTVR
humanp110a	QHALM				
humanp110b	KDYRS				

Comparison of the sequences of p110 α and p110 β . The adapter binding domain is shown in red, the ras binding domain is shown in dark blue, the PIK domain is shown in green and the catalytic domain is shown in bright blue. For further details, see text.

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Appendix

humanp110a	...MPPRPS	SGELWGIHLM	PPRIL.....	.VECLLPNGM	IVTLECLREA
humanp110b	MCFSFIMPPA	MADILDIWAV	DSQIASDGS	PVDFLLPTGI	YIQLEVPREA
humanp110a	TLVTIKHELF	KEARKYPLHQ	LLQDESSYIF	VSVTQEAERE	EFFDETRRLC
humanp110b	TISYIKQLW	KQVHNYPMFN	LLMDIDSYMF	ACVNQFAVYE	ELEDETRRLC
humanp110a	DLRLFQPFK	VIEPVGNREE	KILNREIGFA	IGMPVCFEFD	VKDPEVQDFR
humanp110b	DVRPFLPVLK	LVTRSCDPGE	K.LDSKIGVL	IGKGLHEFDS	LKDPEVNEFR
humanp110a	RNILNVCKEA	VDLRDLNSPH	SRAMYVYPPH	VESSPELPHK	IYNKLDRGQI
humanp110b	RKMRKFSEEK	ILSLVGLSWM	DWLKQTYYPE	HEPS..IPEN	LEDKLYGGKL
humanp110a	IVVIWVIVSP	NNDKQKYTLK	INHDCVPEQV	IAEAIRKCTR	SMLLSSEQLK
humanp110b	IVAVHF....	ENCQDVFSFQ	VSPNMNPIKV	NELAIQKR..	.LTIHGKREDE
humanp110a	LCVLEYQGY	ILKVCGCDEY	FLEKYPLSQY	KYIRSCIMLG	RMPNLKMMAK
humanp110b	VSPYD....Y	VLQVSGRVEY	VFGDHPLIQF	QYIRNCVMNR	ALPHFILVEC
humanp110a	ESLSYQLPMD	CFTMPSYSRR	ISTATPYMNG	ETSTKSLWVI	...NRALRIK
humanp110b	CKIKKMYEQE	MIAIEAAINR	NSSNLPLPLP	PKKTRIIISHV	WENNNPFQIV
humanp110a	ILCATYVNLN	IRDIDKIYVR	TGIYHGGEPL	CDNVNTQRPV	CSNPR.WNEW
humanp110b	LVKGN..KLN	TEETVKVHVR	AGLPHGTELL	CKTIVSSEVS	GKNDHIWNEP
humanp110a	LNVDIYIPDL	PRAARLCLSI	CS....VKGR	KGAK.....EE
humanp110b	LEFDINICDL	PRMARLCFAV	YAVLDKVKTK	KSTKTINPSK	YQTIRKAGKV
humanp110a	HCPLAWGNIN	LFDYDTTLVS	GKMALNLW.P	VPHGLEDLLN	PIGVTGSNPN
humanp110b	HYPVAWVNTM	VDFDKGQLRT	GDIILHSWSS	FPDELEEMLN	PMGTVQTNPY
humanp110a	KE.TPCLELE	F.DWFSSVVK	FPDMSVIEEH	ANWSVSREAG	FSYSHAGLSN
humanp110b	TENATALHVK	FPENKKQPY	YPPFDKIIEK	AAEIASSDS.ANVSS
humanp110a	RLARDNELRE	NDKEQLKAIS	TRDPLSEITE	QEKDFLWSHR	HYCVTI.PEI
humanp110b	RGGKKF....	..LPVLKEIL	DRDPLSQLCE	NEMDLIWLTR	QDCREIFPQS
humanp110a	LPKLLLSVKW	NSRDEVAQMY	CLVKDWPPIK	PEQAMELLDC	NYPDPMVRGF
humanp110b	LPKLLLSIKW	NKLEDVAQLQ	ALLQIWPKLP	PREALELLDF	NYPDQYVREY
humanp110a	AVRCLEKYLT	DDKLSQYLIQ	LVQVLKYEQY	LDNLLVRFL	KKALTNQRIG
humanp110b	AVGCLRQ.MS	DEELSQYLLQ	LVQVLKYEPF	LDCALSRFL	ERALGNRRIG
humanp110a	HFFFWHLKSE	MHNKTVSQRF	GLLLESYCRA	CGMYLKHLNR	QVEAMEKLIN
humanp110b	QFLFWHLRSE	VHIPAVSVQF	GVILEAYCRG	SVGHMKVLSK	QVEALNKLPT
humanp110a	LTDILKQERK	DETQKVQMKF	LVEQMRRPDF	MDALQGLLSP	LNPAHQLGNL
humanp110b	LNSLIKLNVA	KLNRAKGKEA	MHTCLKQSAY	REALSDLQSP	LNPCVILSEL
humanp110a	RLKECRIMSS	AKRPLWLNWE	NPDIMSELLF	QNNIIIFKNG	DDLQDMLTL
humanp110b	YVECKYMDS	KMKPLWLVIN	NKVFGEDSV.	...GVIFKNG	DDLQDMLTL
humanp110a	QIIRIMENIW	QNQGLDLRML	PYGCLSIGDC	VGLIEVVRNS	HTIMQIQ.CK
humanp110b	QMLRLMDLLW	KEAGLDRML	PYGCLATGDR	SGLIEVVSTS	ETIADIQLNS
humanp110a	GGLKGALQFN	SHTLHQWLKD	KNKGEIYDAA	IDLFTRSCAG	YCVATFILGI
humanp110b	SNVAAAAAFN	KDALLNWLKE	YNSGDDLDR	IEEFTLSCAG	YCVASYVLGI
humanp110a	GDRHNSNIMV	KDDGQLFHID	FGHFLDHKKK	KFGYKRERP	FVLTQDFLIV
humanp110b	GDRHSDNIMV	KKTGQLFHID	FGHILGNFKS	KFGIKRERP	FILTYDFIHV
humanp110a	ISKGAQECTK	TREFERFQEM	CYKAYLAIRQ	HANLFINLFS	MMLGSGMPEL
humanp110b	IQQG..KTGN	TEKFRFRQC	CEDAYLILRR	HGNLFITLFA	LMLTAGLPEL
humanp110a	QSFDDIAYIR	KTALADKTEQ	EALEYFMKQM	NDAHGGWTT	KMDWIFHTIK
humanp110b	TSVKDIQYLK	DSLALGKSEE	EALKQFKQKF	DEALRESWTT	KVNWMAHTVR
humanp110a	QHALM				
humanp110b	KDYRS				

