A Structural and Functional Analysis of the Phosphatidylinositol 3-kinase.

by

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Abstract

Phosphatidylinositol 3-kinase has emerged as an important signal transducing molecule which is activated by diverse receptor and non-receptor protein-tyrosine kinases (PTK) and in some cells may be an important mediator in the events leading to mitogenesis. The affinity purification of PI 3-kinase from bovine brain has shown that this enzyme is a tightly associated heterodimer of 85 kDa and 110 kDa subunits. Structural analysis at the protein and cDNA levels revealed two forms of the 85 kDa subunit, p85αwhich corresponds to the material purified from the bovine brain, and another highly related protein termed p85β. Both p85 proteins contain an N-terminal SH3 domain, followed by a BCR domain and two SH2 domains separated by a region predicted to adopt a helical conformation.

The p85 proteins have been expressed in insect cells using the baculovirus expression system and they are shown to form tight complexes with many PTKs when coexpressed in the same cell. The cloning of the catalytic bovine p110 subunit of PI 3-kinase has allowed expression of the p110 subunit in insect cells. This protein was subsequently shown to possess an intrinsic PI 3-kinase activity when expressed alone and to form a stable active complex with p85αand p85\both in vitro and in vivo. The sites of interaction between the two subunits have been mapped to the N-terminal region of p110 to a sequence of 35 amino acids in the inter-SH2 domain of p85. The p85 α /p110 and the p85 β /p110 complexes bind to activated PTKs in a manner that is more selective than the binding of p85αor p85βalone to PTKs. Additional biochemical and mutagenesis studies of the catalytic p110 subunit have demonstrated that this is a novel dual specificity enzyme with an intrinsic protein-serine kinase activity which in turn regulates its PI 3-kinase activity. These studies using the recombinant enzyme have been complemented by the analysis of endogenous PI 3-kinase activation in PDGF-stimulated NIH 3T3 cells.

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Chapter 1: Introduction

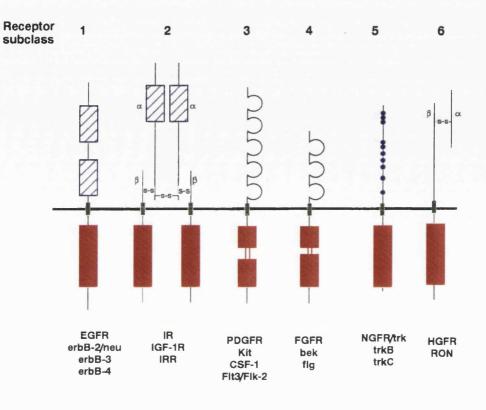
DNA synthesis and cell growth are regulated by a large number of polypeptide growth factors, cytokines and hormones which exert their mitogenic effects by interaction with specific cell surface receptors on responsive cells. The binding of a ligand to its receptor elicits a cascade of events including protein phosphorylation, stimulation of second messenger generating systems, ion fluxes, enhanced DNA synthesis and can culminate with proliferation or differentiation.

One large group of growth and differentiation factors act by binding to and activating surface receptors possessing intrinsic protein-tyrosine kinase (PTK) activity. Among the best characterised are the epidermal growth factor (EGF) and the platelet-derived growth factor (PDGF). The study of these molecules has provided fundamental insights into cell growth, and in particular the mechanisms by which normal regulated proliferation can be subverted in tumorigenic transformation (reviewed in Aaronson et al., 1991). Major advances were the purification and sequencing of PDGF, revealing its homology to the transforming product of the *sis* oncogene which is responsible for tumour formation by the simian sarcoma virus (Doolittle et al., 1983; Waterfield et al., 1983). Indeed, PTKs were originally implicated in growth control by their identification as the transforming gene products of oncogenic viruses, the first being v-Src (Collet and Erikson, 1978). In addition, it later transpired that some oncogenes are altered versions of PTK growth factor receptors, for example the v-erbB oncogene is a truncated version of the EGF receptor (Downward et al., 1984). The close relationship of PTKs with tumourigenesis makes this class of molecules particularly interesting for study.

1.1 Structural features of receptor protein-tyrosine kinases.

Growth factor receptors with PTK activity share a similar overall structure. A large extracellular domain which interacts with a polypeptide ligand is connected by a hydrophobic sequence, which traverses the plasma membrane once, to an intracellular region that contains a PTK catalytic domain (reviewed in Ullrich and Schlessinger, 1990). Variations of these general characteristics have been used to group the receptors into a number of distinct subclasses (Figure 1.1).

Subclass 1: The EGF receptor is the prototype for this subclass which also includes the related neu/Her2/c-erbB2, c-erbB3 and c-erbB4. This class of receptors is characterised by the presence of two cysteine-rich repeats within the extracellular ligand binding



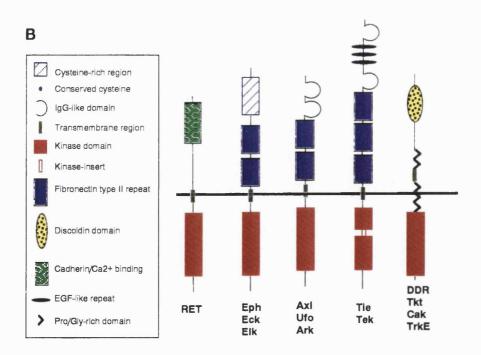


Figure 1.1. Classification of receptor protein-tyrosine kinases

The structural features of receptor protein tyrosine kinases has been used to group the receptors into subclasses 1-6 shown in Panel A.

Panel B shows subclasses of receptors for the ligand has not yet been identified.

domain and a regulatory C-terminal extension that follows the catalytic domain and contains the major sites for autophosphorylation.

Subclass 2: This group is represented by the insulin and insulin-like growth factor receptors and is distinguished by a heterotetrameric $\alpha_2\beta_2$ structure. The receptor molecule is composed of two extracellular α -chains, each containing a single cysteinerich region, which constitutes the ligand binding domain. These are then linked by disulphide bonds to two β -chains which span the plasma membrane and possess intrinsic kinase activity.

Subclass 3: The third class is defined by the PDGF receptors (α and β subtypes), the colony stimulating factor-1 (CSF-1) receptor, the stem cell factor receptor (c-kit) and the Flt/Flk receptors. Distinct features here include five immunoglobulin(IgG)-like repeats in the extracellular domain and a kinase domain-insert region that varies in length between receptors.

Subclass 4: The fourth subtype includes the fibroblast growth factor (FGF) receptors. Similar in structure to receptors from subclass 3, they are typified by an extracellular region that contains three IgG-like repeats and an acidic box of eight amino acids between the two amino-terminal IgG-like loops.

Subclass 5: This is a family of neurotrophic factor receptors called trkA, trkB and trkC. They possess a similar structure to the EGF receptor but the extracellular domain is defined by the presence of conserved cysteine residues.

Subclass 6: The hepatocyte growth factor (HGF) forms the prototype for this subclass which also includes c-sea and c-ron. These receptors are characterised by a β -subunit which spans the membrane and contains the catalytic domain. The β -subunit is disulphide-linked to a smaller α -subunit which remains extracellular.

There are a growing number of receptors that do not have, as yet, identified ligands. These classes of receptor include the eph, elk and eck receptors and the ret family of receptors, others are also shown in Figure 1.1B. With the continued identification of increasing numbers of PTK receptors, it is clear that this grouping is incomplete, however, the classification is still of use for comparison of structure-function relationships between receptors.

1.1.1 Ligand binding

The extracellular ligand-binding domains of the receptor PTKs are characterised by the presence of cysteine residues, the spacing of which defines either IgG-like domains as in the case of the PDGF and FGF receptors, or cysteine rich clusters. Significant progress has been made toward the characterisation of the ligand binding region of the EGF and insulin receptors. Studies based on the internal homology in the primary structures (Yarden and Ullrich, 1988) and electron microscopic analyses (Ullrich and Schlessinger,

1990) propose a four domain model for the organisation of the extracellular portion of the EGF receptor. In this model it is proposed that domain III, which is flanked by the cysteine rich domain, and domain I contribute most of the ligand binding determinants by being situated close together in such a way that the EGF binding region would lie in a cleft formed between them (Lax et al., 1989). The two domains that are formed by the cysteine-rich regions would then lie in contact with each other and close to the plasma membrane. Such a configuration for a ligand binding site is common in many allosteric enzymes where ligand binding alters the interaction between neighbouring subunits, thus allowing transfer of a conformational change (Ullrich and Schlessinger, 1990). Analytical studies with the insulin binding pocket are consistent with a model where the amino-terminal region of the insulin receptor α -subunit, analogous to domain I of the EGF receptor, is implicated in ligand binding (Wedekind et al., 1989).

The extracellular domain also plays a role in regulation of receptor PTK signalling functions. This has been exemplified in v-erbB and v-kit, where deletion of the extracellular domain is found to eliminate the negative control that this structure appears to exert on the cytoplasmic domain (Woolford et al., 1988). Even point mutations within the extracellular domain can lead to intracellular activation, for example mutations at residues 301 and 374 of v-fms (Roussell et al., 1988). These mutations may induce receptor dimerisation and/or a conformational change, equivalent to that triggered by ligand binding.

1.1.2 Transmembrane region

The primary role of the transmembrane domain is to anchor the receptor in the plasma membrane, thereby connecting the extracellular environment with the internal compartment of the cell. Secondary structure prediction suggests that the membrane spanning residues form an α-helix and this is supported by NMR analysis of the transmembrane region of c-erbB2 (Gullick et al., 1992). A more striking function for this domain is exhibited in the *neu* oncogene whose only difference from its non-transforming counterpart is a single amino acid substitution from valine 664 to glutamic acid in the transmembrane region (Bargmann and Weinberg, 1988). This mutation is thought to have a stabilising effect, causing receptor dimerisation and thus constitutive activation of the receptor kinase in the absence of ligand (Sternberg and Gullick, 1990). Similar results have been observed in the insulin receptor; Longo et al. (1992) reported that the mutation Val938-Glu resulted in constitutive activation and Yamada et al. (1992) showed that a chimeric insulin receptor containing the oncogenic neu transmembrane domain was also activated in vitro. However, in spite of the structural similarity between the EGF receptor and neu, substitution of the EGF transmembrane domain with the sequence

analogous to the transforming neu sequence does not constitutively activate the EGF receptor kinase (Carpenter et al., 1991).

1.1.3 Juxtamembrane domain

The juxtamembrane sequences separate the transmembrane region of receptor PTKs from the catalytic domain and are the most divergent between receptor subclasses but conserved between members of the same subclass. The juxtamembrane domain is thought to be involved in receptor transmodulation by heterologous stimuli. For example, mutations in the juxtamembrane sequence of the insulin receptor are observed to have dramatic affects on signalling responses independent of PTK activity. Substitution of Tyr960-Pro in the insulin receptor juxtamembrane region abolishes the receptors ability to phosphorylate its substrate IRS-1 and blocks downstream signalling, without affecting kinase activity (White et al., 1988). This is not surprising since the IRS-1 protein has been shown to act as a discrete docking protein for the binding of distinct substrates to the insulin receptor (discussed in section 1.2). The expression levels of IRS-1 have been shown to strongly correlate with the levels of active insulin receptors (Wilden et al., 1992). In unstimulated human 293-kidney cells and in Chinese hamster Overy (CHO) cells (Backer et al., 1992), but not in NIH 3T3 cells, insulin receptors are tightly associated to IRS-1 (Giorgetti et al., 1993). Amino acid sequence analysis of IRS-1 reveals a protein with an ATP binding site, many potential serine/threonine phosphorylation sites, and at least 20 potential tyrosine phosphorylation sites (Sun et al., 1991) some of which have been shown to be phosphorylated in vivo (Sun et al., 1993). This suggests that the insulin receptor as well as interacting directly with some substrates employs a.

Phosphorylation of the EGF receptor, in particular on threonine 654 in the juxtamembrane region, by protein kinase C (PKC) also results in loss of high affinity EGF binding (Liveneh et al., 1987) and attenuation of its kinase activity (Davis, 1988). Similar results were seen for a chimeric EGF receptor external domain/neu cytoplasmic domain stimulated by the phorbol ester PMA (Lee et al., 1989).

1.1.4 Protein kinase domain

This is the most highly conserved region of all receptors and contains several residues that constitute a typical ATP-binding site, first demonstrated using flourosulphonylbenzoyladenosine (FSBA) treatment (Hanks et al., 1988). The kinase domain catalyses the transfer of the γ -phosphate of ATP to tyrosine residues onto the receptor molecules themselves in an autophosphorylation reaction or onto other substrates. This catalytic domain can be subdivided into highly conserved subdomains numbered I to XI separated by regions of lower sequence identity. The

GXGXXGX(15-20)K sequence is part of a consensus motif that resides in subdomain I, 80-180 amino acids C-terminal to this lie kinase domain consensus sequences DFG (subdomain VI) and APE (subdomain VIII), which together form part of the central core of the catalytic domain (Hanks et al., 1988). These sequences are known to be essential for binding the nucleotide phosphate moieties and for phosphotransferase activity in classical protein kinases (Taylor et al., 1992). Mutation of the lysine residue in the kinase consensus sequence GXGXXGX(15-20)K has been shown to abolish kinase activity in the EGF, insulin and PDGF receptors both in vivo and in vitro (Chou et al., 1987; McClain et al., 1987; Russel et al., 1987; Honegger et al., 1987, Chen et al., 1987; Williams et al., 1987). Further analyses using deletion mutants and linker insertion mutants of the catalytic domain have revealed that the kinase activity of the various receptors was dispensible for their expression and targeting to the cell surface (Liveneh et al., 1986; Prywes et al., 1986; Liveneh et al., 1987). However, the kinase activity was an absolute requirement for signal transduction and induction of both early and delayed responses including mitogenesis and transformation. Although normal in its binding characteristics, a kinase-negative mutant of the EGF receptor was unable to stimulate calcium influx, inositol phosphate formation, Na⁺/H⁺ exchange, c-fos and c-myc expression, S6 ribosomal protein phosphorylation, DNA synthesis and transformation (Honegger et al., 1987; Chen et al., 1987; Moolenaar et al., 1988). This suggests that all receptor PTK signalling activities depend on a functional kinase.

1.1.4.1 Kinase insert region

The kinase domain of subclass 3 and 4 receptor PTKs is divided into two halves by insertions of up to 100 mostly hydrophilic amino acid residues. The kinase inserts of the various receptors vary in length and show only marginal sequence similarity. Their conservation between species however suggests that they play an important role in receptor function. Although it has been demonstrated that the kinase insert region is not required for catalysis (Taylor et al., 1989), it is known to contain a number of autophosphorylation sites, for example, in the human PDGFβ receptor Y740, Y751 and Y771 (Fantl et al.,1992; Kashishian et al., 1992; Kazlauskas and Cooper, 1989). As these are also known substrate binding sites (reviewed in Pawson and Schlessinger, 1993; Section 1.2) it appears that the role of the kinase insert region is to modulate receptor interactions with certain cellular substrates and effector proteins.

1.1.4.2 Carboxy-terminal tail.

The carboxy-terminal tail sequences are among the most divergent between all known receptor tyrosine kinases (Yarden and Ullrich, 1988). Several autophosphorylation sites have been mapped in this region of neu and the EGF receptor (Margolis et al., 1989a; Hazan et al., 1990), insulin (Tornqviist and Avruch, 1988), NGF (Obermeier et al., 1993;

1993a) and PDGF receptors (Ronnstrand et al., 1992). Further, as with the kinase insert sequences, many of these autophosphorylation sites appear to be required for substrate interactions (See section 1.2). Several studies also suggest that the carboxy-terminal tail sequences may exert negative control on receptor tyrosine kinase signalling functions (Roussel et al., 1987; Haley et al., 1989; Velu et al., 1989).

1.15 Receptor activation

Ligand binding to the extracellular domain causes the transmission of a signal which stimulates tyrosine kinase activity of the intracellular domain and in turn initiates a cascade of signalling events. The currently accepted model proposes that ligand binding drives the dimerisation or oligomerisation of receptors (Williams, 1989; Schlessinger, 1988). This process promotes interaction between adjacent cytoplasmic domains leading to their activation. Thus, receptor dimerisation bypasses the requirement for a conformational change to be transmitted through the transmembrane region which would be energetically unfavorable. As oligomerised growth factor receptors possess an elevated PTK activity and enhanced ligand binding affinity (Yarden and Schlessinger, 1987; Boni-Schnetzler and Pilch, 1987), the dimerised form is considered to be the active state of the receptor. Different growth factors or hormones are proposed to utilise different mechanisms to induce receptor dimerisation.

The PDGF molecule is a dimeric growth factor, and the dimerisation of its receptor is thought to be mediated by ligand induced bridging of two PDGF receptors (Hart et al., 1988; Heldin et al., 1988). Two forms of PDGF (denoted A and B types) are known to form AA, BB homodimers and AB heterodimers. Thus, the two forms of the PDGF receptor can be displayed on the cell surface as $\alpha\alpha$ and $\beta\beta$ homodimers and $\alpha\beta$ heterodimers. Quantitative binding studies indicate that the various dimeric forms of the PDGF receptor exhibit differential binding specificity for the various dimeric forms of the PDGF ligand (Reviewed in Heldin, 1990). Hence, heterologous receptor dimerisation can expand the repertoire of receptor ligand interactions and may extend the diversity of signals generated.

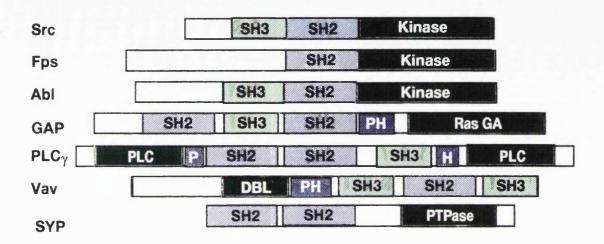
A different model is presented for the EGF and insulin growth factors, where a single growth factor is suggested to bind two receptor molecules and thus, induce receptor dimerisation (Cunningham et al., 1991). Insulin is thought to activate its cell surface receptor by inducing an allosteric transition within a preexisting dimeric structure (Reviewed in White et al., 1994). EGF receptor dimerisation is probably mediated by a conformational change in the extracellular domain (Greenfield et al., 1989) that stabilises the interactions between two occupied receptor molecules (Lax et al., 1991; Hurwitz et al., 1991).

Thus, receptor dimerisation may induce activation of the intrinsic PTK activity (Schlessinger 1988; Ullrich and Schlessinger, 1990, Canals, 1992) which in turn results in autophosphorylation mediated by an intermolecular trans-phosphorylation mechanism (Honegger et al., 1989; 1990; Lammers et al., 1990). Moreover, intermolecular phosphorylation may also occur between two related receptors such as transphosphorylation of c-erbB2 by EGF receptor (King et al 1988) and transphosphorylation between distinct FGF and PDGF receptor subtypes (Bellot et al., 1991; Kelly et al., 1991).

1.2 Targets of activated receptor kinases

Tyrosine autophosphorylation is crucial for normal receptor signalling. The tyrosine autophosphorylation sites on growth factor receptors represent specific binding sites for cytoplasmic target proteins involved in the transmission of the biological signal. Thus a mechanistic link is established between receptor activation and downstream signalling. The molecules that have been found to be involved in these multienzyme complexes include phosphatidylinositol 3-kinase (PI 3-kinase), phospholipase Cγ (PLCγ), the nonreceptor protein-tyrosine kinase pp60^{c-Src}, the serine/threonine kinase Raf, protein tyrosine phosphatases SHPTP1/SHPTP2, the Ras GTPase-activating protein (RasGAP) for Ras, guanine nucleotide releasing factor Vav, and transcription factors such as p91. Adapter proteins such as c-crk, Shc, Nck and Grb2, which possess no intrinsic catalytic activity, have also been found to participate in these complexes. **Tyrosine** phosphorylation and functional regulation of the activity of these intracellular target proteins leads to pleiotropic cellular responses which are essential for mitogenesis or diffentiation. Most receptor-binding proteins are characterised by the presence of Src-homology (SH) 2 domains (Figure 1.2; Discussed in detail in Section 1.3), which are conserved non-catalytic protein modules that recognise specific phosphotyrosine residues present within a distinct recognition motif on activated receptor PTKs and cytoplasmic polypeptides (Reviewed in Koch et al., 1991; Pawson and Gish, 1993). Proteins containing SH2 domains also frequently possess a second discrete module, the SH3 domain (Figure 1.2; Reviewed in Musacchio et al., 1992a), which mediates proteinprotein interactions through the recognition of proline-rich motifs (Ren et al., 1993; Gout et al., 1993). The activation of intracellular biochemical pathways by receptor PTKs, appears in large part to be regulated by the formation of multi-protein complexes mediated by SH2 and SH3 domains and is discussed below.

A. Enzymes



B. Adapter molecules

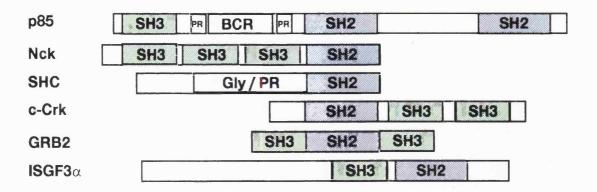


Figure 1.2 Proteins with SH2, SH3 and PH domains. These proteins are divided into those containing a catalytic domain (Panel A) and those that may function as adapter molecules (Panel B). Gly/PR = Glycine/proline-rich region, PR = Proline-rich region, DBL = region of homology with the Dbl protein. BCR = region of homology with the BCR protein. The PH domain of PLC γ is split.

1.2.1 Regulators of phosphoinositide signalling

1.2.1.1. Phosphatidylinositol 3-kinase

The observation that a variety of mitogens rapidly stimulate phosphatidylinositol (PI) turnover and that PI turnover may be constitutively activated in oncogenically transformed cells suggests that these second messengers are involved in the regulation of cell proliferation by growth factors and oncogenes (Michell, 1982; Whitman et al., 1986). In the early 1980s the phosphoinositides of the PI cycle were known to be a source of important second messenger molecules produced through the action of phospholipase C (PLC) on PI 4,5-bisphosphate PI(4,5)P₂ (Figure 1.3). The two second messenger molecules generated by PLC-mediated hydrolysis of this lipid were shown to be diacylglycerol (DAG), which then activates protein kinase C (PKC) (Nishizuka, 1984), and inositol 1,4,5-triphosphate (Ins(1,4,5)P3), which in turn elevates intracellular calcium levels (Berridge and Irvine, 1984). PI(4,5)P₂ is formed biosynthetically by the sequential phosphorylation of PI and PI(4)P by PI 4-kinase and PI 4-phosphate 5-kinase respectively. Subsequently, two distinct PI kinase activities, termed type I and type II, were identified in murine fibroblasts on the basis of their biochemical response to non-ionic detergents and inhibitors such as adenosine (Whitman et al., 1987). The type I activity was found to be inhibited by non-ionic detergents and to be resistant to inhibition by adenosine, while the type II activity was activated by detergents and inhibited by adenosine. Subsequent work showed that the type II activity catalysed the formation of PI(4)P, while the type I activity catalysed the addition of phosphate at the D3 position of the inositol moity of PI to produce PI(3)P. In addition the type I activity was found to specifically associate with activated PTKs (Whitman et al., 1988). This PI 3-kinase activity was also shown to phosphorylate PI(4)P and PI(4,5)P2 to PI(3,4)P3 and PI(3,4,5)P₃ respectively (Auger et al., 1989, 1989a; Lips et al., 1989; Stephens et al., 1989; Brearley and Hanke, 1992). These 3-phosphoinositides are generally absent, or present at very low levels in unstimulated cells, but PI(3,4)P2 and PI(3,4,5)P3 production in particular is rapidly induced following agonist stimulation (reviewed in Irvine, 1992).

1.2.1.1.1 Biological function

PI 3-kinase activity was subsequently identified in immunoprecipitates associated with pp60v-Src (Sugimoto et al., 1984), the polyoma virus middle T antigen/pp60c-Src complex (Whitman et al., 1985), the receptor for PDGF (Whitman et al., 1987), p68v-ros, the transforming protein of the avian sarcoma virus UR2 (Macara et al., 1984) and v-abl from Abelson murine leukaemia virus transformed cells (Fry et al., 1985). Together, these observations established a strong correlation between PI 3-kinase activity and protein-tyrosine kinases involved in mitogenesis and cell transformation.

The notion that PI 3-kinase is functionally important in cell transformation was strengthened by studies of mutant PTKs. For example, mutants of pp60v-Src (Fukui and Hanafusa, 1989) and variants of the Abl oncogene product (Varticovski et al., 1991) that fail to transform cells were shown not to associate with PI 3-kinase. Conversely, pp60c-Src mutants that cause transformation acquire the ability to associate with and activate PI 3-kinase (Chan et al., 1990). For most of the activated oncogene mutants investigated, the ability of the gene product to associate with PI 3-kinase correlated with the ability to transform fibroblasts. Likewise, the analysis of many polyomavirus middle T (mT) mutants that do not transform cells revealed that these mT proteins were also defective in their ability to associate with PI 3-kinase (Whitman et al., 1985; Kaplan et al., 1986, 1987; Courtneidge and Heber, 1987; Serunian et al., 1990; Uleg et al., 1990). Furthermore, a class of polyoma mT mutants were found which associated with PI 3-kinase but failed to transform cells and were subsequently shown to be unable to elevate the levels of cellular 3-phosphoinositides (Druker et al., 1992; Ling et al., 1992).

1.2.1.1.2 Interaction with receptor PTKs

PI 3-kinase, a cytosolic protein in resting cells, was the first enzyme shown to be recruited to a receptor tyrosine kinase in a ligand dependent manner. PI 3-kinase activity was found in complex with the PDGF receptor within one minute following PDGF stimulation of cells (Kaplan et al., 1987; Whitman et al., 1987). PI 3-kinase activity was subsequently shown to coimmunoprecipitate with a number of activated PTKs including the receptors for CSF-1 (Varticovski et al., 1989), EGF (Bjorge et al., 1990), insulin (Endermann et al., 1990; Rudermannn et al., 1990), IGF-1 (Kappeller et al., 1991), NGF (Soltoff et al., 1992), HGF (Graziani et al., 1991) c-kit (Lev et al., 1991; Rottapel et al., 1991) and C-erbB2 (Peles et al., 1992).

PI 3-kinase was first identified as an 85 kDa phosphoprotein using SDS-PAGE gels for analysis. This phosphate labelled protein correlated with the appearance of a PI 3-kinase activity in immunoprecipitates of a number of polyoma mT mutants (Courtneidge and Heber, 1987; Kaplan et al., 1987). The same protein was also shown to appear in parallel with PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates of PDGF stimulated cells and to copurify with the PDGF receptor (Kaplan et al., 1987). Phosphoamino acid analysis revealed that the 85 kDa protein was phosphorylated on both serine and tyrosine residues in polyoma middle T-transformed and PDGF-stimulated cells (Kaplan et al., 1987; Cohen et al., 1990). This protein was also shown to be a direct substrate of pp60c-Src and the PDGF receptor. These studies paved the way for the purification of the enzyme, followed by its cloning and subsequent expression. The data described in this thesis represent a functional analysis of the PI 3-kinase. Thus, data from other labs

addressing the regulation of this enzyme are reviewed in the relevant Discussion Chapters.

1.2.1.2 Phospholipase C

PLC hydrolyses PI(4,5)P₂, which can also serve as a substrate for PI 3-kinase, to produce two second messengers, IP₃ and DAG (Figure 1.3). At least five distinct proteins with PLC activity have been identified and cloned PLC α , PLC β 1, PLC γ 1, PLC γ 2 and PLC δ 1. These isoforms differ in their tissue distribution, substrate specificity and calcium and pH dependency (Suh et al., 1988). PLC β 1 is subject to regulation by G-protein coupled receptors whereas PLC γ 1 is widely expressed and has been shown to be regulated by PTKs (Rhee and Choi, 1992).

1.2.1.2.1 Interaction with receptor PTKs.

PLCy1 was first identified as a substrate for tyrosine phosphorylation in EGF- and PDGFβ- treated cells (Wahl et al., 1989, 1989a; Meisenhelder et al., 1989; Margolis et al., 1989a). The phosphorylation of PLCy was observed within one minute following ligand stimulation of cells and declined thereafter (Margolis et al. 1989a; Peles et al., 1991; Ohmichi et al., 1992; Soler etal., 1993). However, only 1% of the cellular PLCγ molecules were found in association with activated receptor (Margolis et al 1989; Peles et al., Morrison et al., 1990). Ligand stimulated EGF and PDGF receptors were found to coimmunoprecipitate with PLCy, suggesting a tight physical association (Margolis et al., 1989a; 1990a; Morrison et al., 1990). Dimerisation of the receptor was shown to be insufficient to induce association of the EGF receptor with PLCy, since a kinase-negative mutant receptor that underwent normal dimerisation in response to EGF did not associate with PLCy (Margolis et al., 1990). A requirement for kinase activation was demonstrated, since PLCy was found not to associate with a mutant PDGF receptor defective in autophosphorylation (Morrison et al., 1990). Further, cross-phosphorylation of kinase-negative receptor with an active receptor allowed PLCy binding (Margolis et al., 1990). This suggests that tyrosine phosphorylation of the receptor was promoting the binding of PLCy. The site of interaction was localised to the carboxy-terminal tail of both the EGF and PDGF receptors since mutant receptors containing deletions in this region were unable to bind PLCy (Margolis et al., 1990; Seedorf et al., 1992; Valius et al. 1992; Zhu et al., 1992). Moreover, fusion proteins of the PDGF receptor C-terminal tail (Valius et al., 1992), and a cyanogen bromide fragment containing the EGF receptor Cterminal tail (Margolis et al., 1990) were both found to form stable complexes with PLCy, provided the C-terminal tail had been tyrosine phosphorylated. Site-directed mutagenesis was used to define Y1021 of the PDGF receptor and Y992 of the EGF receptor as mediators of binding to the SH2 domains of PLCy (Vega et al., 1992; Ronnenstad et al. 1992; Zhu et al., 1992; Valius et al., 1993). Further, a study examining the relative

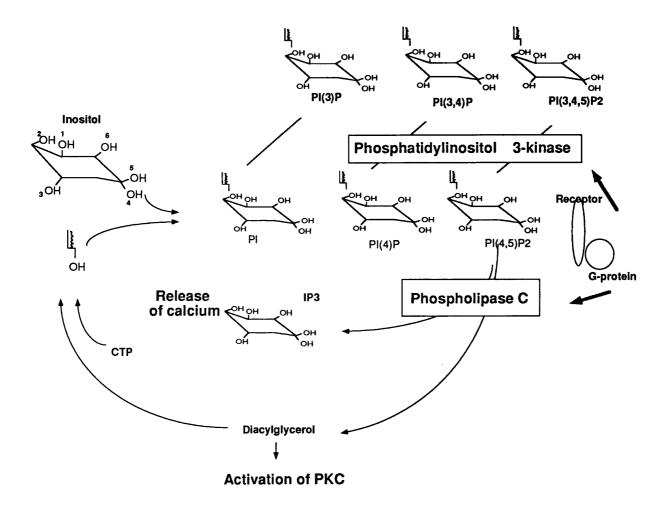


Figure 1.3 The regulation of PI in agonist stimulated cells

protection from dephosphorylation by phosphatases conferred by the SH2 domain of PLCγ binding to autophosphorylation sites, implicated Y992 in high affinity binding, while other phosphotyrosine sequences displayed lower affinity interactions (Rotin et al, 1992).

PLCy has also been found to interact with the activated NGF receptor (Vetter et al., 1991; Ohmichi et al., 1991; 1992) with 100-fold higher affinity than with the activated EGF receptor (Obermeier et al., 1993). Deletion of the C-terminal tail of the NGF receptor was shown to abrogate the binding of PLCy. Moreover, mutation of the autophosphorylation site Y785 specifically diminished binding of PLCy to the NGF receptor and a tyrosine phosphorylated synthetic C-terminal peptide was shown to competitively inhibit association (Obermeier et al., 1993). The FGF receptor has also been shown to specifically bind PLCy via the autophosphorylation site, Y766, in its C-terminal tail (Peters et al., 1992; Mohammadi et al., 1992). Comparison of the PLCy binding sites on these receptors reveals the consensus binding sequence YpL/I/VXP/V/L. Other receptors that have been shown to interact with PLCy include c-erbB2, albeit with a lower affinity than with the PDGF receptor (Fazioli et al., 1991; Segatto et al., 1992), and the HGF receptor using recombinant components in vitro (Bardelli et al., 1992). There are conflicting reports on the association of the c-kit receptor with PLCy. A low affinity association has been described in mast cells (Herbst et al. 1991; Rottapel et al., 1991; Reith et al., 1991), which has been disputed by Peles et al. (1991). Neither insulin (Nishibe et al., 1990; Lavan et al., 1992) nor CSF-1 (Downing et al., 1989) induce complex formation between activated receptors and PLCy and, interestingly, neither contains the consensus motif neccessary for binding.

1.2.1.2.2 Biological function

The observation that phorbol esters that activate protein kinase C (PKC) are mitogens and that inhibitors of PKC can block the activation of DNA synthesis support the possibility that activation of PLCγ, and the subsequent activation of PKC, are important prerequisites for the transmission of a biological signal. The importance of PI hydrolysis in PDGF mediated mitogenesis has been demonstrated using microinjection experiments. Antibodies to the PLC substrate PI(4,5)P₂ were shown to inhibit both PI turnover and PDGF induced cell proliferation (Matuoka et al., 1988). Further, a transforming version of Neu was found to be constitutively associated with PLCγ and mediate its tyrosine phosphorylation (Peles et al., 1991). In accord with these studies, mutants of the PDGF receptor (Y1021F) and EGF receptor (Y992F) that failed to associate with PLCγ were unable to mediate PDGF or EGF dependent production of inositol phosphates and the mitogenic response of these receptor mutants in dog epithelial TRMP cells was diminished by 30% (Valius et al., 1993).

In spite of these results the physiological relevance of these interactions is unclear as several lines of evidence suggest that activation of the PLCy metabolic signalling pathway may not be necessary for PDGF induced mitogenic signalling. Thus, overexpression of PLCy in NIH 3T3 cells, led to enhanced PDGF-induced generation of IP₃, but did not enhance intracellular calcium signalling nor influence PDGF-induced DNA synthesis (Margolis et al., 1990a; Cuadrado et al., 1990). PDGF has also been shown to initiate DNA synthesis in the absence of detectable PLCy activation in C3H10T1/2 mouse fibroblasts (Hill et al., 1990). A PDGF receptor mutant which retained the binding site for PLCy, but lacked binding sites for PI 3-kinase, RasGAP and the Syp phosphatase through mutatagenesis, was still able to elicit a mitogenic response in a hepatoma HepG2 cell line (Valius and Kazlauskas, 1993). Moreover, mutant PDGF receptors (Y1021F) specifically defective in binding PLCy were able to induce a mitogenic response in porcine aortic endothelial (PAE) (Ronnstrand et al., 1992) as well as in NIH 3T3 cells (Seedorf et al., 1992) and could activate Ras in the pro-B cell line BaF3 (Satoh et al., 1993). Consistent with these results, single point mutations of the PLCy association site on the NGF receptor (Y766) resulted in total loss of PLCymediated responses but had no effect on mitogenesis in L6 myoblasts (Peles et al., 1992; Mohammadi et al., 1992). Thus, these results suggest that while activation of PLCy is important for IP3 production, PLCy alone is probably not a prerequisite for the initiation of a mitogenic response.

1.2.2 Protein kinases/phosphatases

1.2.2.1 Non-receptor Src-family protein tyrosine kinases

pp60^{c-Src} is a membrane associated phosphoprotein with intrinsic protein-tyrosine kinase activity. There are currently nine members of the Src gene family which include Fyn, Lyn, Yes, Lck, Blk, Fgr, Hck and Yrk (reviewed in Cooper, 1990; Sudol et al., 1993). A number of these proteins are restricted in their cell and tissue expression. For example Lck in T-lymphocytes and Fgr in monocytes while Src, Fyn and Yes are more widely expressed in different cell types (Cooper et al., 1990). This family of kinases are structurally similar over all but the first 60-80 amino acids of their total length of 500-530 residues. The conserved regions can be divided into five sequence blocks. From the N-terminus these are the extreme N-terminal myristylation signal, the SH3 and SH2 regions, the kinase domain and the C-terminal non-catalytic tail.

1.2.2.1.1 Regulation of Src activity

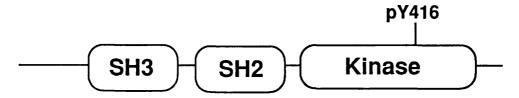
pp60^{c-Src} autophosphorylates at Y416 contained within the kinase domain and is phosphorylated in vivo at a tyrosine residue in the C-terminal tail (Y527) (Courtneidge et al., 1985; Cooper et al., 1986). The oncogenic form pp60^{v-Src} lacks this tyrosine residue

and mutation to a phenylalanine increases the protein-tyrosine kinase activity and the transforming potential of pp60^{c-Src} (Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987). Concomitant with either the lack of or underphosphorylation of Y527 is hyperphosphorylation of Y416 in the catalytic domain (reviewed by Jove and Hanafusa, 1987). In vitro experiments show that C-terminal tail phosphorylation is inhibitory whereas kinase domain phosphorylation is stimulatory for the tyrosine kinase activity (Kmiecik et al., 1988). As the region containing the C-terminal tyrosine is protected from proteases it is probably bound to an internal site in Src (Cantley et al., 1991). Genetic and biochemical data suggest that the inhibition of c-Src kinase activity, induced by phosphorylation of Y527 (Courtneidge et al., 1985; Cooper et al., 1986), requires a physical interaction between the SH2 domain and the tyrosine phosphorylated tail (O'Brien et al., 1990; Hirai and Varius; 1990a, 1990b; Liu et al., 1993; Murphy et al., 1993; Amrein et al., 1993; Gervais et al., 1993). This interaction appears to be of low affinity and this may be reversed by dephosphorylation of Y527 or by intermolecular engagement of the intramolecularly bound SH2 domain. Thus, the maintenance of pp60^{c-Src} as an inactive kinase requires C-terminal phosphorylation which is specifically mediated by the Csk (C-terminal Src kinase) PTK (Figure 1.4) (Okada et al., 1991; Bergman et al., 1992). When overexpressed, Csk inhibits transformation of fibroblasts induced by high levels of Src activity (Sabe et al., 1992). Mutant mice lacking the csk gene die at the neurulation stage, and cell lines established from these mice have increased phosphotyrosine levels and increased activity of Src family kinases (Imamoto and Soriano, 1993; Nada et al., 1993).

1.2.2.1.2 Interaction of Src with receptor PTKs

Although the Src family kinases are intracellular and have no extracellular domain which could directly bind to activators such as growth factors, there is evidence that these kinases are activated by various ligands. pp60^{c-Src} was first found to be phosphorylated when quiescent cells were stimulated with PDGF (Gould and Hunter, 1988). Subsequently, pp60^{c-Src}, pp59^{c-Fyn} and pp62^{c-Yes} were observed to transiently associate with the activated PDGFβ receptor (Kypta et al., 1990). The proteins were then shown to bind directly to the PDGF receptor cytoplasmic domain and this was followed by their phosphorylation and enzymatic activation (Kypta et al., 1990; Twamley et al., 1992). Approximately 5% of the activated receptors were shown to be associated with approximately 5% of the total pool of Src present (Kypta et al., 1990). Essentially the same results have been observed in analyses with the CSF-1 receptor binding of pp60^{c-Src}, pp59^{c-Fyn} and pp62^{c-Yes} which occurs upon CSF-1 stimulation (Courtneidge et al., 1993). Further, this interaction has been shown to take place in insect cells using recombinant components and was shown to be mediated by the SH2 domain

A. Src active



B. Src inactive

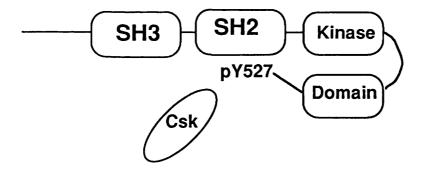


Figure 1.4 Autoregulation of Src.
The binding of the Src SH2 domain to its non-catalytic tail maintains Src in an inactive state. The Csk kinase specifically phosphorylates Y527 in the tail of Src to allow this interaction to occur efficiently.
Activation of Src results in hyperphosphorylation of Y416 in the kinase domain, concomitant with under phosphorylation of Y527.

of pp59c-Fyn. The activated HGF receptor has also been shown to bind pp59c-Fyn in vitro (Bardelli et al., 1992), although such an association was not observed with activated EGF receptor (Courtneidge et al., 1993). The site of interaction of pp60c-Src with the PDGF receptor lies within the juxtamembrane region and was mapped to the autophosphorylation residues Y579 and Y581 using site-directed mutagenesis (Mori et al., 1993). This was substantiated with in vitro experiments through the analysis of the binding of pp60^{c-Src} to phosphopeptides containing these specific tyrosine motifs (Mori et al., 1993). In addition, mutation of Y559 on the CSF-1 receptor, corresponding to Y579 on the PDGF receptor, impairs association with the Src family kinases (unpublished data in Courtneidge et al., 1993). There is evidence to support the notion that pp60c-Src, pp59c-Fyn and pp62c-Yes all bind the same site on the activated PDGF receptor (Kypta et al., 1990; Twamley et al., 1992). A 5-10 fold overexpression of pp59c-Fyn has been shown to result in a reduced association of pp60c-Src and pp62c-Yes with the PDGF receptor (Kypta et al., 1990). In addition, targeted disruption of the c-Yes gene alone or c-Src gene alone in mice does not produce an abnormal phenotype (Soriano et al., 1991), however, simultaneous disruption of c-Src and c-Yes is lethal. This suggests that there is redundancy in c-Src function and c-Yes may commensurate for its loss (unpublished data from Taylor et al., 1993).

1.2.2.1.3. Biological function

The Src family kinases also interact directly with a number of intracellular signalling molecules. These include PI 3-kinase (Sugimoto et al., 1984; Whitman et al., 1985; Pleiman et al., 1993; Taichman et al., 1993; Vogel et al., 1993a), RasGAP (Ellis et al., 1990; Brott et al., 1991), Raf-1 (Morrison et al., 1988), PLCy (Nakanishi et al., 1993), Nck (Meisenheider and Hunter, 1992; Chou et al., 1992) and Shc (McGlade et al., 1992a). A requirement for the Src family kinases in mitogenesis has been demonstrated in vivo. This was shown by microinjection of DNA encoding catalytically inactive forms of pp60c-Src and pp59c-Fyn proteins which inhibited PDGF-stimulated entry of cells into S-phase (Twamley et al., 1993). In addition, deletion of the src gene in osteoclasts results in cells unable to resorb bone efficiently and thus osteopetrosis (Soriano et al., 1991). The op (osteopetrosis) mutation also causes osteopetrosis and is contained in the gene encoding CSF-1. This suggests that CSF-1 may be required for differentiation of mature osteoclasts acting through the CSF-1 receptor which binds and activates pp60c-Src (Courtneidge et al., 1993). This may explain the requirement for Src activity in these cells. Experiments involving mutation of the Src binding sites on the PDGF receptor will shed further light on the requirement for Src in mitogenesis.

1.2.2.2 Raf

The c-Raf gene was first identified as the normal cellular counterpart of v-Raf, the transforming gene of murine sarcoma virus 3611 (Rapp et al., 1988). The protein product of the c-Raf gene, designated Raf-1, is a 70-75 kDa phosphoprotein which has intrinsic serine/threonine kinase activity. Sequence analysis of the protein suggests a two-domain structure with a kinase domain occupying the carboxyl terminal of the molecule and a potential regulatory domain making up the remainder of the protein. The regulatory domain contains two highly conserved regions. CR1 is made up of a cysteine-rich region which is thought to be involved in binding zinc ions whose structure is related to the regulatory domains of PKC which bind DAG; CR2 is a serine/threonine rich region which becomes phosphorylated concomitant with Raf-1 activation (Heidecker et al., 1992). The integrity of this N- terminal region is crucial for normal regulation of Raf-1 activity. The v-Raf protein is essentially a deletion of the regulatory amino terminal half of the Raf-1 molecule. A variety of amino terminal deletions of Raf-1 generated in vitro are also transforming (Wasylyk et al., 1989; Heidecker et al., 1990). While the c-Raf-1 gene is ubiquitously expressed, two related genes A-Raf and B-Raf are found to exhibit tissue specific expression (Stephens et al., 1992).

1.2.2.2.1 Biological function

There is much evidence for the involvement of Raf-1 in mitogenic signal transduction pathways. Firstly, Raf-1 activated by mutation induces transcriptional transactivation (Jamal et al., 1990; Wasylyk et al., 1989), DNA synthesis (Smith et al., 1990) and cellular transformation (Rapp et al., 1983; Heidecker et al., 1990). Expression of the activated Raf-1 oncogene in PC12 cells results in neuronal differentiation (Wood et al., 1993). In addition, the activation of many growth factor receptors results in the rapid hyperphosphorylation of the Raf-1 kinase.