Comparison of the sequences of p110 α and p110 β . The adapter binding domain is shown in red, the ras binding domain is shown in dark blue, the PIK domain is shown in green and the catalytic domain is shown in bright blue. For further details, see text.

p45
p85a	.MSAEGYQYR	ALYDYKKERE	EDIDLHLGDI	LTVNGKSLVA	LGFSDBGQEAR
p55a
p85b	MAGPEGFQYR	ALYPFRRERP	EDLELLPGDV	LVSRAALQA	LGVAEGGERC
p45
p85a	PEEIGWLNKY	NETTGERGDF	PGTYVEYIGR	KKISPPTPKP	RPPRPLPVAP
p55a
p85b	PQSVGWMPGL	NERTRQRGDF	PGTYVEFLGP	VALARPGPRP	RGPRPLPARP
p45
p85a	GSSKTEADVE	QQALTLPLDLA	EQFAPPDIAP	PLLKLVLEAI	EKKGLECSTL
p55a
p85b	RDGAPEP...	..GLTLPLDLP	EQFSPPDVAP	PLLVKLVLEAI	ERTGLDSESH
p45
p85a	YRTQSSSNLA	ELRQLLDCDT	PSVDLEMIDV	HVLADAFKRY	LLDLPNPVIP
p55a
p85b	YRPELPAPRT	DW.....	SLSDVDQWDT	AALADGIKSF	LLALPAPLVT
p45
p85a	AAVYSEMISL	APEVQSSEY	IQLLKKLIRS	PSIPHQYWLT	LQYLLKHFFK
p55a
p85b	PEASAEARRA	LREAAGP...	...VGPALP	PTLPLHRALT	LRFLQLHLGR
p45
p85a	LSQTSSKNLL	NARVLSEIFS	PMLFR.....FSA	ASSDNTENLI
p55aMYNT.....VWN	MEDLDLEYAK
p85b	VARRAPALGP	AVRALGATFG	PLLLRAPPPP	SSPPPGGAPD	GSEPSDFPA
p45MH.NLQT	LPPKPPK...	.PTTVANNSM	NNNMSLQDAE
p85a	KVIEILISTE	WNERQ.PAPA	LPPKPPK...	.PTTVANNGM	NNNMSLQNAE
p55a	TDINCGTDLM	FYIEM.DPPA	LPPKPPK...	.PTTVANNGM	NNNMSLQNAE
p85b	LLVEKLLQEH	LEEQEVAPPA	LPPKPPKAKP	APTVLANGG.	.SPPSLQDAE
p45	WYWGDISREE	VNEKLRDTAD	GTFLVRDAST	KMHGDYTLTP	RKGGNNKLIK
p85a	WYWGDISREE	VNEKLRDTAD	GTFLVRDAST	KMHGDYTLTL	RKGGNNKLIK
p55a	WYWGDISREE	VNEKLRDTAD	GTFLVRDAST	KMHGDYTLTL	RKGGNNKLIK
p85b	WYWGDISREE	VNEKLRDTPD	GTFLVRDASS	KIQGEYTLTL	RKGGNNKLIK
p45	IFHRDGKYG	SDPLTFNSV	ELINHYRNES	LAQYNPKLDV	KLLYPVSKYQ
p85a	IFHRDGKYG	SDPLTFSSV	ELINHYRNES	LAQYNPKLDV	KLLYPVSKYQ
p55a	IFHRDGKYG	SDPLTFSSV	ELINHYRNES	LAQYNPKLDV	KLLYPVSKYQ