1.2.2.2.2 Interaction of Raf with receptor PTKs.

Raf kinases are inactive in quiescent cells. Increased phosphorylation on serine and threonine residues has been observed in a number of independent studies upon stimulation of cells by EGF (Morrison et al., 1988; App et al., 1991; Oshima et al., 1991), CSF-1 (Baccarini et al., 1990), NGF (Oshima et al., 1991; Stephens et al., 1992), FGF [acidic] (Morrison et al., 1988), insulin (Blackshear et al., 1990; Izumi et al., 1991), steel growth factor (Lev et al., 1991) and PDGF (Morrison et al., 1989; Baccarini et al., 1990). Only a small fraction (~1%) of the total pool of Raf-1 becomes phosphorylated (App et al., 1991), coincident with a 2-6 fold increase in activity of the Raf kinase (Blackshear et al., 1990; Izumi et al., 1991; Oshima et al., 1991; App et al., 1991; Stephens et al., 1992). Phosphorylation of the Raf protein has been observed within 2 minutes and shown to reach a maximum by 10-15 minutes, with the Raf protein remaining phosphorylated

above the basal level for up to two hours (Baccarini et al., 1990; Blackshear et al, 1990; App et al., 1991; Oshima et al. 1991).

The Raf-1 protein has been shown to coimmunoprecipitate with overexpressed EGF receptor (App et al., 1991), moreover, an EGF receptor point mutant lacking PTK activity was found to be inactive in binding to Raf-1 (App et al., 1991). A direct interaction of the Raf-1 protein and a PTK has also been observed with the PDGFβ receptor (Morrison et al., 1989). This association was demonstrated in vivo by immunoprecipitation and in vitro using purified recombinant components. This interaction, like that with the EGF receptor, was shown to require an active receptor kinase both in vivo and in vitro (Morrison et al., 1989). It should be noted that Raf-1 does not contain SH2 domains, but is still observed to bind receptors in a phosphotyrosine dependent manner. This suggests the possible presence of an unidentified adapter protein which may mediate the interaction between Raf-1 and the phosphorylated receptor.

1.2.2.3 Protein-tyrosine phosphatases

Protein tyrosine phosphorylation appears to be the principal biochemical mechanism by which growth factors and hormones regulate the steps that lead to cellular proliferation. Activated growth factor receptors stimulate intracellular signalling pathways by physically associating with and/or phosphorylating downstream regulatory cytoplasmic proteins. In normal cells the levels of tyrosine phosphorylation and thus the extent and duration of the signals transmitted must also be regulated by the presence of protein tyrosine phosphatases (PTPases) which remove phosphate from phosphotyrosine residues.

A large number of PTPases have been identified through molecular cloning and like the PTK family they too form a family with diverse structures and a conserved phosphatase domain. PTPases are conserved in evolution from bacteria to mammals (Reviewed in Fischer et al., 1991). Amino acid sequence analysis of cDNA clones defines conserved regions within the phosphatase domain interspersed by other regions that are more divergent. Mammalian PTPases are divided into intracellular and transmembrane classes. Transmembrane PTPases such as CD45 (Charbonneu et al., 1988) and leukocyte antigenrelated (LAR) PTPase (Streuli et al., 1988), with intracellular catalytic domains and distinct extracellular domains resemble in structural organisation the transmembrane tyrosine kinases such as the EGF receptor; these receptor-like PTPases may be capable of initiating transmembrane signalling in response to as yet unidentified ligands, presumably by modulating their PTPases activity. Further insights into the regulation and function of intracellular PTPases came when activated growth factor receptors were found to coimmunoprecipitate with SH2 domain-containing phosphatases termed SHPTP1 (Yi et

al., 1993) and SHPTP2 (Feng et al., 1993; Lechleider et al., 1993a; Vogel et al., 1993). Amino acid sequence analysis of SHPTP1 revealed significant homology with the gene product of the *Drosophila* corkscrew (*csw*) locus (Perkins et al., 1992). This gene is thought to act downstream of the PDGF receptor-like torso and in concert with the *Drosophila* c-Raf homologue, D-Raf (Ambrosio et al., 1989) to positively transduce signals generated by the torso receptor PTK (Casanova et al., 1989; Sprenger et al., 1989).

1.2.2.3.1 Association with receptor PTKs

SHPTP1 (PTP1C/HCP/SHP) is expressed primarily in haematopoietic cells and at levels ~fifty fold lower in some epithelial cells (Yi et al., 1993). It has been shown that SHPTP1 becomes phosphorylated on tyrosine, and transiently associates with, ligand activated c-kit (Yi et al., 1993) or a chimeric Neu under the control of the EGF receptor external domain (Vogel et al., 1993) by its SH2 domain. In addition, the CSF-1 receptor was observed to induce tyrosine phosphorylation of the SHPTP1 protein in stimulated macrophages (Yeung et al., 1992). Interestingly, the SHPTP1 gene has been mapped to region p12-13 of chromosome 12, which is a location associated with rearrangement in ~10% of cases of acute lympocytic leukemia in children.

The SHPTP2 phosphatase (also called PTP1D/SYP/PTP2C) is ubiquitously expressed. It can be coimmunoprecipitated with the EGF and PDGF β receptors from ligand stimulated cells (Feng et al., 1993; Lechleider et al., 1993a; Vogel et al., 1993) and has also been found in association with the c-kit receptor and a chimeric Neu under the control of the EGF receptor external domain (Vogel et al., 1993). In addition, upon insulin stimulation, SHPTP2 binds to a tyrosyl-phosphorylated 185 kDa protein called the insulin receptor substrate-1 [IRS-1] (White et al., 1985), both in vivo (Kuhne et al., 1993) and in vitro (Lechleider et al., 1993). A phosphopeptide containing Y1172 and surrounding residues of IRS-1 was subsequently identified as a high affinity binding site for the SHPTP2 protein in vivo (Sun et al., 1993).

Using a number of PDGF receptors with phosphorylation site mutants the major site of interaction for the SHPTP2 protein and its SH2 domain was identified as Y1009 (Valius et al., 1993; Lechleider et al., 1993b; Kaklauskas et al., 1993). Further, a phosphopeptide comprising this tyrosine and surrounding residues were able to compete for binding. In addition, the YLRV sequence on the EGF receptor (communicated in Pawson and Schlessinger, 1993) has been suggested as a site which mediates its binding to SHPTP2. Comparison of the residues surrounding the phosphotyrosine suggest the consensus sequence pYV/L/IXV/L/I for high affinity binding.

An unidentified phosphotyrosine phosphatase has also been shown to be functionally coupled with the activated HGF receptor (Moruzzi et al., 1993). However, whether this represents another member of the SHPTP family of phosphatases remains to be determined. Thus, receptor bound phosphatases may act as potential regulators of PTK activity by dephosphorylating and thereby downregulating either the receptor with which they are associated or other receptor bound signalling molecules.

1.2.2.3.2 Biological function

SHPTP2 has been shown not to be necessary for relay of a mitogenic response when assayed using a Y1009 mutant PDGF receptor (Valius et al., 1993; Ronnenstrand et al., 1992), although it appeared to be required for the activation of Ras in response to PDGF in TRMP cells (Discussed further in Section 1.62, Valius and Kazlauskas-cell, 1993; Li et al.,1994). However, a function has been suggested for the SHPTP1 protein following the identification of its encoding mammalian gene, hcph. Mice homozygous for loss-of-function mutations in hcph have a phenotype termed motheaten, in which monocyte/macrophages and erythroid cells are overproduced (Tsui et al., 1993; Shultz et al., 1993). Motheaten mice also have defects in lymphoid development, possess elevated levels of CD5+ autoreactive B-cells, and as heterozygotes are prone to develop lymphomas. These results raise the possibility that SHPTP1 serves to limit tyrosine kinase mitogenic signalling, and as such may function as a tumour suppressor gene.

1.2.3 Regulators of G-protein function

1.2.3.1 GTPase activating protein

It is well established that mitogenesis induced by the activation of receptor PTKs requires the P²¹Ras (Ras) protein (Feig and Cooper, 1988, Discussed further in section 1.6.2). Ras is a guanine nucleotide binding membrane protein which possesses a weak intrinsic GTPase activity and cycles between an active GTP-Ras form and inactive GDP-Ras form (Figure 1.5). RasGAP is one potential target protein that can stimulate the intrinsic GTPase activity of normal Ras proteins to convert them to their inactive GDP bound form. There is evidence suggesting that RasGAP functions as a negative regulator of Ras and may thus be important for regulating signalling responses in stimulated cells (Discussed further in section 1.6.3.1). For example, RasGAP is unable to bind and downregulate oncogenic Ras proteins and thus as a result oncogenic Ras is hyperactive and constitutively in the GTP bound form (Trahey and McCormick, 1989). In addition,

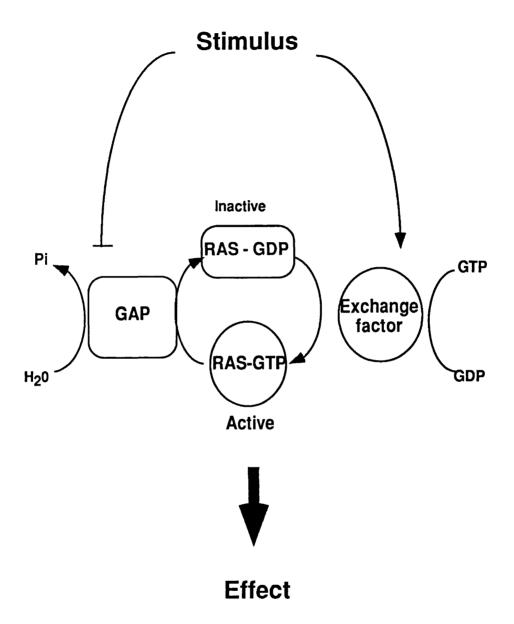


Figure 1.5 Cycling of p21Ras between GTP and GDP bound states

overexpression of full length RasGAP or its catalytic domain has been shown to increase the fraction of cellular Ras in the inactive GDP-bound state and result in morphological reversion of v-Src transformed cells (Declue et al., 1991, 1991a).

1.2.3.1.1 Interaction of GAP with receptor PTKs

RasGAP is a 120 kDa protein that is ubiquitously expressed (Trahey and McCormick, 1989; Gibbs et al., 1988). The regulatory activity of RasGAP has been localised to its C-terminal region (Marshall et al., 1989) which has also been shown to interact with the effector region of Ras (Marshall et al., 1989). RasGAP is predominantly located in the cytosol, however stimulation of cells with PDGF or EGF can induce rapid phosphorylation of RasGAP and its translocation to the cell membrane (Molloy et al., 1989; Kazlauskas et al., 1990; Ellis et al., 1990). Approximately 10 % of the total RasGAP molecules were found to complex directly with PDGF receptor inducing tyrosine phosphorylation of GAP within one minute of exposure to ligand, a level which declined thereafter (Molloy et al., 1989; Kazlauskas et al., 1990). Complex formation between the activated EGF receptor and RasGAP was found to be a process having a 10 fold lower affinity than that observed with the PDGF receptor and also more transient (Serth et al., 1992; Soler et al. 1993).

The interaction between RasGAP and an activated receptor could be reconstituted in vitro using purified components (Kaplan et al., 1990) and was shown to require a kinase-active receptor (Kazlauskas et al., 1990; Kaplan et al., 1990). Using mutant receptors the kinase-insert of the PDGF receptor (Kaplan et al., 1990) and the C-terminal tail of the EGF receptor (Margolis et al., 1990; Soler et al., 1993) were found to mediate the interaction with RasGAP. Mutagenesis further identified Y771 as the specific site of interaction on the human PDGFB receptor for GAP in vivo (Kashishian et al., 1992) and in vitro (Kazlauskas et al., 1992). Moreover, using a number of phosphotyrosine containing peptides derived from the PDGF receptor, a phosphopeptide containing Y771 was identified as the binding site for the SH2 domain of RasGAP. This phosphopeptide was able to block association in competition assays (Fantl et al., 1992). The binding site for RasGAP on the EGF receptor was mapped using a series of deletion mutants. The association was found not to be mediated by a unique autophosphorylation site but by multiple perhaps compensatory sites. An EGF receptor containing a single point mutation at Y1168 exhibited the lowest affinity for binding to RasGAP, and individual point mutants of the four other known autophosphorylation sites displayed lower affinity binding (Soler et al., 1993). Similar experiments have shown that both p85 and PLCy compete for binding to the activated EGF receptor (Wood et al., 1992; Rotin et al., 1992; Zhu et al., 1992), suggesting overlapping substrate-binding sites. In contrast, specific, non-overlapping binding sites in the PDGF receptor have been described for PLCy,

RasGAP and p85 (Fantl et al., 1992; Kazlauskas et al., 1992-embo; Kashishian et al., 1992; Kazlauskas et al., 1993). This highlights a difference between PDGF and EGF induced signalling mechanisms for which an explanation is presently unclear.

RasGAP was not initially identified as a substrate for the insulin receptor (Molloy et al., 1989; Lavan et al., 1992), however, subsequent analyses in the presence of the specific phosphotyrosine inhibitor phenylarsine oxide (PAO) have shown that RasGAP can directly associate with the insulin receptor (Pronk et al., 1993). Similarly, Zhang et al., (1993) showed that RasGAP transiently associates with the insulin receptor and is rapidly released from the complex after its phosphorylation on tyrosine residues. It is intriguing that RasGAP interacts directly with the insulin receptor and not the IRS-1 molecule as do other substrates such as PI 3-kinase (Skolnik et al., 1993; Lavan et al., 1992). The HGF receptor was been shown to interact with RasGAP using recombinant components in vitro (Bardelli et al., 1992). In addition, the RasGAP protein was found to be a poor substrate for the CSF-1R and this interaction did not appear to be mediated by the kinase insert region (Reedjik et al., 1990). There is conflicting evidence as to the existence of an interaction between RasGAP and the NGF receptor (Ohmichi et al., 1991; Obermeier et al., 1993), and neither c-kit nor the FGF receptors have been shown to phosphorylate or associate with RasGAP (Molloy et al., 1989; Rottapel et al., 1991; Reith et al. 1991; Herbst et al., 1992).

1.2.3.1.2 GAP forms a complex with p190 and p62 in stimulated cells

Analysis of RasGAP immune complexes from EGF, CSF-1 or insulin stimulated cells revealed the presence of two phosphoproteins termed p62 and p190 (Ellis et al., 1990; Bouton et al., 1991; Reedjik et al., 1990; Pronk et al., 1993). Cytoplasmic RasGAP was found to be tightly bound to p190 in nearly stoichiometric amounts in mitogenically stimulated cells and cells transformed by activated PTKs. In addition, p190 was phosphorylated mainly on serine residues with very little phosphorylation on tyrosine (Ellis et al., 1990; Boutin et al., 1991; Moran et al., 1991). The activity of RasGAP complexed to p190 was shown to be ~4 fold lower than that of monomeric RasGAP (Moran et al., 1991), suggesting that kinase activation might inhibit RasGAP by promoting its interaction with p190. However in contrast, a stoichiometric interaction between RasGAP and p190 has also been observed independent of ligand stimulation (McGlade et al., 1993). RasGAP associated-p62 is a minor protein that rapidly becomes highly phosphorylated on tyrosine residues upon EGF stimulation of cells (Bouton et al., 1991; Moran et al., 1991). The function of this protein in multimeric RasGAP signalling is unknown, although the phosphorylation of p62 on tyrosine does correlate with the transforming activity of a number of mutants of v-fps and c-abl (Koch et al., 1989; Muller et al., 1991).

The genes encoding p62 and p190 have been cloned (Wong et al., 1992; Settleman et al., 1992). The p62 protein shows homology with a putative hnRNP protein (GRP33) and was found to bind DNA and RNA, suggesting a role in DNA/RNA metabolism (Wong et al., 1992). Moreover, p62 was shown to bind to the SH2 domain of RasGAP in vitro, an interaction mediated by the phosphorylation of tyrosine residues on p62 (Wong et al., 1992). Analysis of the p190 clone revealed three regions of homology to known sequences (Settleman et al., 1992). At the N-terminus there are three sequence motifs indicative of a GTP-binding site in other proteins. The C-terminus contains a region with homology to n-chimaerin and RhoGAP, consistent with the finding that p190 displays GAP activity on Rho family members in vitro (Settleman et al., 1992a). A sequence similarity to the transcriptional repressor of the reported glucocortoid receptor gene (GRF-1) was found in the middle portion of the protein. The physiological significance of these domains remains to be determined but these results do provide a possible mechanistic link between the plasma membrane generated signals and nuclear responses. Indeed, subcellular fractionation studies showed ~25% of the total pool of p190 to be associated with the cell nucleus (Settleman et al., 1992).

1.2.3.1.3 Biological function.

RasGAP does not appear to play an essential role in eliciting a mitogenic response in growth factor stimulated signalling as determined using specific receptor point-mutants (Kazlauskas et al., 1992; Fantl et al., 1992; Valius and Kazlauskas, 1993; Declue et al., 1993; Satoh et al., 1993). However, a most interesting observation was the identification of a domain within neurofibromin, the protein product of the NF1 gene, that has 30% sequence similarity with RasGAP. Expression of this domain reveals that it can act as a RasGAP in vitro (Xu et al., 1990a; Martin et al., 1990). A striking sequence similarity was also observed with the yeast RasGAP homologues IRA1 and IRA2. Further, NF1 has been shown able to complement the loss of IRA function in S. cerevisiae (Xu et al., 1990, 1990a) suggesting that NF1 may be the mammalian homologue of IRA1 and IRA2. Patients with Neurofibromatosis type I lack the NF1 gene and are characterised by the development of benign and sometimes malignant tumours that originate in the neural crest. Cells derived from malignant NF1 tumours express very low levels of neurofibromin (Basu et al., 1992; DeClue et al., 1992). Perhaps as a result Ras accumulates in its GTP-bound state and contributes to the transformed state of the cells through interactions with unknown effectors. Similarly, mutations in IRA1 or IRA2 resulted in phenotypes similar to those found in the activated yeast Ras2^{val-12} [equivalent to activated mammalian Ras^{val} 12] (Tanaka et al., 1989). This identifies NF1 as a tumour suppressor, as its loss of function contributes to tumour growth and has interesting implications for the physiological functions of GAPs in mammalian cells.

1.2.3.2 Vav

Vav was initially detected as an oncogene which was shown to be activated in vitro by replacement of its normal amino terminus with coding sequences from pSV2neo (Katzav et al., 1989). Subsequent studies have shown that truncation of the Vav proto-oncogene activates the transforming capacity of Vav (Coppolla et al., 1991; Katzav et al., 1991). Overexpression of the full-length Vav in fibroblasts is also weakly transforming (Coppolla et al., 1991; Katzav et al., 1991). Mutagenesis of cysteine residues within the protein kinase C-like zinc finger/phorbol ester binding site abrogates transforming activity, suggesting that this region is essential for the oncogenic activity of Vav (Coppolla et al., 1991). Antibodies to the Vav protein recognise a 95 kDa protein which is found expressed exclusively in the haematopoietic cell lineages (Katkav et al., 1989; Adam et al., 1992).

1.2.3.2.1 Interaction of Vav with receptor PTKs

Vav has been found to be constitutively phosphorylated on tyrosine residues in murine macrophages and fibroblasts which ectopically overexpress human Vav (Margolis et al.. 1992; Bustelo et al., 1992). Truncated, oncogenenically activated Vav is also tyrosine phosphorylated (Hu et al., 1993a). Tyrosine phosphorylation was shown to be enhanced in fibroblasts by treatment of cells with EGF or PDGF (Bustelo et al., 1992). In addition, the rate of Vav phosphorylation in stimulated cells has been shown to parallel that of the receptor itself, reaching maximal levels within one minute after the addition of ligand (Bustelo et al., 1992). Further, direct complex formation between Vav and the activated PDGF and EGF receptors was demonstrated both in vivo and in vitro and shown to be mediated by the Vav SH2 domain (Margolis et al., 1992; Bustelo et al., 1992). Tyrosine phosphorylated Vav has also been found in association with the activated c-kit receptor in mast cells and the activated HGF receptor in haemopoietic cells (Alai et al., 1992). However, tyrosine phosphorylation of Vav induced by CSF-1 was not detected in murine macrophage cells expressing the CSF-1 receptor (Margolis et al., 1992), nor by NGF in PC12 cells (Suen et al., 1993).

1.2.3.2.2 Vav is an exchange factor

The Vav protein has been found to contain a guanine nucleotide exchange motif (Adams et al., 1992). Guanine nucleotide exchange or releasing factors (GNRF/GRF) are responsible for stimulating the rate of guanine nucleotide exchange on Ras (Reviewed in Downward; 1992; Boguski and McCormick, 1994) causing release of GDP and uptake of GTP, a process which leads to the activation of Ras. The exchange motif in Vav displays significant sequence similarity to the CDC24 GNRF of *S. cerevisiae*, the Bcr breakpoint cluster region protein, RasGRF and the dbl oncoprotein which has guanine nucleotide exchange activity on CDC42Hs (related to the Ras-like proteins Rac and Rho; Hart et al.,

1991) (Adam et al., 1992). Indeed, Vav immunoprecipitates from T-cell lysates were found to contain a GRF activity that increased after stimulation of the T-cell antigen receptor. Further, the Vav-GRF domain was found to be active in vitro. Tyrosine phosphorylation of Vav was found to parallel GRF after TCR-CD3 ligation in Jurkat cells. In addition, Lck was also shown to be present in these immunoprecipitates (Gulbins et al., 1993). Thus, the presence of an active GRF domain in Vav and its selective expression in haematopoietic cells suggests that Vav may represent a haematopoietic cell specific GRF, by analogy with RasGRF which is expressed only in the brain (Shou et al., 1992). Further, purified Vav was found to display GRF activity against Ras in vitro (Gulbins et al., 1994) and transformed NIH 3T3 cells have been found to contain ~10 fold more GRF activity than control cells suggesting that an unregulated GRF activity of Vav may be responsible for cellular transformation.

1.2.4 Transcription factors

1.2.4.1 p91

An interesting development in the study of signal transducing mechanisms came with the finding that a transcription factor, termed p91, is activated by EGF stimulation of cells and concomitantly translocated to the nucleus. This data demonstrated a direct connection existed between growth factor receptor tyrosine kinases at the membrane and gene transcription at the nucleus.

The p91 protein was originally defined as a component of a family of proteins that serve as DNA binding and transcription activating factors (Schindler et al., 1992). Treatment of responsive cells with interferon- α (IFN- α) was shown to induce the formation of a tyrosine phosphorylated multimeric complex consisting of three SH2 domain-containing protein subunits p91, p84 and p113. This complex was shown to be translocated into the nucleus upon ligand stimulation of cells where it combined with a 48 kDa subunit to form a complex termed ISGF-3 α . ISGF-3 α is a DNA binding complex specific for the IFNstimulated response element (ISRE) present in the promotors of IFN- α -responsive genes (Fu et al., 1990; David et al., 1993; Gutch et al., 1992). IFN-γ is known to act through a similar mechanism, although the factor responsible, IFN-y activation factor (GAF), consists solely of the 91 kDa subunit of ISGF-3 (Shuai et al., 1992; 1993a). PDGF and EGF stimulation of cells is known to activate a nuclear DNA binding factor which is termed the SIS-inducing factor (SIF) (Wagner et al., 1990). SIF can bind a regulatory element of the c-fos gene promotor termed the SIS-inducible element (SIE) (Wagner etal., 1990; Sadowski et al., 1993). Thus SIE is sufficient to confer PDGF responsiveness to the *c-fos* gene promotor (Wagner et al; 1990). A number of elegant experiments showed p91 to be a necessary component of the SIF complex (Fu et al., 1993;

Silvennonien et al., 1993; Sadowski et al., 1993; Larner et al., 1993; Ruff-Jamison et al., 1993a; Shuai et al., 1993a).

1.2.4.1.1 Interaction of p91 with receptor PTKs

EGF stimulation of cells was found to cause tyrosine phosphorylation of p91 within one minute of exposure to ligand, and the level of p91 phosphorylation was shown to be maintained for up to an hour (Fu et al., 1993; Silvennonien et al., 1993; Sadowski et al., 1993; Ruff-Jamison et al., 1993a). Concomitant with tyrosine phosphorylation of p91 was the rapid translocation of p91 to the nucleus (Fu et al., 1993; Sadowski et al., 1993; Ruff-Jamison et al., 1993a). Further the activated PTK receptor was found to coimmunoprecipitate with p91, suggesting a tight physical association of the protein with receptor (Fu et al., 1993). A requirement for tyrosine phosphorylation of the receptor was demonstrated using a mutant EGF receptor devoid of the C-terminal tail and another in which all the autophosphorylation sites had been mutated to phenylalanine (Silvennonien et al., 1993). In this case only cells expressing the wild type receptor showed stimulation of p91 in cells responsive to EGF. Indeed, antiphosphotyrosine antibodies were found to inhibit the activation of p91 in stimulated cells (Silvennonien et al., 1993). Consistent with these data, the SH2 domain of p91 was shown to bind the phosphorylated EGF receptor (Fu et al., 1993; Ruff-Jamison et al., 1993a) and EGF and PDGF activated p91 has been shown to stimulate SIE dependent transcription in vitro (Fu et al., 1993; Sadowski et al., 1993a). All these results clearly demonstrate the requirement of receptor autophosphorylation for binding to and activation of p91, which is then translocated to the nucleus to form an active transcriptional complex with a nuclear DNA-binding factor. Similar results have been obtained by stimulation of cells with PDGF, CSF-1 and GM-CSF (Silvennonien et al., 1993; Larner et al., 1993), but not for insulin or the FGF receptor (Silvennonien et al., 1993).

1.2.3.2.2 Eps8

Also of interest was the cloning of a protein termed eps8, that coimmunoprecipitated with the activated EGFR, and was found to contain a putative nuclear targeting sequence and no SH2 domains (Fazzioli et al., 1993). Although eps8 was distinctly phosphorylated on tyrosine following stimulation with EGF, FGF and CSF-1 and was found in direct association with the EGF receptor, a phosphotyrosine requirement for eps8 binding with receptor has not been clearly demonstrated. However, overexpression of eps8 in cell lines resulted in increased mitogenic response to EGF, implicating the EPS8 gene product in the control of mitogenic signals.

1.2.5 Non-catalytic adapter proteins

This class of proteins that have been identified as substrates for activated receptor PTKs lack any known intrinsic catalytic activity and consist almost entirely of non-catalytic SH2 and SH3 domains. These proteins include Grb2, Shc, Crk, Nck, and the p85 protein for PI 3-kinase and serve as adapters by coupling PTKs to downstream targets that the receptors themselves are unable to bind directly. For example the p85 protein is the regulatory subunit for PI 3-kinase (see Chapters 3 and 4). Similarly, Grb2 was recently identified as the adapter protein that associates with receptor PTKs and mediates Ras signalling.

1.2.5.1. Grb2

The human Grb2 protein was originally cloned using the tyrosine phosphorylated C-terminal tail of the EGF receptor as a high affinity probe to screen expression libraries (Lowenstein et al., 1992). The rat (ASH) and murine Grb2 have subsequently been cloned (Matuoka at al., 1992; Suen et al., 1993). Amino acid sequence analysis showed Grb2 to be composed of a single SH2 domain, flanked by two SH3 domains. The absence of a catalytic domain suggests Grb2 functions as an adapter molecule (Lowenstein et al., 1992). Grb2 was found to be ubiquitously expressed in tissues and specific antisera recognised a 25 kDa protein in human, murine and rat cell lines (Lowenstein et al., 1992; Matuoka at al., 1992; Suen et al., 1993).

1.2.5.1.1 Interaction of Grb2 with receptor PTKs.

Association of Grb2 with the activated EGF, PDGF and CSF-1 receptors has been detected (Lowenstein et al., 1992; Matuoka at al., 1992; Suen et al., 1993; Van der Greer et al., 1993). This interaction was shown to be strictly dependent on ligand activation and receptor autophosphorylation. Despite stable complex formation both in vitro and in vivo, Grb2 was a poor substrate for the activated EGF, PDGF or CSF-1 receptors and not phosphorylated on tyrosine, threonine or serine when expressed at physiological levels (Lowenstein et al., 1992; Suen et al., 1993; Van der Greer and Hunter, 1993). However, it was possible to phosphorylate Grb2 on tyrosine residues in cells overexpressing both the receptor and Grb2 (Lowenstein et al., 1992). Insulin stimulation of cells resulted in complex formation involving the Grb2 protein, not directly with the insulin receptor but with one of its substrates namely IRS-1 (Tobe et al., 1993; Skolnik et al., 1993). Complex formation between Grb2 and IRS-1 was found to be rapid, beginning as early as 10 seconds following ligand binding and again Grb2 was not observed to be phosphorylated on tyrosine (Tobe et al., 1993; Skolnik et al., 1993). Using a number of phosphopeptides containing the tyrosine phosphorylation sites on the IRS-1 protein which were phosphorylated both in vivo and in vitro, Y895 was identified as a high affinity binding site for the Grb2 protein and its SH2 domain. Further, this phosphotyrosine-containing peptide was able to inhibit complex formation between Grb2 and IRS-1 in vitro (Skolnik et al., 1993; Sun et al., 1993). Comparative analyses defined Y1068 on the human EGF receptor and Y697 on the murine CSF -1 receptor as the mediators of their interaction with the SH2 domain of Grb2 (Skolnik et al., 1993; Liu et al., 1993; Buday and Downward, 1993; Van der Greer and Hunter, 1993). Examination of the Grb2 binding sites reveals they share the common motif YXNX. Neither the FGF nor the NGF receptors were able to induce complex formation with Grb2 (Lowenstein et al., 1992; Suen et al., 1993).

1.2.5.1.2 Biological function.

It is of great interest that the amino acid sequence of Grb2 is 58% identical to the protein encoded by the Caenorhabditis elegans sem 5 gene (Clarke et al., 1992). Sem 5 was identified as a gene that regulates vulval development and sex myoblast migration in C. elegans, based on evidence that mutations in sem 5 disrupt these processes. Similar defects in vulval development are caused by mutations in the C. elegans genes let 23 (which is EGF receptor-like) and let 60 (Ras-like). This implicates the proteins encoded by the let 23, let 60 and sem 5 genes in the same signalling pathway (Figure 1.9). The finding that activated let 60/Ras can rescue vulval development in sem 5 mutants validates this hypothesis (Aroian et al., 1990; Horvitz and Sternberg, 1991). Consistent with this model in C. elegans, experiments involving microinjection of Grb2 and Ras together into quiescent fibroblasts show that they stimulate mitogenesis, whereas these two components have no effect when introduced on their own (Lowenstein et al., 1992). Similarly, overexpression of Grb2 alone in PC12 cells did not induce neuronal differentiation, in contrast to overexpression of the Ras oncogene which led to the formation of neuritic outgrowths (Suen et al., 1993).

1.2.5.2 Shc

The *Shc* gene encodes three widely expressed proteins of 46, 52 and 66 kDa which are composed of a C-terminal SH2 domain and an N-terminal glycine/proline-rich region (Pelicci et al. 1992). These proteins appear to be well conserved between mammalian species and between cell types of different embryological origin (Pelicci et al., 1992). No catalytic activity has been assigned to the Shc proteins to date and thus they may mediate protein-protein interactions.

1.2.5.2.1 Interaction of Shc with receptor PTKs

EGF stimulation of cells is found to result in stable association with the activated receptor and tyrosine phosphorylation of Shc proteins both in vivo and in vitro (Pelicci et al., 1992; Ruff-Jamison et al., 1993; Pronk et al., 1994). A lower amount of Shc was

observed to associate with the activated PDGF receptor (Pelicci et al., 1992). Shc proteins were also found to be associated with and phosphorylated by activated c-erbB2 (Segatto et al., 1993), and the region of binding was asssigned to the C-terminal tail of the receptor. The stoichiometry of binding was shown to involve only 2-3% of the total population of Shc proteins which could be coimmunoprecipitated with 5-10% of the total number of receptors present. This is consistent with studies describing the proportion of other signalling molecules observed to interact with activated receptors (Kypta et al., 1990; Margolis et al., 1989; Kazlauskas et al., 1990). Exposure of fibroblasts transiently expressing a chimeric EGF/NGF receptor, containing the external domain of the EGF receptor fused to the cytoplasmic NGF receptor domain, revealed Shc proteins to become phosphorylated and associate directly with activated receptors (Obermeier et al., 1993a; Suen et al., 1993). Further, using a combination of phosphopeptides and point-mutated receptors Y490 in the juxtamembrane region of the NGF receptor was identified as the binding site for the Shc protein and its SH2 domain (Obermeier et al., 1993a). Y₁₁₅₅LRV has been suggested to be the binding site for Shc on the EGF receptor (Communicated in Pawson and Schlessinger, 1993). The addition of steel factor or CSF-1 to quiescent cells results in tyrosine phosphorylation of the Shc protein although binding of the protein to the CSF-1 receptor was not detected (Cutler et al., 1993; Van der Greer., 1993).

Insulin can stimulate tyrosine phosphorylation of the Shc proteins (Kovacina and Roth, 1993; Pronk et al., 1993; 1994), although unlike Grb2, the Shc proteins were unable to form complexes with IRS-1. However, Shc was found to immunoprecipitate as part of a complex comprising the activated insulin receptor and Grb2 (Skolnik et al., 1993a). In addition, Shc was also found to coimmunoprecipitate with the EGF receptor and Grb2 (Lowenstein et al., 1992; Rozakais-Adcock et al., 1992). Further, Grb2 has been found in direct association with Shc in cells, although there is conflicting evidence as to whether formation of such a complex was induced by ligand-activation of receptors (McGlade et al., 1992a; Cutler et al., 1993; Rozakis-Adcock et al., 1992; Pronk et al., 1993; Van der Greer et al., 1993). More definitive evidence for the existence of Shc and Grb2 as a complex came with the identification of Y317 on the Shc protein as the specific site of interaction with the Grb2 SH2 domain (Skolnik et al., 1993a). This result implicated Shc in signalling through Ras (Lowenstein et al., 1992; Clarke et al., 1992). Indeed, overexpression of Shc in PC12 cells induced Ras dependent neurite outgrowth which could be blocked by the expression of a dominant-negative Ras mutant (Rozakis-Adcock et al., 1992). This indicated that Shc, like Grb2, functions upstream of Ras. In addition, the overexpression of Shc in fibroblasts results in a transformed phenotype (Pelicci et al., 1992) suggesting a role for Shc in the regulation of growth in mammalian cells.

1.2.5.3 c-Crk

The oncogene product of the CT10 virus, p47gag-crk, is a fusion protein of a retroviral gag protein and c-Crk which contains one N-terminal SH2 domain followed by two SH3 domains. The oncogenic v-Crk protein has been found to be associated with both the EGF and PDGF receptors (Birge et al., 1992; Fajardo et al., 1993) as well as a broad range of tyrosine phosphorylated proteins, including the cytoskeletal protein paxillin (Matsuda et al., 1990; Mayer at al., 1990; Borge et al., 1993). The function of Crk is unknown, however, despite the absence of a kinase domain, cells transformed by v-Crk have an elevated level of phosphotyrosine, by a mechanism that is yet to be clarified (Mayer et al., 1988). It is possible that this protein exerts its transforming potential by binding phosphotyrosine containing proteins via its SH2 domain and disturbing an equilibrium of phosphorylation and dephosphorylation. Binding of the full-length Crk to the phosphorylated EGF receptor has been shown to result in significant protection against dephosphorylation by a cellular phosphatase activity (Birge et al., 1992; Rotin et al., 1992). Interestingly, microinjection of Crk, was found to induce neuronal differentiation of the rat pheochromocytoma cell line PC12 through activation of p21^{Ras} (Tananka et al., 1993). Moreover, Crk has been reported to bind the guanine nucleotide exchange factor C3G (Tanaka et al., 1994). This suggests that c-Crk functions in the same biochemical pathway as the Grb2/SEM 5/ASH proteins, with the common property of activating Ras-related proteins.

1.2.5.4 Nck

Nck was fortuitously cloned as a result of screening of a human melonoma cDNA library (Lehmann et al., 1990). Amino acid sequence analysis showed Nck to be composed of three SH3 domains followed by one C-terminal SH2 domain. The Nck protein has since been found to be widely expressed in cell lines from human, murine and rat origins (Park et al., 1992; Li et al., 1992a). Overexpression of human Nck protein in rat fibroblasts leads to cell transformation and these cells form tumours in nude mice (Chou et al., 1992; Li et al., 1992a). These results identify Nck as a potential oncogene and suggests that it may play a role in the control of cell proliferation.

The Nck protein isolated from quiescent cells is phosphorylated on serine residues suggesting that Nck is phosphorylated by an extrinsic protein serine kinase activity since it possesses no intrinsic catalytic activity (Chou et al., 1992; Li et al., 1992a; Meisenhelder and Hunter, 1992). Stimulation of cells with EGF, PDGF or NGF causes phosphorylation of Nck on tyrosine, serine and threonine residues (Chou et al., 1992; Li et al., 1992a; Meisenhelder and Hunter, 1992; Park et al., 1992), which was shown to be maximal after 1-3 minutes and lasted for at least 60 minutes (Li et al., 1992a). Nck was shown to form a tight complex both in vivo and in vitro with the activated EGF and

PDGF receptors and tyrosyl phosphorylated IRS-1 (Li et al., 1992a; 1993; Sun et al., 1993), suggesting a phosphotyrosine requirement for the association. Indeed, complex formation was shown to be mediated by the SH2 domain of Nck (Li et al., 1993). Competition experiments using phosphopeptides containing tyrosine residues derived from the IRS-1 protein, identified Y147 as a mediator of the interaction of Nck with IRS-1 (Li et al., 1993). However, this site has not been shown to be an in vivo phosphorylation site (Sun et al., 1993). Site-directed mutagenesis was used to identify Y751 in the kinase insert region of the PDGF β receptor as a binding site for the Nck protein. Mutation of this residue eliminated the binding of Nck to the PDGF receptor both in vivo and in vitro and PDGF stimulated phosphorylation of Nck was not detected in these experiments (Nishimura et al., 1993). It is of interest that Y751 is also one of the two possible binding sites on the PDGF receptor necessary for PI 3-kinase binding (Fantl et al., 1992; Kashishian and Cooper et al., 1992). This suggests that any downstream defects associated with a mutation at Y751 are not necessarily solely due to the elimination of PI 3-kinase binding, but may also be due to the inability of Nck to bind. Although the CSF-1 receptor is from the same sub-family of PTKs as the PDGF receptor, it failed to bind Nck (Van der Greer and Hunter, 1993).

The experiments described above illustrate that multiple SH2 domain-phosphotyrosine interactions follow receptor PTK activation. The sites of interaction of these various signalling molecules on receptor PTKs is summarised in Figure 1.6. Whether these proteins can exist as multimeric signalling complexes remains a matter for debate. It is however clear that in most cases the interaction between receptor and SH2 domain is rapid, transient and of high affinity (Panayotou et al., 1993). In the following section the nature of the interaction between SH2 domains and receptor PTKs is discussed in more detail to shed more light on the mechanism of activation of these signalling molecules.

1.3 SH2 domains: mediators of the interaction between activated receptors and second messenger signalling molecules

Study of the primary structure of these receptor-associated proteins described above shows that they are multimodular proteins which are comprised of a number of discrete domains (Figure 1.2). In particular SH2 domains of these molecules are thought to be critical in mediating their interaction with the phosphotyrosine residues of activated PTKs. SH2 domains were first described as a sequence motif present in the N-terminal region of the Src family of PTKs - SH1 being the catalytic domain (Sadowski et al., 1986; Pawson et al., 1988; Mayer et al., 1988). These non-enzymatic domains of ~100 amino acids were subsequently identified in a diverse array of intracellular proteins

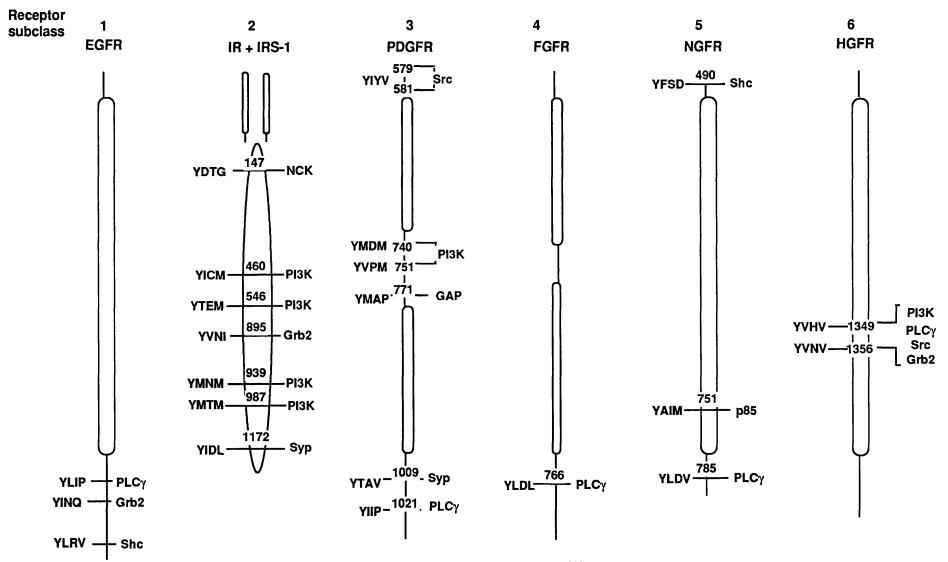


Figure 1.6 The peptide sequences on different receptor types that have been shown to be important for binding of various signalling molecules.

(Reviewed in Pawson 1988; Koch et al., 1991) (Figure 1.2). The SH2 domain was found to behave as a discrete protein module, able to fold independently and confer common recognition properties to diverse proteins. This was demonstrated by their ability to retain their function when expressed alone as recombinant domains. It has been shown that the isolated SH2 domains of PLCγ, RasGAP and pp60c-Src synthesised in bacteria were all able to bind to activated PDGF receptors in vitro (Anderson et al., 1990; Margolis et al., 1990, 1990a; Kazlauskas et al., 1992; Mohammadi et al., 1992; Obermeier et al., 1993). Subsequently, the SH2 domains of Vav (Margolis et al., 1992; Bustelo et al., 1992), SHPTP2 (Feng et al., 1993; Lechleider et al., 1993a; Vogel et al., 1993); Shc (Pelicci et al., 1992; Ruff-Jamison et al., 1992; Obermeier et al., 1993a; Grb2 (Lowenstein et al., 1992; Liu et al., 1993; Skolnik et al., 1993); Nck (Li et al., 1992a; 1993; Sun et al., 1993); p91 (Fu et al., 1993) were shown to mediate their interaction with activated receptors in vitro. Further, these biochemical studies showed that individual SH2 domains were effective in competing with the intact protein to bind receptors in vitro.

1.3.1 Function of SH2 domains

Mutational and biochemical analyses performed on the v-Crk SH2 domain and the abl PTK demonstrated that the SH2 domain was of primary importance in mediating their binding to phosphotyrosine containing proteins (Matsuda et al., 1991;1993; Mayer et al.. 1991). The direct binding of substrates to activated receptors has been shown to be neccessary, as SH2 domain containing proteins have significantly higher Vmax/Km values for tyrosine phosphorylation than substrates lacking a SH2 domain (Sierke et al., 1993). Thus, the physical complex formed between a receptor PTK and an SH2 domaincontaining signalling protein enhances substrate phosphorylation and hence promotes signal transduction from these proteins. High association rates for SH2 domain interactions have been shown to be coupled with fast dissociation rates (Panayotou et al., 1993a; Felder et al., 1993), demonstrating high affinity albeit transient interactions. High affinity binding of an SH2 domain was shown to require that the phosphotyrosine be embedded within a specific amino acid sequence (Cantley et al., 1991). For example, as described in Figure 1.6 the activated PDGF receptor binds a number of SH2 domaincontaining proteins on specific autophosphorylation sites. Inhibition studies using small phosphotyrosine containing peptides have shown that molecules containing only five residues can effectively block SH2 domain mediated binding to activated receptors (Fantl et al., 1992).