p85b	VFHRDGHYGF	SEPLTFCSV	DLINHYRHES	LAQYNAKLD	RLLYPVSKYQ
p45	QDQVVKEDNI	EAVGKKLHEY	NTQFQEKSR	YDRLYEYTR	TSQEIQMKRT
p85a	QDQVVKEDNI	EAVGKKLHEY	NTQFQEKSR	YDRLYEYTR	TSQEIQMKRT
p55a	QDQVVKEDNI	EAVGKKLHEY	NTQFQEKSR	YDRLYEYTR	TSQEIQMKRT
p85b	QDQIVKEDSV	EAVGAQLKVY	HQQYQDKSR	YDQLYEYTR	TSQELQMKRT
p45	AIEAFNETIK	IFEEQCQTQE	RYSKEYIEKF	KREGNEKEIQ	RIMHNDKLL
p85a	AIEAFNETIK	IFEEQCQTQE	RYSKEYIEKF	KREGNEKEIQ	RIMHNYDKLK
p55a	AIEAFNETIK	IFEEQCQTQE	RYSKEYIEKF	KREGNEKEIQ	RIMHNYDKLK
p85b	AIEAFNETIK	IFEEQCQTQE	KCSKEYLERF	RREGNEKEMQ	RILLNSERLK
p45	SRISEIIDS	RRLEEDLKKQ	AAEYREIDKR	MNSIKPDLIQ	LRKTRDQYLM
p85a	SRISEIIDS	RRLEEDLKKQ	AAEYREIDKR	MNSIKPDLIQ	LRKTRDQYLM
p55a	SRISEIIDS	RRLEEDLKKQ	AAEYREIDKR	MNSIKPDLIQ	LRKTRDQYLM
p85b	SRIAEIHESR	TKLEQQLRQA	ASDNREIDKR	MNSLKPDLMQ	LRKIRDQYLV
p45	WLTQKGVQRK	KLNEWLGREN	.TEDQYSLVE	DDEDLPHHDE	KTWNVSSNR
p85a	WLTQKGVQRK	KLNEWLGREN	.TEDQYSLVE	DDEDLPHHDE	KTWNVSSNR
p55a	WLTQKGVQRK	KLNEWLGREN	.TEDQYSLVE	DDEDLPHHDE	KTWNVSSNR
p85b	WLTQKGVQRK	KINEWLGIKN	ETEDQYALME	DEDDLPHHDE	RTWYVGINR
p45	NKAENLLRGK	RDGTFLVRES	SKQGCYACSV	VVDGEVKHCV	INKTATGYGF
p85a	NKAENLLRGK	RDGTFLVRES	SKQGCYACSV	VVDGEVKHCV	INKTATGYGF
p55a	NKAENLLRGK	RDGTFLVRES	SKQGCYACSV	VVDGEVKHCV	INKTATGYGF
p85b	TQAEEMLSGK	RDGTFLIRES	SQRGCYACSV	VVDGDTKHCV	IYRTATGFGF
p45	AE PYNLYSSL	KELVLHYQHT	SLVQHNDSLN	VTLAYPVYQA	QRR.....
p85a	AE PYNLYSSL	KELVLHYQHT	SLVQHNDSLN	VTLAYPVYQA	QRR.....
p55a	AE PYNLYSSL	KELVLHYQHT	SLVQHNDSLN	VTLAYPVYQA	QRR.....
p85b	AE PYNLYGSL	KELVLHYQHA	SLVQHNDALT	VTLAHPVRAP	GPGPPPAAR

Comparison of the sequences of p85 α (and its splice variants) and p85 β . SH2 domains are shown in red, BH domain in green and the SH3 domain in blue. For further details, see text.