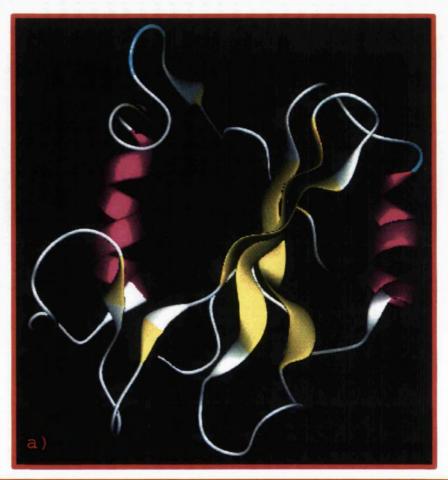
The residues at positions +1 and +3 (with respect to phosphotyrosine) appear to provide specificity for most SH2 domains. This has been addressed using various complementary techniques. Firstly, comparison of different receptor autophosphorylation sites that are

responsible for binding of a specific protein identified short phosphotyrosine containing sequences that are necessary for high affinity binding. A second approach systematically addressed the binding specificity of SH2 domains for different phosphopeptide sequences. A semi-random peptide library was used to determine the optimal sequences for tight binding to specific SH2 domains (Sonyang et al., 1993). Thus, the C-terminal SH2 domain of PLCy selected a sequence similar to that biochemically demonstrated as the PLCy binding sites pY(LI)IP on the PDGF and EGF receptors (Ronnestad et al., 1992; Valius et al., 1992), whereas the specific sequence recognised by the N-terminal SH2 domain of PLCy resembled that of the known PLCy binding site on the FGF receptor (Mohammadi et al., 1993). Alternatively, the Grb2 protein showed a very strong preference for asparagine at the +2 position but less discrimination at the +1 and the +3 positions, which is consistent with the biological data. However, it still remains possible that additional residues either amino or carboxyl terminal to the four amino acid sites described will play a role in phosphotyrosine recognition by SH2 domains. For example the C-terminal SH2 domain of PLCy contacts peptide residues at the +5 and +6 positions (Pascal et al., 1994).

In addition to binding phosphotyrosine containing sequences, it has been suggested that SH2 domains under certain circumstances may also bind specific phosphorylated serine sequences and hence alter regulation (Pendergast et al., 1991; Muller et al., 1992). In chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (ALL), a translocation involving chromosomes 9 and 22 disrupts the ABL and Bcr loci, leading to the formation of a chimeric Bcr-ABL oncogene. This gene encodes a fusion protein in which the amino terminal Bcr region activates the linked ABL tyrosine kinase. It has been suggested that Bcr becomes highly phosphorylated on serine and binds the ABL SH2 domain (Pendergast et al., 1991) thereby deregulating ABL kinase activity. Subsequent studies have revealed that the SH2 domain of PLCγ, RasGAP and pp60c-Src were able to interact with phosphorylated serine residues in a phosphotyrosine independent manner albeit with reduced affinity (Muller et al., 1992). Similarly, the SH2 domains of p85 have been found to associate with IRS-1 in resting cells, when IRS-1 is mainly phosphorylated on serine and threonine residues (Lavan et al., 1992).

1.3.2 Structural analysis of the SH2 domain

The elucidation of the tertiary structures of the Src, Abl, Lck, C-terminal SH2 domain of PLC γ and the N-terminal SH2 domain of p85 (Waksman et al., 1992, 1993; Booker et al., 1992; Overduin et al., 1992, Eck et al., 1993; Pascal et al., 1994) provided a foundation for the detailed study of SH2 domain-phosphotyrosine complex formation. The structure of the domain is characterised by a central 3-stranded anti-parallel β -pleated sheet flanked by two α -helices packing into each face of the sheet (Figure 1.7). Several conserved



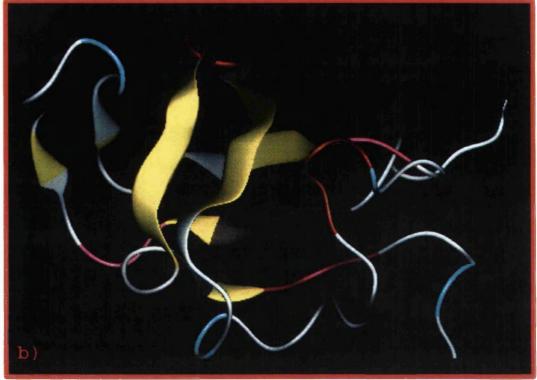


Figure 1.7 A ribbon diagram of a) p85 N-terminal SH2 domain (Booker et al 1992) and b) p85 SH3 domain (Booker et al 1993). Yellow represents $\beta\text{--sheet}$ and cyan indicates $\alpha\text{--helical}$ structures.

The figure was generated using QuantaTM (courtesy of M.J.J M.Zvelebil)

residues (Koch et al., 1991; Russel et al., 1992) which have been shown by site-directed mutagenesis to be important for recognition (Mayer et al., 1992; Marengene and Pawson 1992), cluster at the surface of the molecule and mediate the binding of the phosphopeptide (Waksman et al., 1992). In particular, examination of the crystal structure of the SH2 domain of v-Src complexed with a phosphopeptide pYEEI, predicted to form a high affinity interaction (Sonyang et al., 1993), reveals that the phosphopeptide takes part in six primary interactions. The phosphotyrosine and the isoleucine residues are tightly bound by two pockets resembling a two pronged plug engaging a two holed socket. In contrast, using the phosphopeptide pYQPD which has a ten fold lower affinity (Liu et al., 1993), only the phosphotyrosine maintains a significant contact with the SH2 domain and the peptide chain is extended away from the surface of the molecule (Waksman et al., 1992). Taken together these results suggest that SH2 domains of different signalling molecules bind to distinct high affinity tyrosine phosphorylated sites and that the specifity of this interaction is inherent to the sequence of the SH2 domain and the residues that surround the phosphorylated tyrosine on the receptor.

It is interesting to note that p85, PLCy, SHPTP1/SHPTP2 and RasGAP all contain two SH2 domains. It has been postulated that two SH2 domains in PI 3-kinase are required to maintain a high affinity interaction and activate the PI 3-kinase (Kasishian et al. 1992; Reedijk et al., 1992; Carpenter et al., 1993; Ponzetto et al., 1993). As discussed above, the amino and the carboxy terminal SH2 domains of PLCy exhibit different specificities of interaction with receptors (Valius et al., 1992; Mohammadi et al., 1992) and might have distinct structural features (T. Pawson, unpublished observation). In addition, the EGF receptor and p62 bind only the N-terminal SH2 domain of RasGAP (Moran et al., 1990; Koch et al., 1992b). Indeed, sequence analysis and mutagenic studies have shown that the RasGAP C-terminal SH2 lacks critical residues which are otherwise well conserved and contribute to the high affinity binding exhibited by the N-terminal SH2 (Marengere and Pawson, 1992). Similarly, only the N-terminal SH2 domain of SHPTP2 is shown to interact directly with activated PDGF receptor (Leichleder et al., 1993). However, as all these studies were performed using isolated recombinant domains, their behaviour in vitro cannot necessarily be used to interpret the situation in vivo or within the context of the entire protein.

1.4 SH3 domains: proline-rich binding modules

The Src homology 3 (SH3) is a discrete region of 50-75 residues often present in conjunction with SH2 domains in a large number of proteins (Figure 1.5) (Mayer et al., 1988; Reviewed in Pawson and Schlessinger, 1993; Musacchio et al., 1992a). Unlike

SH2 domains, SH3 domains are also present in yeast suggesting an evolutionary more primitive function that arose before the elaborate machinery involved in regulating multicellular growth. However, although this conserved element is present in a diverse array of proteins, a specific physiological function remains to be assigned.

1.4.1 Function of the SH3 domain

Initial studies identified SH3 domains in a number of cytoskeletal proteins such as myosin 1, fodrin, spectrin, and an actin binding protein from yeast, ABP-1 (Drubin et al, 1990; Rodaway et al., 1987). Several lines of evidence have been used to suggest an involvement of SH3 domains in regulating the interaction of signalling molecules within the cytoskeleton. The SH3 domain of pp60^{c-Src} was found to associate with a number of cellular proteins present mainly in the particulate and detergent insoluble cell fractions. In particular paxillin, a vinculin binding protein, was identified as a Src-SH3 domain binding protein. Paxillin is a component of focal adhesion contacts, where the actin cytoskeleton is linked to the extracellular matrix via integrin receptors and cytoskeletal proteins such as vinculin (Cherievert et al., 1992). Indeed, Src has been found associated with the cytoskeleton in transformed cells (Hamaguchi et al., 1987). Further, the SH3 domain of PLCγ and Grb2 localised to the microfilament network and membrane ruffles respectively when microinjected into cells (Bar-Sagi et al., 1993). In yeast, alterations of a SH3 domain-containing protein were found to lead to conditional viability with defects in cytoskeletal reorganisation (Bauer et al, 1993).

As well as having a role in targeting proteins to their site of action, a body of evidence also implicates SH3 domains in the regulation of G-protein function. Screening of expression libraries with an isolated SH3 domain of the ABL PTK led to the cloning of partial cDNAs encoding possible SH3-binding proteins, termed 3BP1 and 3BP2 (Cicchetti et al., 1992). DNA sequence analysis revealed a region of 3BP1 with similarity to the C-terminus of Bcr (Shtivelman et al. 1985), the neuronal protein nchimaerin (Hall et al., 1993), RhoGAP (Diekmann et al., 1991) and P190 (Settleman et al., 1992a). Bacterially expressed Bcr homology domains from these proteins have been shown to stimulate the GTPase activity of the Ras-related guanine nucleotide binding proteins, Rac and Rho. This is intriguing since the Rho and Rac proteins have been implicated in controlling membrane ruffling and formation of actin stress fibres and focal contacts (Ridley and Hall 1992; Ridley et al., 1992). More compelling evidence for the involvement of SH3 domains in G-protein regulation came with the identification of the GTPase dynamin as an SH3 domain binding protein. A panel of recombinant SH3 domains were shown to selectively bind dynamin and comitant with this interaction was activation of the intrinsic GTPase activity of dynamin (Gout et al., 1993).

Another system in which SH3 domain-containing proteins have been observed to play a regulatory role is the neutrophil oxidative burst. In neutrophils, agonist stimulation leads to the activation of the membrane bound cytochrome-b oxidase and generation of superoxide. Two cytosolic factors, p47-phox and p67-phox, each of which contain two SH3 domains (Leto et al., 1990) have been implicated in regulating this system. These SH3-containing proteins are absent in some patients with chronic granulomatous disease and cause their neutrophils to lose the ability to generate superoxide; a process which normally is used to kill microorganisms (Segal et al., 1989). In vitro reconstitution experiments have revealed the requirement for a RacGTP-binding protein (Abo et al., 1991) for the stimulation of oxidase activity. This suggests that the SH3 domains of p47phox and p67phox may be involved in regulating Rac function.

These results implicate SH3 domains in first targeting of proteins to subcellular compartments and in the regulation of the activity of small G-proteins. Interestingly some G-proteins can regulate the assembly of multimolecular complexes that are directly linked to the organisation of polymerised actin. For example, the adapter protein Grb2 has been shown to play a role in signalling via Ras (Lowenstein et al., 1992; Discussed in Section 1.62) and Ras has been shown to be preferentially localised in membrane ruffles (Bar-Sagi et al., 1988). In addition, expression of an N-terminal RasGAP construct consisting solely of the SH2 and SH3 domains in Rat-1 cells correlated with observed changes in the cytoskeleton and in adhesion properties of these cells, which were typified by the observed disruption of actin stress fibres, a reduction in focal contacts and an impaired ability to adhere to fibronectin (McGlade et al., 1993). Moreover, the Nterminal RasGAP construct was shown to be constitutively bound to p190 in vivo which contains a Rho/Rac GAP domain. This probably inactivates the Rho/Rac family members to give the observed cellular phenotype. The two postulated functions of SH3 domain-containing proteins are also seen to converge in yeast. The product of the S. cerevisiae gene Bem 1, which contains two SH3 domains interacts with the protein product of the Bud 5 gene which encodes a putative guanine nucleotide exchange factor related to the CDC25 protein (Chant et al., 1991), and the product of the Bem 2 gene which encodes a protein containing a domain with sequence similarity to RhoGAP. These genes are required for organisation of the cytoskeleton resulting in polarisation and hence assembly of the bud site.

1.4.2 Structural analysis of the SH3 domain

These functional studies have been complemented by the rapid elucidation of tertiary structures for a number of SH3 domains. Solution structures of SH3 domains from pp60^{c-Src} (Yu et al., 1992), PLC γ (Kohda et al., 1993) and p85 α (Booker et al., 1993; Koyana et al., 1993; Yu et al., 1994), together with crystal structures of α -spectrin

(Musacchio et al., 1992) and pp59^c-Fyn (Noble et al., 1993) have revealed striking structural similarities between these SH3 domains despite relatively limited sequence conservation. The structures show that the amino and carboxyl terminal residues are in close proximity to each other indicating that SH3 domains are independent structural modules. The basic structure is composed of between five and eight β -strands which form a barrel-like structure. The conserved aliphatic and aromatic residues present in SH3 domains form a hydrophobic pocket and conserved carboxylic amino acids are located in loops adjacent to this pocket. Initial analyses using 3BP1 localised the SH3 domain binding site to a ten amino acid proline-rich region (Ren et al., 1993) with a potential consensus motif XPXXPPPZXP (where X is any amino acid and Z is a hydrophobic amino acid). The subsequent screening of a biased combinatorial library of proline-rich peptides with the Src and PI 3-kinase SH3 domains together with mutagenesis studies and a solution structure of the PI 3-kinase SH3 domain complexed with ligand has led to the conclusion that SH3 domains recognise distinct proline-rich motifs possessing the left-handed type II polyproline helix conformation (Yu et al., 1994). Two proline residues maintain a direct interaction with the SH3 domain while other prolines in the ligand appear to promote the formation of a left-handed type II polyproline helix.

1.5 Pleckstrin domains: A novel mediator of protein-protein interactions

The pleckstrin homology (PH) domain was originally detected as an internal sequence duplication in pleckstrin, a 47 kDa protein which is the major substrate of PKC in platelets (Tyers et al., 1988). Subsequent searches of protein sequences have revealed the presence of this 100 amino acid domain in many proteins involved in cellular signalling, which merits its inclusion alongside SH2 and SH3 domains (Mayer et al., 1993; Haslam et al., 1993). Several proteins that function as regulators of small GTP-binding proteins have been found to contain PH domains (Figure 1.5). These include RasGAP (Trahey et al., 1988), guanine nucleotide releasing factor for Ras (GRF) (Shou et al., 1992), the exchange factor Son of Sevenless (Sos) (Simon et al., 1992), the SH3 binding protein 3BP2 (Cicchetti et al., 1993), CDC24, a guanine nucleotide exchange factor from S. cerevisiae (Adams et al., 1992), the Bcr protein, DBL the human oncoprotein homologue of CDC24 (Hart et al., 1991), Vav (Adams et al., 1992), and the GTPase dynamin (Obar et al., 1990). It is intriguing that the presence of the PH domain in GRFs and RasGAPs is mirrored by a tendency of the PH domain to be located adjacent to the CDC24 homology domain and such a topological relationship might imply functional interdependence. The PH domain has also been identified in a number of protein kinases including members of the Rac family of serine/threonine kinases such as Akt (Bellacosa et al., 1991), a murine Tec tyrosine kinase (Mano et al., 1993), and the β -adrenergic receptor kinase (Benovic et al., 1989). Other proteins that contain PH domains include the EGF receptor binding protein GRB7 (Margolis et al., 1992a), the cytoskeletal protein spectrin and the insulin receptor substrate IRS-1. (Many other proteins containing this domain have been mentioned in the review of Musacchio et al., 1993 and Shaw et al., 1993).

1.5.1 Structural analysis of the PH domain

Several of these PH-domain containing proteins also contain SH2 and SH3 domains. For example, PLC γ , RasGAP, GRB7, Vav, spectrin, and the Tec related kinase ATK. The PH domain appears to be far more divergent in sequence conservation than SH2 and SH3 domains. This is reflected in the lower level of sequence similarity between the 40 or so pleckstrin domains thus far identified, which varies from 4% to 25% (Musacchio et al., 1993 and Shaw et al., 1993). As with the Src homology domains, the PH domain does not appear to occupy a specific position in the host molecule suggesting that it is functionally independent. Only the pleckstrin and RasGRF proteins are found to have more than one copy of this domain. The presence of the PH domain in the yeast secretory protein sec 7 (Haslan et al., 1993), as well in invertebrates such as the *C. elegans* Unc-104 (a kinesin-like molecule) indicates a long evolutionary history as has observed for SH3 domains. Secondary structure prediction distinguishes five conserved subdomains which are predicted to adopt a folded structure comprising 7-8 β -strands, most likely in one or two β -sheets and a single α -helix (Musacchio et al., 1993).

1.5.2 A functional role for the PH domain.

A function for the PH domain has not yet been defined, however, there are a number of possibilities. Several proteins that contain the PH domain function in conjunction with GTP-binding proteins. Experimental evidence (Inglese et al., 1993; Koch et al., 1993; Touhura et al., 1994) suggests that the PH domain of β ARK may be responsible for binding the $\beta\gamma$ complex of a heterotrimeric G-protein via a coiled-coil interaction. This binding was shown to be inhibited by a synthetic peptide which begins at the conserved tryptophan residue of the PH domain (Koch et al., 1993). Further, GST-fusion proteins of a number of pleckstrin domains were shown to bind $\beta\gamma$ subunits to varying extents (Touhura et al., 1994). Thus, PH domains may function to couple some host proteins to $\beta\gamma$ subunits of heteromeric G-proteins. Indeed, the localisation of β -spectrin in the cytoskeleton could be appropriate to allow interaction with membrane bound G-protein $\beta\gamma$ subunits. Further, both dynamin and Unc 104 are known to have a function in membrane intracellular transport. There is also evidence emerging for the function of the PH domain in other protein-protein interactions. An Arg28-Cys28 mutation in the PH domain of the ATK tyrosine kinase (homologous to TecA) was shown to result in

numerous cellular defects including aberrant responses to activating signals (Rawlings et al., 1993). Although at present fragmentary, the picture emerging suggests that the PH domain may be a key component of signalling mechanism.

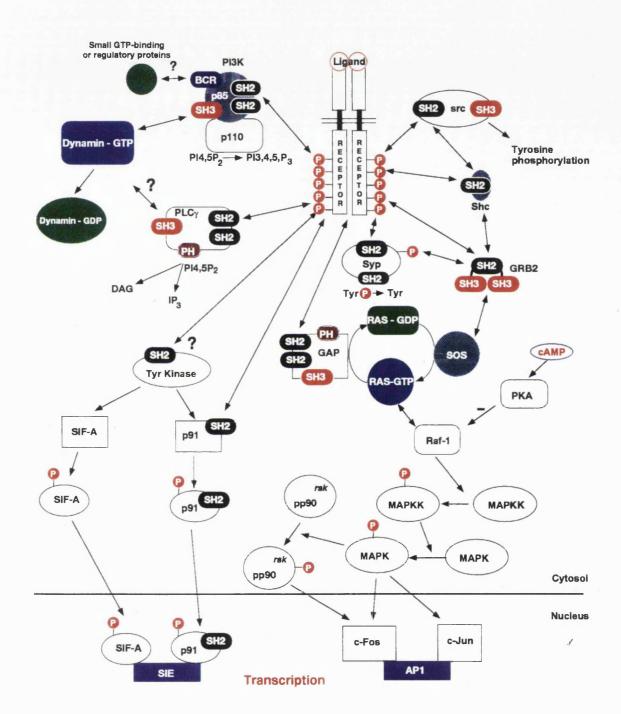
1.6 The transmission of a signal from the membrane to the nucleus

Ligand stimulation results in receptor dimerisation and autophosphorylation on specific tyrosine residues. This in turn results in the recruitment of a number of signalling molecules. There are three basic mechanisms by which these second messenger proteins which have been described in section 1.2 may become activated following ligand stimulation of cells. These are translocation to the plasma membranes with consequent access to substrate, and association with, and phosphorylation by, activated protein-tyrosine kinases (PTKs). These mechanisms are not mutually exclusive and probably synergise to result in a cascade of events, culminating with a biological response. The signalling cascades initiated upon activation of these receptor recruited signalling molecules are summarized in Figure 1.8 and shall be discussed in this section.

1.6.1 Mechanisms for activation of signalling molecules

1.6.1.1 Translocation.

Growth factor stimulation of cells results in the physical translocation of receptor substrates from the cytosol to the plasma membrane. In turn this brings them into close proximity with their substrates or with other proteins with which they interact. For example, the phospholipid substrates of both PI 3-kinase and PLCy reside in the membrane. Indeed, subcellular fractionation studies have revealed that 70% of the total pool of PLCy redistributes to membrane-associated fraction within 1 minute of exposure of cells to growth factor (Todderud et al., 1990; Kim et al., 1990). These results suggest that the membrane association of PLCy is a significant event in second messenger signal transduction. Indeed, the binding of PLCy to receptors appears to be crucial for stimulation of PLCy activity, even if PLCy has already become phosphorylated (Valius et al., 1993; Mohammadi et al., 1992; Vega et al., 1992). In addition, the targets of RasGAP, Vav, Grb2 and Shc are small membrane-bound GTP-binding proteins. Consistent with this, intraperitoneal injection of EGF results in the translocation of Shc from its cytosolic location to the plasma membrane of liver cells (Ruff-Jamson et al., 1992). Hence, regulation of enzymatic activities appears in part to result from subcellular relocalisation with consequent and essential access to membrane associated substrate.



Figure~1.8.~Schematic~showing~the~different~signal~transduction~pathways~activated~upon~ligand~stimulation~of~cells.~.

1.6.1.2 Binding.

The physical binding of the SH2-domain containing substrates to the activated receptor upon translocation is also likely to exert some regulatory control. Indeed, the physical association between the SH2 domain of a signalling protein with pY-containing sites may alter the conformation of the signalling proteins directly stimulating their activity. A phosphopeptide containing the pYEEI consensus sequence required for high affinity binding of Src (Waksman et al., 1993; Sonyang et al., 1993) was found to directly increase the activity of pp60^{c-Src}. Crystallographic analyses of the Src SH2 domain complexed with this phosphopeptide revealed significant localised changes in structure compared to the uncomplexed SH2 domain. In addition, a 5-10 fold activation of the SYP (SHPT1D/PTP2C/SHPTP2) phosphatase has been observed upon binding of a phosphotyrosylpeptide comprising the region around Y1009 of the PDGF receptor (Leichleider et al., 1993). Thus, it seems likely that these interactions induced changes in structure which are sufficient to induce an allosteric activation of catalytic subunits.

1.6.1.3 Phosphorylation

The physical binding of substrates such as PLC₁ to receptor PTKs can markedly lower the Km for their phosphorylation making the SH2 domain-containing proteins preferred substrates of receptor PTK activity; a process which may contribute to their activation. Tyrosine phosphorylation has been shown to be particularly pertinent to the activation of PLCy. The time course of PLCy phosphorylation in intact cells has been shown to coincide with that of EGF-induced IP3 formation (Wahl et al., 1988a; Todderud et al., 1990). In addition, ligand stimulated cells overexpressing PLCy displayed an increase in the levels of both tyrosine-phosphorylated PLCy and inositol phosphates, compared with a cell line expressing physiological level of PLCγ (Margolis et al., 1990a; Sultzman et al., 1991). Phosphorylation of PLCγ on tyrosine by an activated EGF receptor stimulates its catalytic activity four-fold both in intact cells and in vitro. Moreover, dephosphorylation of the activated enzyme by a tyrosine-specific phosphatase results in a decrease of its catalytic activity (Nishibe et al., 1990). These studies were complemented by the identification of Y783 and to a lesser extent Y1254 on PLCy as phosphorylation sites essential for its activation (Kim et al., 1991). A possible mechanism by which tyrosine phosphorylation may activate PLC_{γ} has been suggested by the observations that PLC_{γ} localises to the actin cytokeleton (Bar-Sagi et al., 1993) and that PI(4,5)P2 is found in complex with the actin binding protein profilin (Goldschmidt-Clermont et al., 1991). A PIP2-profilin association disrupts the profilin-actin complex, thereby increasing the amount of monomeric actin available for polymerisation (Lassing and Lindberg, 1985; Goldschmidt-Clermont et al., 1991). Profilin associated PI(4,5)P2 is recognised only by the phosphorylated form of PLCy (Goldschmidt-Clermont et al., 1991). Thus, tyrosine phosphorylation of PLCy by an activated receptor PTK may trigger the hydrolysis of PI(4,5)P2 and thus result in the release of profilin. Free profilin would then form a complex with actin thereby inhibiting actin polymerisation.

The assembly of IFN/EGF activated transcription complexes has also been shown to require tyrosine phosphorylation. Complex formation between activated receptors and ISGF-3 was specifically disrupted by treatment with a protein-tyrosine phosphatase and this effect was found to be inhibited in the presence of vanadate (Fu et al., 1992; Fu and Zhang, 1993, Shulai et al., 1993; Igarashi et al., 1993; David et al., 1993). In particular, phosphorylation of p91, contained within this transcription complex has been shown to be critical for nuclear translocation, DNA binding and gene activation. Y701 was identified as the site of phosphorylation on p91 and mutation of this tyrosine residue blocked translocation of p91 to the nucleus. Further, phosphorylation of p91 has been shown to be a requirement for dimerisation of p91 proteins, mediated through specific SH2 domain phosphotyrosyl peptide interactions. These p91 homodimers are then able to bind specific DNA sequences directing transcription (Shuai et al., 1994).

The evidence for activation of RasGAP by phosphorylation has thus far remained unclear. The level of tyrosine phosphorylation of RasGAP has been shown to correlate with the transforming activity of the EGF receptor and pp56^{lck} (Soler et al., 1993; Ellis et al., 1991). In addition, v-Src mutants that failed to phosphorylate RasGAP and p62 also demonstrated poor transforming activity (Moran et al., 1990). In contrast, no difference in the activity of phosphorylated and unphosphorylated formsof GAP has been detected in vitro (Serth et al., 1992).

The kinase activity of pp60^{c-Src} increases 3-5 fold following PDGF stimulation of quiescent fibroblasts (Ralson and Bishop, 1985; Gould and Hunter, 1988; Kypta et al., 1990) and of isolated membranes (Walker et al., 1993). It has been suggested that tyrosine phosphorylation of pp60^{c-Src} may itself be responsible for contributing directly to the increased enzymatic activity (Twamley et al., 1992; Courtneidge et al., 1993; Walker et al., 1993). SHPTP2 was also found to be phosphorylated on tyrosine in cells overexpressing the PDGF and the EGF receptors (Vogel et al., 1993; Feng et al., 1993) and this tyrosine phosphorylation correlated with an enhancement of its catalytic activity. Interestingly, some reports have suggested that tyrosine phosphorylation of substrates may be responsible for release of the complexed protein from the activated receptor. For example PLCγ dissociates more rapidly following its phosphorylation by the EGF receptor (Margolis et al., 1990a). This may be a consequence of the PLCγ SH2 domain binding in an intramolecular manner to an internal tyrosine residue on PLCγ which becomes phosphorylated by the activating receptor.

The Raf-1 serine/threonine kinase is also a component of multienzyme complexes although it lacks SH2 domains. Activation of its catalytic activity upon recruitment to the activated receptor will presumably utilise a different mechanism to that proposed for SH2 domain-containing substrates. Nevertheless, ligand stimulation of cells has been shown to induce a 3-5 fold increase in the level of Raf-1 kinase activity, concomitant with hyperphosphorylation of Raf-1 on serine residues. Serine residues 43, 259 and 621 have been identified as the major sites for PKC phosphorylation on Raf1 in PDGF treated cells (Morrison et al., 1993). Furthermore, treatment of the activated Raf1 kinase with phosphatase was found to greatly reduce its serine-kinase activity (Kovacina et al., 1990). In addition, evidence exists for a five-six fold increase in Raf1 serine kinase activity in response to tyrosine phosphorylation by the PDGF receptor both in vitro and in vivo (Morrison et al., 1989).

1.6.2 Activation of the Ras signalling pathway.

The first Ras proteins discovered were 21 kDa proteins encoded by the oncogenes of the Harvey and Kirsten rat Sarcoma viruses. The proteins termed p21v-Ha-Ras and p21v-Ki-Ras are responsible for the transforming ability of these retroviruses (Harvey, 1964; Kirsten and Mayer, 1967). A third, closely related transforming protein, p21^{N-Ras} was later discovered as an oncogene product in a neuroblastoma (Hall et al., 1983). Normal p21c-Ras (Ras), the product of the proto-oncogene, is a guanine nucleotide binding membrane protein. Mutational analysis of Ras has defined an effector region encompassing residues 32-40 (Willumson et al., 1986; Sigal et al., 1986; 1986a). Mutations in this region have been shown to impair Ras transforming activity, presumably by blocking the interaction of Ras with its downstream targets. Although this region is essential for transformation, mutations in this locality do not alter intrinsic Ras activity, nor do they effect its membrane localisation.

The function of Ras differs in mammalian tissues according to cell type, but it is established that Ras plays a critical role in mediating tyrosine kinase stimulated cell proliferation and differentiation. Microinjection of a Ras neutralising monoclonal antibody into quiescent cells blocks the mitogenic response of the cells to serum, PDGF and EGF (Mulcahy et al., 1985) and reverts the transformed morphology of v-Src, v-Fms and v-Fes transformed fibroblasts (Smith et al., 1986). Conversely, constitutively active Ras proteins substitute for the action of ligand activated PTK receptors and induce mitogenic signal transduction and DNA synthesis or differentiation in various cell types

(Hagag et al., 1986; Szeberenyi et al., 1990; Kremer et al., 1991; Wood et al., 1992; Thomas et al., 1992).

In all eukaryotic organisms examined Ras homologues have been identified which act as key signal transducing elements in receptor activated pathways (Figure 1.9). The cell cycle of *S. cerevisiae* is controlled by Ras proteins which are responsive to the nutritional status of the environment. In *C. elegans* Ras is required for vulval development and photoreceptor cell development in *Drosophila melanogaster* is also Ras dependent. In particular, understanding Ras function is important since activated Ras oncogenes have been shown to play a role in the development of at least 30% of all cancers.

A large number of extracellular stimuli have been shown to activate Ras proteins as determined by measuring an increase in the amount of Ras-bound GTP relative to GDP in cells metabolically labelled with ³²Pi. These include: GMCSF and steel factor in mast cells (Duronio et al., 1992; Satoh et al., 1991), NGF and EGF in PC12 cells and rat-1 fibroblasts respectively (Muroya et al., 1992; Buday and Downward, 1993) PDGF and insulin in various fibroblast cell lines (Burgering et al., 1991; Gibbs et al., 1990; Satoh et al., 1990a/b). Ras proteins are biologically inactive in the GDP-bound state and are activated by the exchange of GDP for GTP (Figure 1.4) (Bourne et al., 1991). In principle, the level of Ras-GTP may be enhanced either by stimulation of guanine nucleotide exchange by a exchange factor (GNRP) or inhibition by a GAP molecule (Rogge et al., 1992; Gaul et al., 1992). For example, in two instances the stimulation of Ras has been linked to a decrease in GAP activity: phorbol ester or T-cell receptor agonist treatment of T-cells (Downward et al., 1990, 1990a) and erthropoietin treatment of human erythroleukemia cells (Torti et al., 1992). In other cell types an increase in nucleotide exchange activity has been associated with the activation of Ras: PC12 cells treated with the differentiation-inducing factor NGF (Li et al., 1992), wild-type or Ras overexpressing rat-1 fibroblasts treated with EGF (Buday et al., 1993; Medema et al., 1993), or insulin (Medema et al., 1993). The mechanism by which certain growth factors can stimulate the rate of exchange of nucleotide on p21c-Ras has until recently remained unclear, although a requirement for tyrosine kinase activity was observed (Buday et al., 1993).

1.6.2.1 Activation of the Ras signalling pathway in *Drosophila*

The genetic dissection of signal transducing pathways in *C. elegans* and *D. melanogaster* has now provided detailed insights into the transmission of a signal from a receptor protein-tyrosine kinase to Ras via specific protein-protein interactions (Figure 1.9). In *Drosophila*, the sevenless gene encodes a transmembrane receptor protein-tyrosine kinase called Sevenless that is most related to the human c-Ros protein (Hafen et al., 1987). The only known function of sevenless is for the proper development of the *Drosophila* eye

	D. Melanogaster	Mammalian cells	C. elegans	S. Pombe
Receptor PTK	Sevenless /Torso	PDGF	Let-23	G-protein coupled receptor
Adapter	Csw Drk	SHPTP2 Grb2	Sem-5	ND
Guanine Nucleotic Releasing factor	de Sos	Sos	ND	Ste 6
Guanine Nucleotic Activating factor	de Gap1	RasGAP	ND	gap1
Guanine Nucleotic binding protein	de Ras1	p21Ras	Let-60	ras1
Ser/Thr kinase	D-raf	Raf1	Lin-45	byr2
Ser/Thr kinase	Dsor1	MKK	Mk-1/2	byr1
Ser/Thr kinase	DmERKA	MK	Lin 31	spk1
Transcription Factors	Sina	c-jun	ND	pap1

Figure 1.9 Molecules conserved through evolution in signalling cascades. See text for details. ND, not yet determined.

(Tomlinson and Ready, 1986). The eye consists of approximately 800 repeated units called ommatidia, each of which contains a central core of eight photoreceptors that is surrounded by 12 accessory cells. During development, the cells of each ommatidium are recruited to their proper fates by a series of inductive interactions by which previously determined cells apparently instruct their uncommitted neighbours to adopt a specific cell fate (Reviewed in Banerjee and Zipursky, 1990). The R7 photoreceptor is the last of the photoreceptors to be recruited and its specification is dependent on the local activation of the Sevenless receptor tyrosine kinase in the R7 precursor cell (Hafen et al., 1987;). In the absence of functional Sevenless protein, the R7 precursor does not develop into a photoreceptor cell, but rather into a non-neuronal cone cell (Tomlinson and Ready, 1986). Conversely, the constitutive activation of Sevenless kinase activity in the R7 and other ommatidial precursor cells results in the formation of multiple R7 cells in each ommatidium (Basler et al., 1991). The response of ommatidial precursor cells to either a partially functional, or a constitutively active Sevenless kinase is dosage dependent. This observation was exploited to identify components genetically downstream of Sevenless and establish that both Drosphilia GAP1 and Ras1 played essential roles in signalling from the Sevenless receptor (Figure 1.9) (Rogge et al., 1991; Rogge et al., 1992; Gaul et al., 1992; Simon et al., 1991). Subsequently, the son of sevenless (Sos) and the Drk gene products were identified as also acting downstream of the Sevenless and the Drosophila EGF receptor homologue (DER), (Rugge et al., 1991 cell; Simon et al., 1991/1993; Olivier et al., 1993). Sos has a central domain related to the S. cerevisiae CDC25 protein which acts as a Ras GNRP (Simon et al., 1991; Bonfini et al., 1992). The C-terminal sequence of Sos is relatively rich in proline residues and represents an SH3 binding site (Simon et al., 1991).

The *Drk* gene encodes a widely expressed 24 kDa protein containing a single SH2 domain and two flanking SH3 domains with extensive homology to the sem-5 protein of *C. elegans* and mammalian Grb2 (Clarke et al., 1992; Lowenstein et al., 1992). Genetic analyses suggested that *Drk* function was essential for signalling by the Sevenless receptor PTK (Simon et al., 1993; Olivier et al., 1993). Mutants of the *Drk* gene product were unable to activate Ras, although expression of constitutively activated Ras could bypass the normal requirement for Drk function (Olivier et al., 1993; Simon et al., 1993). Moreover, sequencing of the *Drk* gene from *Drosophila* heterozygous for this mutation identified point mutations in the SH2 domains (Olivier et al., 1993). The recombinant wild type Drk protein was demonstrated to bind the activated EGF receptor and Sevenless both in vivo and in vitro (Olivier et al., 1993; Simon et al., 1993). Consistent with the binding being mediated by the SH2 domain, incorporation of the Drk mutation into a recombinant wild type Drk protein abrogated its ability to bind activated receptors (Olivier et al., 1993). Further, the recombinant Drk protein was found to bind Sos in

vitro (Olivier et al., 1993; Simon et al., 1993), suggesting that the function of Drk is to couple activated receptor PTKs, which it binds with its SH2 domains, to Sos by its SH3 domain.

1.6.2.2 Activation of the Ras signalling pathway in *Caenorhabditis*

In the nematode worm *C. elegans*, differentiation of vulval precursor cells requires an inducible signal from the anchor cell which triggers a signalling cascade within vulval progenitor cells (Horvitz et al., 1991). Genetic evidence indicates that a receptor PTK, Let 23 transmits the signal through a Ras protein encoded by the *let 60* gene (Figure 1.9). Upstream of Let 60 and downstream of Let 23 is the product of the *sem-5* gene (Clarke et al., 1992). A plausible explanation for the role of the sem 5 protein is that it binds to the activated Let 23 receptor PTK through its SH2 domain and a downstream Sos-like exchange factor through its SH3 domains. In accordance, mutations in the SH2 and either SH3 domains of sem 5 give rise to a vulvaless phenotype (Clarke et al., 1992; Pawson, 1992).

1.6.2.3 Activation of the Ras signalling pathway in mammals

Biochemical studies in mammalian cells provide a consistent and complementary picture to the conclusions drawn from the genetic analyses in C. elegans and Drosophila, revealing that the main components of this pathway have been highly conserved through evolution. In mammals the Grb2 protein is the human homologue of the Sem 5 and Drk proteins. Indeed, both Grb2 and Drk are able to rescue sem 5 loss of function mutations in C. elegans (Pawson and Schlessinger, 1993). Further, Drk cDNA was cloned using the phosphorylated tail of the EGF receptor as a probe to screen Drosophila expression libraries (Olivier et al., 1993). The murine (mSos1 and mSos2) and human homologues (hSos) of the *Drosophila Sos* gene are also known (Bowtell et al., 1992; Li et al., 1993). It has been shown that the mammalian Sos protein possesses guanine nucleotide releasing activity for Ras (Chardin et al., 1993; Egan et al., 1993; Li et al., 1993) and that overexpression of Grb2 enhances this activity (Gale et al., 1993). Indeed, the CDC25 related catalytic domain of hSos has been demonstrated to complement loss of CDC25 function in yeast (Liu et al., 1993). Further, overexpression of the Drosophila Sos protein in mammalian Rat-1 cells results in cellular transformation (Egan et al., 1993). These studies strengthen the notion that Sos proteins, initially discovered as signalling components in fly eye development, are so well conserved in evolution that they can participate in activating a mammalian mitogenic pathway.

Hence, the interactions between mammalian tyrosine kinases, Grb2, mSos and Ras have been analysed. Mammalian Sos was found to associate with Grb2 both in vivo and in vitro (Egan et al., 1993; Rozakis-Adcock et al., 1993; Liu et al., 1993; Buday and

Downward, 1993; Skolnik et al., 1993a; Baltensperger et al., 1993), and consistent with complex formation taking place in vitro, the interaction was found to take place independent of ligand stimulation (Rozakis-Adcock et al., 1993; Liu et al., 1993). The Grb2 protein has been demonstrated to bind the proline-rich C-terminal tail of Sos (Egan et al., 1993; Rozakis-Adcock et al., 1993; Liu et al., 1993). Both SH3 domains of Grb2 were implicated in coordinately interacting with the Sos protein as mutations in either SH3 domain, corresponding to loss of function sem 5 alleles (Lowenstein et al., 1992), were found to reduce the affinity of Grb2 binding to Sos in vitro (Egan et al., 1993; Rozakis-Adcock et al., 1993; Liu et al., 1993). Indeed, both SH3 domains were shown to bind different sites on the Sos protein (Egan et al., 1993). Consistent with this, the Sos protein is found to contain four proline-rich sequences which are very similar to the binding consensus for other SH3 domains already described (Ren et al., 1993; Gout et al., 1993). Synthetic peptides corresponding to three proline-rich sequences were found to compete for binding of Grb2 to a C-terminal Sos fragment (Rozakis-Adcock et al., 1993; Liu et al., 1993) suggesting that they may correspond to additional Grb2 binding sites. Further, a ten amino acid C-terminal proline-rich fragment of Sos (VPPPVPPRRR) was able to mediate directly the binding of Grb2 (Liu et al., 1993) and in addition blocked the binding of Grb2 to Sos (Liu et al., 1993), suggesting that this proline-rich sequence is the ligand of at least one of the SH3 domains of Grb2.

The Grb2-Sos complex, present as an inactive complex in the cytosol of unstimulated cells, was found to associate with the activated EGF receptor within one minute of exposure of cells to ligand (Egan et al., 1993; Rozakis-Adcock et al., 1993; Liu et al., 1993; Buday and Downward, 1993; Gale et al., 1993). Indeed, fractionation of quiescent fibroblasts demonstrated that the Sos protein is almost exclusively localised in the cytoplasmic fraction and in response to EGF treatment, a major proportion of the Sos protein is translocated to the particulate fraction. The SH2 domain of Grb2 bound to residues surrounding Y1068 on the EGF receptor (Liu et al., 1993; Skolnik et al., 1993), and a phosphopeptide corresponding to this site was found to block both the assembly of the complex and EGF stimulation of nucleotide exchange on Ras in a permeabilised cell system (Buday and Downward, 1993). Thus, the adapter protein Grb2 binds the activated EGF receptor through its SH2 domain, thereby translocating Sos to the plasma membrane in close vicinity to its substrate Ras. A direct interaction of the Grb2-Sos complex with the PDGF receptor has not been documented although the binding of SHPTP2 to the PDGF receptor has been shown to activate the Ras signalling pathway (Valius and Kazlauskas, 1993). These findings were shown to be significant when it was found that the interaction between the PDGF receptor and the Grb2-Sos complex is mediated via the SHPTP2 phosphatase (Li et al., 1994). It is intriguing that the PDGF receptor homologue Torso has also not been reported to bind Grb2 directly (Perrimon, 1993). Moreover,

overexpression of the product of the activated Ras1 allele has been shown to compensate for the lack of the corkscrew tyrosine phosphatase (csw) suggesting that csw might regulate Ras in Drosophila embryos (Lu et al., 1993). There is also evidence to suggest that stimulation by EGF, PDGF, FGF or insulin leads to phosphorylation of Sos on serine and threonine residues (Rozakis-Adcock et al, 1993). Despite the activation of Ras following EGF stimulation, no change in the guanine-nucleotide releasing activity of Sos is detected (Buday and Downward, 1993). This may indicate that the translocation of Sos to the plasma membrane may enable activation of Ras simply by increasing its local concentration rather than by altering Sos activity.

The Shc protein has also been implicated in Ras signalling pathways. The v-Src tyrosine kinase signals through Ras proteins in Rat-2 fibroblasts but does not bind detectably to Grb2 (Rozakis et al., 1993), however, these cells are found to express Shc (McGlade et al., 1992a). Hence, in these v-Src transformed cells the Shc adapter protein may replace the EGF receptor as the phosphotyrosine target and presumably connect v-Src to the Ras signalling pathway ((Egan et al., 1993; Rozakis-Adcock et al., 1992; 1993; McGlade et al., 1992a). The Shc protein was not found to coimmunoprecipitate with the EGF receptor-Sos complex from stimulated A431 and PC12 cells, despite its being expressed, suggesting that Shc was not neccesary for Sos-EGF receptor complex formation. Similarly, in Rat-1 fibroblasts, Shc was not found to bind the activated CSF-1 receptor, although Shc/Grb2 complexes were observed in resting cells and CSF-1 stimulation resulted in tyrosine phosphorylation of Shc proteins (Van der Greer and Hunter, 1993). A Shc/Grb2/IRS-1 complex has been detected in cells stimulated by insulin (Baltensperger et al., 1993; Skolnik et al., 1993, 1993a). Hence, stimulation with insulin leads to association of the Grb2-Sos complex with two tyrosine phosphorylated proteins, IRS-1 and Shc, although the significance for the presence of Shc in these multiple complexes is presently unknown.

Also intriguing is the observation that the Grb2 protein was also found in direct complex with Bcr-Abl both in vivo and in vitro (Pendergast et al., 1993). This interaction was found to be mediated by the SH2 domain of the Grb2 protein binding Y177 on the Bcr-Abl protein which lies within the YXNX Grb2 binding consensus sequence (Sonyang et al., 1993). Moreover, the Grb2/Bcr-Abl interaction was demonstrated to be necessary for activation of the Ras signalling pathway. In addition, a Bcr-Abl protein lacking Y177 was severely impaired in its ability to transform Rat-1 fibroblasts or induce Ras activation. This suggests that inhibiting the association between Grb2 and Bcr-Abl may represent a potential therapeutic approach to CML.

At first sight a number of proteins, such as GAP, which are also implicated in transducing signals from receptors to Ras are omitted from the scenario described above. However, it is conceivable that the accumulation of RasGTP following Grb2 mediated activation may be compromised by high levels of RasGAP activity, so that effective signal transmission occurs only when RasGAP is inhibited. It is also possible that Grb2 or Sos proteins are modified by other signals emitted from activated receptors to compromise or increase their ability to bind. All these possibilities can now be examined. The mammalian Ras activator RasGRF also appears without a niche. This protein has a more restricted tissue distribution than the Sos proteins (Martegani et al., 1992; Shou et al., 1992), and it also does not contain the proline-rich sequences that couple Sos to Grb2 and hence to receptor PTKs. Presumably, RasGRF is recruited to the membrane by a different mechanism in response to incoming signals, the nature of which has yet to be determined. These findings suggest that Ras may be activated via diverse mechanisms in different cell types presumably with different tyrosine kinases employing alternate pathways.

1.6.3 Downstream effectors of Ras

1.6.3.1 GAP

Ras-GTP must exert its biological effects by interacting with one or more cellular molecules, presumably through its effector domain. A Ras effector has been identified in the yeast *S. cerevisiae*, where Ras activates an adenylyl cyclase complex (Toda et al., 1985). However, Ras does not activate adenylyl cyclase in higher eukaryotes (Birchmeir et al., 1985; Beckner et al., 1985). The observation that mutations in the Ras effector domain prevent RasGAP binding to Ras led to the suggestion that RasGAP may function as a downstream effector of Ras (Adari et al., 1988; Cales et al., 1988). Consistent with this notion, RasGAP is able to block the opening of potassium ion channels in response to muscarinic stimulation of atrial membranes in vitro, a process which requires Ras function (Martin et al., 1992). It has also been reported that RasGAP may function as a downstream effector of Ras in oocyte maturation (Dominguez et al., 1991; Duchesne et al., 1993). In addition, the N-terminal, non-catalytic domain of RasGAP was shown to direct interactions with cellular phosphoproteins such as p190 in vivo and thereby exert an effector function which modulated the cytoskeleton and cell adhesion (McGlade et al., 1993).

However, the proposed effector function of RasGAP is a topic of considerable debate since an equally convincing body of evidence has demonstrated only a negative regulatory function for RasGAP in the Ras signal transduction pathway. For example, ectopically expressed RasGAP inhibited transformation by overexpressed normal Ras

suggesting that in this case RasGAP was functioning primarily as a negative regulator of Ras (Zhang et al., 1990). In *Drosophila*, loss of function mutations in the RasGAP homologue *GAP1*, increase signal transduction through a Ras-dependent pathway, suggesting that GAP1 is not the effector of Ras in this case (Gaul et al., 1992). IRA1 and IRA2, the *S. cerevisiae* homologues of RasGAP have been proposed to function upstream of the Ras1 and Ras2 genes downregulating Ras activity (Tanaka et al., 1989; 1990). Thus, although RasGAP may mediate Ras activity in some situations it cannot be the only effector of Ras. Indeed, the complex roles of Ras in different cell types and organisms suggests multiple targets.

1.6.3.2 Raf

The serine/threonine kinase Raf has been positioned downstream of Ras in several studies of signal transduction pathways. Indeed, there is a growing body of evidence suggesting that Raf is a direct effector of Ras, that is both necessary and sufficient for Ras stimulated signal transduction. Drosophila Raf (DRaf) function is necessary for R7 cell specification and can induce R7 development in the absence of Sevenless function (Dickenson et al., 1992). Further, genetic evidence implicates Raf as acting downstream of Ras since the activation of the Ras pathway was suppressed upon introduction of dominant-negative Raf into flies carrying activated Ras genes (Dickenson et al., 1992). Similar results have been observed for the PDGF receptor -like Torso which triggers terminal cell fate development in the Drosophila embryo (Sprenger and Nusselein-Volhard, 1992). Here D-Raf activity was also shown to be required downstream of Torso (Ambrosio et al., 1989) and Ras (Lu et al., 1993). In Xenopus, both Ras and Raf are required for FGF induced mesoderm formation (Whitman and Melton, 1992; MacNicol et al., 1993). In C. elegans, Ras and Raf are both required for signalling through Let 23 (Han et al., 1993). Similarly, in NIH 3T3 cells the essential role of c-Raf-1 in the transduction of proliferative signals initiated by receptor PTKs and transmitted through c-Ras is demonstrated by the ability of dominant-negative c-Raf-1 mutants (Kolch et al., 1991; Bruder et al., 1992) to block DNA synthesis and growth stimulated by serum, the phorbol ester TPA or by Ras oncogenes. Further evidence for the central role of Raf-1 as a downstream effector of Ras is the ability of v-Raf expression to overcome the growth arrest effects produced by microinjection of inhibitory anti-Ras antibodies (Smith et al., 1986; Huleihel et al., 1986). Studies using activated Raf protein (Woods et al., 1993) and dominant negative Ras mutants have also implicated Raf in Ras-dependent NGF induction of PC12 cell differentiation (Woods et al., 1993).

The most compelling evidence directly linking the Ras and Raf proteins in a mitogenic signalling cascade came from a series of experiments utilising a variety of recombinant expression systems. Firstly, Raf was identified as a binding protein using a yeast two-

hybrid system to screen proteins that interacted with Ras, (Votjek et al., 1993), secondly, Raf was shown to bind an immobilised Ras protein used to probe brain cytosol (Zhang et al., 1993), and finally, using recombinant bacterially expressed fusion proteins, Raf and Ras were shown to interact directly in vitro (Warne et al., 1993; Votjek et al., 1993; Zhang et al., 1993). The amino terminal cysteine-rich regulatory region of Raf has been shown to bind directly to Ras with high affinity. The binding was found to be strictly dependent on the Ras protein being in the active GTP-bound conformation rather than the inactive GDP-bound state. As anticipated the interaction with Raf was mediated by the effector region of Ras, furthermore Raf was found to act as a potent competitor with the RasGAP and NF1 proteins for binding to Ras. Indeed, Raf itself displayed weak GTPase-stimulating activity towards Ras (Warne et al., 1993; Votjek et al., 1993; Zhang et al., 1993). However in these studies, a reduced affinity interaction with GTP-bound Ras was observed using the full-length Raf protein. This suggests the presence of an as yet unidentified protein that may increase the affinity of interaction in mammalian cells, presumably by exposing the N-terminus of Raf for binding to Ras. Indeed, GTP-bound Ras has been shown to interact with Raf present in cell lysates (Moodie et al., 1993).

1.6.4 Effectors of Raf

Despite the remarkable diversity of mitogens that activate quiescent cells, their immediate targets and the signalling pathways stimulated, there is an enormous unity in the response: virtually all mitogens lead to the rapid activation of a 42 kDa and a 44 kDa mitogen-activated protein kinases (MAPK) termed ERK2 and ERK1 respectively (extracellular signal-related kinases). MAP kinases exist in a dephosphorylated form in quiescent or unstimulated cells and become activated upon phosphorylation of both a tyrosine and a threonine residue. The phosphorylation sites have been identified as threonine 183 and tyrosine 185 in mammalian 42 kDa MAP kinase (Payne et al., 1991) and these residues lie within the consensus sequence TEY. This sequence is conserved in MAP kinases from yeast, *Drosophila* and mammals. The activation of MAP kinase by phosphorylation is performed by a single dual specificity MAPK kinase (MKK) which is unusually selective in its substrate specificity (Matsuda et al., 1992; Reviewed in Rudermann 1993). MAPK kinase itself is positively regulated by phosphorylation on serine residues indicating the existence of a MAPKK kinase (MKKK).

The direct introduction of oncogenic Ras into fibroblasts, *Xenopus* oocytes or the overexpression of active Ras in fibroblasts and PC12 cells in the absence of growth factors leads to rapid and persistent MAP kinase activation (Leevers and Marshall, 1992; Thomas et al., 1992; Wood et al., 1992; Pomerance et al., 1992). Conversely, MAP kinase activation is blocked by the introduction of dominant negative Ras mutants or

overexpression of RasGAP (Woods et al., 1992; Thomas et al., 1992; Nori et al., 1992). Several reports indicate that addition of the Ras to cell free extracts prepared from mature *Xenopus* oocytes activates the endogenous MAP kinase (Shibuya et al., 1992; Itoh et al., 1993). Such activation requires Ras that has been fully processed at its carboxyl terminus and is in the GTP-bound state (Itoh et al., 1993). Further, a mutation in the Ras effector domain, which abolishes Ras transforming activity prevents *Xenopus* MAP kinase activation (Pomerance et al., 1992). Consistent with this overexpression of Grb2 in cells enhanced MAP kinase activation and a dominant-negative form of Ras blocked insulin-induced activation of MAP kinase (Skolnik et al., 1993a).

These data all identify Ras as an upstream regulator of MAP kinases and therefore suggest the existence of a Ras activated kinase cascade. A signalling link between Ras and Raf has been established and consistent with this Raf may act as an upstream regulator of MAP kinases. Indeed, there is a body of evidence directly implicating Raf-1 as a positive regulator of MAP kinase activation. Transient expression of activated c-Raf or transformation by v-Raf can lead to persistent MAP kinase activation (Howe et al., 1992); protein complexes from stimulated cells, containing MAPK kinase activity and Raf-1 protein, were dependent on the activity of Ras (Moodie et al., 1993); Raf immunoprecipitated from stimulated cells can reactivate MAPK kinase and this activation of Raf can be stimulated in vitro using purified recombinant components (Howe et al., 1992; Kyriakis et al., 1992; Dent et al., 1992; MacDonald et al., 1993 Schaap et al., 1993). Moreover, a Drosophila gene (Dsor1) encoding a MAPK kinasehomologue has been isolated as a suppressor of Drosophila Raf-1 loss of function mutations (Tsuda et al., 1993) hence demonstrating the connection between Raf and MAP kinase in a physiological environment. Further, a Drosophila MAP kinase, ERK-A, encoded by the rolled locus was shown to be required downstream of the Sev signal transduction pathway (Figure 1.9) (Brunner et al., 1994; Biggs III et al., 1994). The MAP kinase phosphorylation cascade is also found to be conserved in yeast (Figure 1.9). Genetic studies in S. pombe have revealed the existence of spk1, a MAP kinase homologue, byr 1 the equivalent of MAPK kinase, and byr 2 which serves an equivalent role to the mammalian MAPKK kinase, and this pathway is also activated by Ras. However, it should be noted that although byr 2 shows striking homology to the two MAPKK kinases STE11 and BCK present in S. cerevisiae, byr 2 from S. pombe does not show high sequence homology to Raf-1 in vertebrates. By anology with yeast, there are presumably several distinct kinds of MAPKK kinases present in vertebrate cells.

The activation of MAP kinase leads ultimately to the phosphorylation of known nuclear proto-oncogenes including c-Fos, c-Jun, and c-Myc (reviewed in Roberts, 1992) and

hence transcription and mitogenesis. These pathways are represented schematically in Figure 1.8.

More recently a number of papers were published describing cross talk between two major signalling pathways; namely the Ras and the cyclic adenosine 3' 5'-monophosphate (cAMP) pathways. cAMP was the first signalling messenger to be identified and its role in regulating physiological processes is well established (Reviewed in Cohen 1988). Hormone receptors increase the intracellular concentration of cAMP by increasing the level of the free alpha subunit of the GTP-binding protein Gs ($G\alpha_S$ GTP), which in turn activates adenylyl cyclase (Reviewed in Choi et al., 1993). However, the precise role of cAMP in regulating cell growth and proliferation remains a matter of debate. In some cells, such as Swiss 3T3 cells and thyrocytes, cAMP is a mitogenic messenger and promotes the G1 to S phase transition in the cell cycle (Dumont et al., 1993). In contrast, cAMP inhibits the proliferation of other fibroblast cell lines, T-cells and some cells transformed by Ras and Src (Pastan et al., 1975). Increased concentrations of cAMP were found to block activation of Raf-1, MAPK kinase and MAP kinase in a number of cell types (Wu et al., 1993; Cooke and McCormick 1993; Graves et al., 1993; Burgering et al., 1993). The full significance of this remains to be determined although its of great potential as this is the first time the Ras pathway has been physiologically blocked in vivo which may allow construction of methods of inhibiting abnormal cell growth of those cancers in which the Ras pathway is overactive.

1.7 Conclusion

A simple preliminary mapping of signalling pathways has been defined in particular for the 'Ras pathway' (Figure 1.8). However, the situation in vivo is clearly more complex and cross-talk and divergence exists between major pathways. PI 3-kinase does not appear to function in the 'Ras-signalling pathway', although precedent suggests that this signal-transducing molecule is important for cellular proliferation (Fantl et al., 1992; Valius and Kazlauskas, 1993) and plays a role in transformation (Uleg et al., 1990; Serunian et al., 1990). These properties, as well as its ubiquitous expression and association with activated growth factor receptors suggests it merits further study. In the following Chapters a biochemical investigation of the subunit structure of PI 3-kinase, together with a functional analysis of the regulation of the enzyme are described. The biological implications of the presence of PI 3-kinase in a particular cell type are discussed with respect to the function of this enzyme.

Chapter 2: Experimental procedures

2.1 GENE CLONING TECHNIQUES

2.1.1 Preparation of DNA

2.1.1.1 Precipitation of Nucleic acids with ethanol

Nucleic acids were precipitated by the addition of 0.1 volumes of 3 M sodium acetate [pH 5.2] and 2.5 volumes of ethanol that had been stored at 20°C. The samples were mixed thoroughly and then incubated on dry ice for 10 min or on ice for 20 min, followed by centrifugation at 10 000 g for 20 min at 4°C. The nucleic acids were collected and washed with 70% (v/v) ethanol, dried under vacuum and resuspended in TE to the required concentration.

2.1.1.2 Phenol/chloroform extraction

Phenol and chloroform denature proteins and are thus used to extract proteins from DNA solutions. TE saturated phenol was prepared according to Sambrook et al., (1988) and mixed 1:1 with chloroform:isoamylalcohol 24:1. The volume of the DNA solution was adjusted to 200 µl and an equal volume of phenol and or chloroform:isoamylalcohol added. The phases were mixed well by vortexing for several minutes and separated by centrifugation at 10 000 g for 5 min. The upper aqueous phase was transferred to a fresh tube leaving behind the material at the interphase. A phenol extraction was always followed by a chloroform extraction to remove any residual phenol and the DNA precipitated with ethanol as described above.

2.1.1.3 Miniprep of plasmid DNA

This procedure was used to prepare small amounts of DNA that could be used for rapid identification purposes. A single bacterial colony was used to innoculate 2 ml of L-Broth supplemented with the appropriate antibiotic(s) and the culture incubated with shaking at 37° C overnight. 1.5 ml of this culture was then transferred to an eppendorf tube and the bacterial cells were pelleted by centrifugation at $10\,000\,g$ for 3 min and the remaining culture was used to make a glycerol stock [12.5% (w/v)]. The cell pellet was then resuspended in $50\,\mu$ l of TNE ($10\,m$ M Tris-HCL [pH 8.0], $100\,m$ M NaCl, $1\,m$ M EDTA). The DNA was then purified from proteins by a phenol/chloroform extraction followed by ethanol precipitation. The purified DNA was finally dissolved in $20\,\mu$ l of water containing $50\,\mu$ g/ml RNase.

2.1.1.4 Midiprep of plasmid DNA

This is a variation of the maxiprep procedure and was used to prepare good quality DNA for small scale sequencing. A 5 ml culture of LB and antibiotics was innoculated with a single bacterial colony or from a bacterial culture and then incubated with shaking at 37 °C overnight. The bacterial cells were pelleted by centrifugation at 2000 g for 5 min and then

resuspended in 100 ml GTE (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl [pH 8.0]) and left for 5 min at room temperature. The DNA was denatured and RNA hydrolysed with the addition of 150 μ l of ice-cold 5M potassium acetate and gentle mixing by occasional inversion on ice for 5 min. The DNA was separated from other cell debris by centrifugation at 10 000 g for 5 min and the supernatant transferred to a fresh tube. Any remaining RNA was removed by adding RNase to a final concentration of 20 μ g/ml and incubation at 37°C for 20 min. The DNA was purified free of other proteins by phenol/chloroform extraction and ethanol precipitation and then dissolved in 16 μ l of water and subjected to PEG precipitation by the addition of 4 μ l of 4 M NaCl and 20 μ l of 13% PEG (w/v). The sample was mixed well and then incubated on ice for 20 min. Nucleic acids were precipitated by centrifugation at 10 000 g for 10 min after which the supernatant was removed and the pellet washed with 1 ml of 70% (v/v)ethanol. Finally the pellet was dissolved in 100 μ l of water, after which it was subjected to a phenol/chloroform extraction and ethanol precipitation.

2.1.1.5 Maxiprep of plasmid DNA

This procedure was used to produce large amounts of transfection quality DNA. 250 ml of L-Broth were innoculated with a 2 ml L-Broth culture that had been innoculated with a single colony the previous night. These 250 ml cultures were then incubated with shaking at 37°C. Bacterial cells were pelleted by centrifugation at 3000 g for 20 min and resuspended in 10 ml of GTE (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl [pH 8.0]), 1.25 ml of 20 mg/ml of lysozyme solution, and the mixture incubated on ice for 10 min. 20 ml of a solution containing 0.2 M NaOH and 1% SDS (w/v) was added, the tube inverted several times and incubated on ice for 10 min. The DNA was purified from proteins by precipitation using 10 ml of 5M potassium acetate on ice for 15 min and then centrifugation at 8000 g for 10 min at 4 °C. The supernatant was transferred to a clean tube and nucleic acids precipitated by the addition of 0.6 volumes of isopropanol and centrifugation at 8000 g for 20 min. The DNA pellet was then washed with 70% (v/v)ethanol and resusupended in 5 ml of TE containing 5 g of CsCl. 200 µl of ethidium bromide (10 mg/ml) was added and the solution subjected to centrifugation at 8000 g for 10 min to remove any cellular debris. The supernatant was then transferred to Beckman polyallomer quick seal ultracentrifuge tubes. The tubes topped up with the TE/CsCl solution (ideal weight 9.3-9.9 to ensure the correct density gradient is achieved) and centrifuged overnight at 200 000 g. Closed circular plasmid DNA was isolated by drawing out the lower of the two florescent bands visible on UV illumination (the upper band containing chromosomal DNA). The plasmid DNA was rebanded to remove any contaminating chromosomal DNA by centrifugation for 4 h at 275 000 g. The ethidium bromide was extracted with equal volume of water saturated isopropanol, the upper pink organic phase discarded and the extraction repeated until the aqueous phase was colourless. The DNA was precipitated at -20 °C on the addition of 3 volumes of ethanol and nucleic acids were collected by centrifugation for 20 min at 11 000 g. The DNA was finally washed with 70% (v/v) ethanol, dried under vacuum and resupended in TE.

2.1.2 Preparation of competent E. coli.

A 2 ml LB-culture was innoculated with a single E. coli colony and grown overnight at 37 °C with shaking. This was then used to innoculate 10 ml of nutrient broth at a dilution of 1/100. After growth to OD₆₀₀=0.4, the cells were pelleted by centrifugation at 4 °C for 10 min at 2000 g, resuspended in 5 ml of ice-cold 50 mM calcium chloride by shaking, and left on ice for 15 min. The cells were then spun again as above and suspended in 400 μ l of ice-cold CaCl₂ (50 mM) and stored on ice until used.

To make a large stock for long term storage at -70 °C, the same procedure was used with a 50 ml bacterial culture. Cells were pelleted and resuspended in 1/3 of the culture volume in a buffer containing 100 mM KCl, 50 mM CaCl₂, 10% (v/v) redistilled glycerol and 10 mM potassium acetate that had been sterilised through a 0.2 μ m filter. The mixture was incubated on ice for 10 min after which the cells were centrifuged at 1000 g at 4 °C for 5 min and then resuspended in 1/12.5 of the original culture volume. 200 μ l aliquots of the cell suspension were then flash frozen in a dry-ice ethanol bath for 5 min and then stored at -70 °C for several weeks.

2.1.3 Transformation of E. Coli

The DNA was added to competent cells, mixed by gentle pipetting and incubated on ice for 30 min to allow the DNA to be absorbed onto the cells. The cells were then induced to take up the DNA by heat-shock at 37°C for 5 min, after which 0.5 ml of L-Broth was added and the cells incubated at 37 °C with shaking for 30 min. The cells were then gently spread onto a pre-dried agar plate containing the relevant antibiotic selection and incubated at 37 °C overnight.

2.1.4 Subcloning Restriction fragments

2.1.4.1 Digestion of DNA with Restriction endonucleases

DNA was restricted according to the manufacturers specifications. Generally, restriction enzymes can be divided into 3 catagories based on their buffer requirements:

	low salt	medium salt	high salt
NaCl	0	50 mM	100 mM
Tris-HCl [pH 7.4]	10 mM	10 mM	50 mM
MgCl2	10 mM	10 mM	10 mM
DTT	1 mM	1 mM	1 mM

2.1.4.2 Ligation of DNA fragments

Ligation of DNA fragments was generally performed in 20 µl final volume. The DNA was ligated in a 1:1 ratio when cloning a restriction fragment into a vector, mixed with 2 µl of 10X ligation buffer (660 mM Tris-HCl [pH 7.6], 66 mM MgCl₂, 100 mM DTT, 6.6 mM ATP) and the volume adjusted to 20 µl with water. 1 unit of T4 DNA ligase was used and the reaction incubated at 16°C overnight, after which the reaction was terminated by chloroform extraction and the DNA ethanol precipitated prior to further processing.

2.1.4.3 Phosphatase treatment of restriction fragments

To prevent self ligation of digested vector in a ligation reaction, the vector was first was dephosphorylated. Briefly, the vector was digested with the appropriate restriction enzyme, extracted with chloroform, ethanol precipitated and resuspended in medium restriction enzyme salt buffer. To this was added 0.2 units of calf intestinal alkaline phosphatase (Bohringer), SDS to a final concentration of 0.2% (w/v) and the sample incubated at 37°C for 30 min. The reaction was then terminated by the addition of EGTA to a final concentration of 20 mM to chelate all Mg²⁺ ions and heat inactivation of enzyme at 68 °C for 15 min. The DNA was then phenol/chloroform extracted twice and ethanol precipitated.

2.1.4.4 Electrophoresis of DNA fragments

DNA molecules are fractionated according to size when run on an agarose gel. 1% (w/v) gels were generally used although 2% (w/v) gels were employed for analysis of DNA fragments less than 500 bp in size, and 0.7% (w/v) gels for DNA larger than 10 Kb. The appropriate volume of 1% agarose (w/v) in TAE buffer (0.004M Tris-Acetate, 0.001M EDTA) was made and heated to allow the agarose to dissolve. The agarose solution was cooled to 60 °C and the ethidium bromide added to a concentration of 1 μ g/ml. 0.1X total volume loading buffer (0.25% (w/v) xylene cyanol, 0.25% (w/v) bromophenol blue, 15% (w/v) Ficoll type 400 in water) was added to the samples and electrophoresis performed in TAE at 5V/cm against standard molecular weight markers (Boehringer).

2.1.4.5 Polyacrylamide electrophoresis

DNA fragments of less than 1 Kb in length were purified by polyacylamide gel electrophoresis (PAGE) in general at an acrylamide concentration of 9% (w/v). 15 ml of a solution of acrylamide:bis 30:1 was added to 5 ml of 10X TBE and the volume adjusted to 50 ml with water. Polymerisation was initiated on the addition of 125 μ l of 10% (w/v) ammonium persulphate and 10 μ l of TEMED. Electrophoresis was performed against DNA size markers (Gibco-BRL) until the samples had migrated the required distance.

2.1.5 Purification of DNA

2.1.5.1 Electroelution of DNA from agarose gels

Used mainly for large fragments of DNA. The band corresponding to the DNA fragment of interest was localised using a long-wave length (300-360 nm) UV lamp and an agarose slice

extracted using a sharp scalpel. The slice was then placed into dialysis tubing containing 400 µl of 0.5X TBE. The dialysis bag was immersed into a shallow layer of 0.5X TBE in an electrophoresis tank. Current was then passed through the bag (100 V for 2 h or 20 V overnight), after which the polarity was reversed briefly to release the DNA from the wall of the dialysis bag. The buffer was then carefully recovered from the bag and the DNA containing solution chloroform extracted and ethanol precipitated.

2.1.5.2 Using a nitrocellulose column

This method was fast and efficient and used on a more routine basis. DNA was resolved on a low melting point agarose gel and the band of interest was excised in the same way as described above and placed into an eppendorf. To extract the DNA from the agarose the sample was placed on dry ice for 10 min followed by incubation at 37 °C for 10 min. The melted agarose was then carefully transferred to a COSTAR spin column which has a 0.22 µm nitrocellulose acetate membrane. The sample was centrifuged for 30 min, after which the DNA solution that passed through the column was ethanol precipitated.

2.1.5.3 Gel purification via phenol extraction

This procedure was used to purify small DNA fragments.

Following excision of band from a low melting point gel, the gel slice was placed onto an eppendorf tube with an equal volume of TE (approx. 300 μ l) and incubated at 65°C for 10 min. The aqueous phase was then extracted with TE-saturated phenol that had been preheated to 65°C three times after which the aqueous phase was subjected to a phenol/chloroform extraction, followed by a chloroform extraction and then ethanol precipitation.

2.1.5.4 Electroelution from PAGE gels

Following electrophoresis, the gel was soaked in TBE containing 1 μ g/ml ethidium bromide for 10 min and the DNA fragments visualised by UV illumination. The required fragment was then electroeluted from the acylamide using the same procedure as described for agarose gels in section 2.1.5.4.

2.1.5.5 Oligonucleotide purification by denaturing PAGE

Oligonucleotides were purified from denaturing acrylamide gels for use as radioactively labelled probes and primers. The oligos were synthesised using Applied Biosystems 380B model and then purified on a 15% (w/v) polyacrylamide-2M urea gel (1.5 mm thick).

The gel was prepared as:

Water	4 ml
30% (bis 1%) (w/v) polyacylamide	25 ml
10X TBE	5 ml
urea	21 g
15 % APS	235 µl
TEMED	<u>23.5 μl</u>
	50 µl

The gel was pre-electrophoresed in 1X TBE at 200 V for 30 min and the oligonucleotide resuspended in formamide loading buffer (99% (v/v) formamide, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol, 1 mM EDTA), loaded onto the gel at approx. 20 μ g per well. Electrophoresis was performed at 29 mA until the bromophenol blue front had migrated 2/3 of the length of the gel. The oligonucleotide was visualised by UV shadowing against a thin layer silica chromotography plate (Merck 60F254 (Cat No. 5735) illuminated using a short wave UV lamp. The band of interest was excised from the gel and placed into an eppendorf tube, crushed in 300 μ l of TE and incubated overnight at 37 °C. The supernatant was then harvested by centrifugation for 5 min and the crushed gel washed again with another 300 μ l of TE. The two supernatants were combined and then passed through a siliconised glass wool mass to ensure no acrylamide was present. The oligonucleotide was then precipitated using 0.1 volume of 3 M sodium acetate [pH 5.2] and 4 volumes of ethanol on dry ice for 30 min. The pellet was washed using 100% (v/v) ethanol and then the sample dried, suspended in 10 μ l sterile water and the concentration checked by spectrophotometry at λ 260.

2.1.6 Detection of DNA using radioactive probes

2.1.6.1 The radioactive labelling of DNA

Firnberg and Vogelstein (1983) introduced the use of random sequence hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length. The unique property of the Klenow DNA polymerase I to prime only in the 3'-5' direction and an absence any 5'-3' exonuclease activity ensures that labelled nucleotides incorporated by the polymerase are not subsequently removed as monophosphates. Thus, this approach generates a stable probe of high specific activity. A multiprime DNA labelling kit marketed by Amersham was used to label probes using this approach (RPN 1601).

A DNA fragment was labelled as follows: 25 μ g of DNA was denatured by heating to 100 °C for 2 min in a boiling water bath and then chilled on ice. The reaction was then set up in an eppendorf tube on ice as follows:

11.5 µl	containing 25 ng of denatured DNA
5 μl	soln 2 (dATP, dGTP and dTTP in Tris-HCl [pH 7.8], MgCl $_2$ and $\beta\text{-}$
	mercaptoethanol).
2.5 µl	primer (hexanucleotides)
5 μl	dCTP (α -32P 0.5 μ Ci)
<u>1 µl</u>	1 unit DNA polymerase I Klenow fragment
25 μl	

The contents were gently mixed by pipetting and then incubated at 37 °C for 30 min. Unincorporated $[\alpha^{-32}P]dCTP$ was removed by passing the reaction through a Sephadex G50 column as described below.

5' end labelling of oligonucleotides

T4 polynucleotide kinase catalyses the transfer of the γ -phosphate of ATP to the 5' hydroxyl-terminus of the DNA strand. The oligonucleotides were synthesised on the Applied Biosystems DNA synthesiser, purified as described in section 2.1.5.5 and then 5' labelled according to the following protocol:

1-5 pmol

oligonucleotide

 $2 \mu l$

kinase buffer

50 μCi [γ^{32} P]ATP (10 mCi/ml Amersham)

10 units T4 polynucleotide kinase

20 µl final vol.

The above components were mixed and incubated at 37 °C for 45 min. The reaction was terminated by the addition of EDTA to give a final concentration of 20 mM, and boiling at 65°C for 2 min. The labelled oligonucleotide was then used for hybridisation studies.

2.1.6.3 Removal of unincorporated radionucleotide from a random priming reaction reaction

Unincorporated radioactively labelled nucleotide was removed from a random priming reaction by passing the reaction mix through a spun Sephadex G50 column. The nucleotides are retarded in the Sephadex matrix whereas the labelled DNA molecules are collected in the eluate. The Sephadex G50 beads were pre-swollen in column running buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl). The bottom of a 1 ml syringe was blocked with a small quantity of siliconised glass wool and the syringe filled to capacity with the G50 suspension. The column was packed by centrifugation for 5 min at 500 g and equilibrated by the addition of 100 µl of column running buffer containing 0.1% (w/v) SDS and centrifugation as before. This procedure was repeated until 100 µl of buffer had eluted. The volume of the reaction mix was increased to 100 µl by the addition of column running buffer containing 0.1% (w/v) SDS, and the sample loaded on the column. The radioactively labelled DNA molecules were collected upon centrifugation, the DNA denatured by incubation at 95°C for 5 min and then stored on ice until required.

2.1.6.4 Removal of unincorporated nucleotide from end labelled oligonucleotides

For end-labelled oligonucleotides a similar strategy was employed where the 5' labelled oligonucleotide was purified by passing the reaction mix through a DE52 column and the oligonucleotide eluted from the matrix using 1 M NaCl. A 1 ml gilson tip was used as a column. The end of the tip was loosely packed with siliconised glass wool and then 100 µl of preswollen DE52 was added on top. This mini column was then equilibrated using several volumes of a buffer containing 0.2 M NaCl in TE to remove the unincorporated nucleotides. The oligonucleotide was eluted in 3x 0.5 ml of buffer containing 1 M NaCl in TE and the specific activity of the probe calculated.

2.1.6.5 Hybridisation of DNA immobilised on nitrocellulose filters using radiolabelled restriction fragments

Bacteria were either streaked directly onto nitrocellulose paper or a direct lift of the bacteria colonies on the plate was made by placing the nitrocellulose on the agar plate for 3 min. The filter paper was orientated by making holes in the agar and paper using a needle. The bacteria were then lysed by laying the filter colony side up for 2 min on a piece of Whatman 3 MM paper soaked in Denaturing solution (0.5 M NaOH, 1.5 M NaCl). This was then neutralised by incubation for 5 min on a piece of Whatman 3 MM soaked in Neutralising solution (1.0 M Tris-HCl [pH 7.4], 1.5 M NaCl). After washing the filter in 3x SSC and 0.1% (w/v) SDS to remove any residual agar, the filter was allowed to air dry colony side up for approximately 30 min. The DNA was then irreversibly bound either by baking under vacuum at 80°C for 2 h or by cross-linking using UV light for 90 sec in a UV Stratalinker 2400. As an optional step the filter could be washed in a buffer containing 3x SSC and 0.1% (w/v) SDS at 65 °C for 1 h to remove any excess protein. The filter was then prehybridised in a buffer containing 50% (v/v) formamide, 5X SSC, 5X Denhardts, 1 mM Sodium pyrophosphate, 50 mM sodium phosphate, 0.1 mM ATP, 0.1% (w/v) SDS and 0.1 mg/ml sheared sonicated salmon sperm DNA, to block any sites on the nitrocellulose that might bind the probe non-specifically. The filter was sealed in a plastic bag with the minimum volume of pre-hybridisation buffer required to cover the filter, air bubbles were removed, and the filters incubated overnight. The filters were washed 4 X 5 min at room temperature in 3X SSC, 0.1% (w/v) SDS and then for 30 min at 65 °C with one change of buffer. If the background radioactivity on the filters remained high, the stringency of the wash buffer was increased to 1X SSC, 0.1% (w/v) SDS and the incubation continued for a further 30 min with one buffer change. A final wash using 0.1% (w/v) SDS was performed if the background still remained high and then the filters air dried and exposed to Kodak (XAR 5) film at -70 °C against an intensifying screen.

2.1.6.6 Hybridisation of filters using oligonucleotides

The oligonucleotide was labelled to high specific activity with $[\gamma^{32}P]ATP$ and T4 polynucleotide kinase and purified as described in section 2.1.5.5. The filters were prehybridised for 2-4 h at 5-10 C< tm for the oligonucleotide in prehybridisation buffer (6x SSC, 5x Denhardt's, 0.1% (w/v) SDS and 10 µg/ml sheared and sonicated salmon sperm DNA). The probe was added and the incubation continued overnight at the same temperature. The filters were washed at increasing stringency monitoring the level of radioactivity between each wash, starting with 6x SSC, 0.1% (w/v) SDS for 10 min at room temperature, followed by 2x SSC, 0.1% SDS for 10 min at room temperature, and finally if necessary 2x SSC, 0.1% (w/v) SDS at 40°C for 30 min. The filters were then exposed to Kodak (XAR 5) film at -70°C.

If reprobing of the filters was required they were placed in TE and 50% (v/v) formamide at -70°C for 1 h, and then prehybridised and hybridised as already described.

2.1.7 Double stranded sequencing

The sequencing reaction

The chain-termination method of sequencing DNA involves the in vitro synthesis of a DNA strand by a DNA polymerase I, using a single stranded DNA template. Synthesis is initiated from a unique site in the template where the oligonucleotide primer anneals. The synthesis reaction is terminated when a nucleotide analogue is incorporated. These analogues are 2',3'-dideoxynucleotide 5'-triphosphates (ddNTPs) that do not support continued DNA synthesis, since they lack the 3'OH which is involved in the elongation reaction. When the correct ratio of dNTPs and one of the four ddNTPs is achieved, enzyme catalysed polymerisation will terminate in the proportion of the population of DNA molecules where the ddNTP was incorporated. Four separate reactions, each involving a different ddNTP, will generate complete sequence information. A radioactively labelled nucleotide is included in the reaction so that the population of labelled molecules can be visualised by autoradiography following separation by electrophoresis.

The sequencing kit from Phamacia LKB was employed for all sequencing reactions with double stranded DNA template. The relevent oligonucleotide primer was then annealed to the template and then enzyme catalysed extension of this primer occurs in two stages. This was because T7 DNA polymerase was used as opposed to the Klenow fragment and T7 DNA polymerase uses dideoxynucleotides very readily and thus they are excluded in the first labelling reaction, in which extension is initiated using all 4 deoxynucleotides, one of which is radiolabelled. This was then followed by four separate termination reactions each start with 1/4 of the labelling reaction and including a single dideoxynucleotide in addition to the four deoxynucleotides. Midiprep DNA was used in sequencing reactions as care needs to be taken to remove all proteins that may interfere in sequencing.

Double stranded DNA was denatured by adding to a microcentrifuge tube;

8 µl water containing

2-3 µg DNA template

 $2 \mu l$

1M NaOH

10 µl

This mixture was vortexed gently, briefly centrifuged and the tube was incubated at room temperature for 10 min. The DNA was then precipitated with the addition of;

 $3 \mu l$

3 M sodium acetate [pH 4.8]

 $7 \mu l$

distilled water

 $60 \mu l$

100% (v/v) ice-cold ethanol

and placed on dry ice for 15 min. The precipitated DNA was collected by centrifugation and the pellet washed with ice-cold 70 % (v/v) ethanol, dried and then resuspended in 10 μ l distilled water. The primer was annealed to the denatured DNA by an optimal primer concentration contained in 2 μ l of water added to the denatured DNA with 2 μ l of annealing buffer. The annealing reaction was allowed to proceed for 20 min at 37°C and then cooled to room temperature. The labelling reaction was performed by the following components being added to the primer/DNA reaction tube.

3 μl labelling mix (a mixture of deoxynucleotides)

 $1 \mu l (10 \mu Ci)$ [α³⁵S]dATP

2 μl (3 units) diluted DNA polymerase

The components were mixed and the contents collected at the bottom of the tube by a brief centrifugation and incubated at room temperature for 5 min. For each labelling reaction, 4 tubes were labelled GATC and to each was added 2.5 μ l of the appropriate dideoxynucleotide, ie ddATP, ddGTP, ddCTP, ddTTP. To each of these tubes 4.5 μ l of the labelling mix was added. The components were mixed by gentle agitation and then incubated at 37°C for 5 min. The reaction was then terminated with the addition of 5 μ l of stop solution (95% (v/v) formamide, 20 mM EDTA, 0.05%`(w/v) bromophenol blue and 0.05% (w/v) xylene cyanol FF), the reactions heated to 75 °C for 2 min and then 2-3 μ l loaded onto a 6% (w/v) sequencing gel.

The preparation of 7.6 M urea sequencing gels.

The sequencing reactions were analysed on 6% (w/v) acrylamide, 7.6 M urea gels as follows.

50 g urea

15 ml 40% (w/v) acrylamide/2 % (w/v) Bis acyrlamide

44 ml distilled water

This mixture was passed through a 0.2 µm filter and then made upto 100 ml with the addition of 10 ml 10X TBE. 400 µl of 10% (w/v) ammonium persulphate and 40 µl TEMED were added to initiate polymerisation and the gel poured immediately between the glass plates. The gel was pre-electrophoresed in TBE at 40 W for 30 min. The samples were boiled for 3 min in order to separate the labelled sequenced strand from the template strand and 2-3 µl loaded into the wells. Electrophoresis was performed at 40 W (~2000 V) until the bromophenol blue in the sample had migrated to the bottom of the gel. The gel was then fixed for 20 min in 10% (v/v) acetic acid, 10% (v/v) methanol, dried under vacuum for ~20 min at 80°C and then autoradiographed against Kodak (XAR 5) film.

2.1.8 Polymerase chain reaction (PCR)

PCR rections were performed in 50 μ l volumes containing 15 mM Tris HCl [pH 8.8], 60 mM KCl, 2.25 mM MgCl₂, 0.25 mM dNTPs (Pharmacia), 0.2 pmol/ μ l of each primer and 0.05 U/ μ l of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) and covered with a couple

of drops of mineral oil (Sigma Chemical Co. Ltd.). Template DNA was provided by 1-10 ng plasmid DNA and amplication was performed on a Perkin Elmer Cetus thermal cycler. Samples were denatured (94°C, 30 s), annealed (50°C, 30 s) and extended (72°C, 60 s) for 30 cycles followed by a 5 min extension at 72°C. The mineral oil was extracted with chloroform and the PCR products were resolved by TAE/agarose gel electrophoresis and purified as described in section X.

2.2 CELL CULTURE METHODOLOGY

2.2.1 Preparation of Insect Tissue Culture Medium

2.2.1.1 TNM-FH

Grace's medium (Gibco) was supplemented with 10% (v/v) fetal calf serum (FCS), 3.3 g/l lactalbumin hydrolysate (Difco) and 3.3 g/l TC yeastolate (Difco).

2.2.1.2 IPL4-1

Powdered medium (for 60 L) obtained commercially from J. R. Scientific Inc. was dissolved in 50 L of deionised, distilled water (for the complete chemical composition of this medium see Weiss and Vaughn 1986). To this medium 39.72 g of CaCl₂ and 21 g of NaHCO₃ were each dissolved separately in 3 L of deionised distilled water and added to the main solution. To this was added 2 ml of ZnSO₄ (3.5 g/l) and 60 ml of AlCl₃ (3.6 g/l) as trace elements and a lipid emulsion containing 105 µl cod liver oil, 249 µl Tween-80, 45 mg cholestrol, 21 ml d-Tocopherol Acetate, 1 ml ethanol and 10 g pluronic polyol-F68. The solution was then titrated to pH 6.2 with the addition of 5 M NaOH (approximately 200 ml) before the addition of 240 g of yeast extract which had been dissolved in 2 L of distilled water and filtered using the Minitan (10 000 Da cut off). The volume of medium was made up to 60 L with the addition of approximately 1680 ml of deionised, distilled water and the medium filtered through a RHS PALL filter, after which it was stored at 4°C until use when it was supplemented with 10% (v/v) heat inactivated FCS, 1% (v/v) fungizone and 0.1% (v/v) gentamycin where required.

2.2.2 General cell culture techniques and seeding densities for mammalian cells.

A porcine aortic endothelial cell line stably expressing the human PDGF β -receptor (ZNR)(Claesson-Welsh *et al.*, 1988; Westermark *et al.*, 1990), SGBAF-1 cell line was established by transfection of bovine adrenal cortex zona fasciculata cells with pSV3neo as previously described for other cell types (Whitley et al., 1987), A431 cells, COS-1 cells and NIH 3T3 cells were all maintained in Dulbecco's modified Eagles medium (DMEM)

containing 10% (w/v) FCS and penicillin (60 IU/ml), streptomycin (100 μ g/ml) and glutamine (29.2 mg/ml). The porcine aortic endothelial cells (ZNR) containing the human PDGF β -receptor were also grown in the presence of 100 μ g/ml G418. Cells were routinely grown in 6 cm petri dishes and incubated in a humidified atmosphere of 5% (v/v) CO₂ 95% (v/v) air at 37°C. For subculture cells were washed twice with PBS and then incubated in the presence of 2 ml of 1X trypsin-EDTA for 4-5 min at 37°C. 10 ml of DMEM containing 10% (v/v) FCS was then added to inactivate the trypsin and the cells harvested by centrifugation at 200 g for 5 min. Cells were seeded at a ratio of 1:20 in 10 ml fresh medium and then subcultured 4-5 days later. If cells were to be stimulated, they were starved in DMEM/0.5% (v/v) FCS for 36 h and then stimulated with saturating concentration of growth factor (~100 ng of PDGF) for 7 min or as otherwise stated and then immediately washed with PBS and lysed.

2.2.3 General cell culture techniques and seeding densities for insect cells.

Spodoptera frugiperda (Sf9) cells were maintained as described in Summers and Smith (1987). All culture medium was incubated at 27°C prior to use and the cells routinely incubated at 27°C. The culture medium used throughout was IPL4-1 (J. R. Scientific) supplemented with 10% (vv) FCS. Routine culture was performed in the absence of antibiotics although 1% (v/v) fungizone and 0.1% (v/v)gentamycin were added for amplication of virus stocks. Sf9 cells have a doubling time of 18-24 h in IPL4-1 containing 10% (vv) FCS and thus needed to be subcultured at least twice a week. Confluent cells were routinely subcultured in a 75 cm² flask, by tapping the side of the flask gently against a surface, with minimal foaming, to dislodge cells and 0.5 ml of medium transferred to another flask containing 9.5 ml of fresh IPL4-1 media. The flask was rocked gently to distribute the cells evenly and was then incubated at 27°C.

2.2.3.1 Infection of Sf9 cells

Vessel	cell density	min. virus vol.	final vol.
25cm ² flask	4.5 X 10 ⁶	1 ml	5 ml
75cm ² flask	2×10^{7}	2 ml	10 ml
150cm ² flask	4.5 X10 ⁷	4 ml	20 ml

Table 2.1: Appropriate seeding densities for typical vessel sizes

Cells were counted and seeded into flasks or dishes at the appropriate density acording to Table 1 and allowed to attach for 1 h at 27°C. For virus amplification, cells were allowed to attach in a 25 cm² flask, incubated with 0.5 ml of the original plaque purified virus and then the cells incubated for 4 days. The virus was harvested by centrifugation at 3000 g and then 1 ml used to infect cells in a 75 cm² flask. After harvesting 4 days post-infection, 1 ml of

this virus stock was used to infect cells in a 150 cm² flask. Virus from this amplification was then used to build up a passage 5 virus stock which could then be titred for optimum protein production. To optimise protein production a number of infections were set up using varying dilutions of the virus stock in 25 cm² flasks, ie. from 1/10000 through to 1 to be harvested either 2 or 3 days post infection. Cells were harvested by gently hitting the flask against a surface without causing foaming to dislodge the cells. The media was then spun at 1000 g for 4 min, the supernatant decanted and the cells washed once in PBS and then again subjected to centrifugation. These cells were then lysed as described in section 2.3 and virus titred by infecting cells at different dilutions of virus and protein production examined by means described in Section 2.3.

2.2.3.2 Purification of extracellular virus (ECV) DNA

The following procedure was adapted from that of Smith and Summer (1982).

Relatively pure viral DNA can be obtained from extracellular virus particles (ECV). Sf9 cells infected with AcMNPV (at 2 X 10⁶ cells/ml) will yield about 1 µg of purified ECV DNA per ml of culture medium 48 h post infection. Duplicate cultures of Sf9 cells were set up in 150 cm² flasks as already described and the infected cells pelleted 48 h post-infection by centrifugation for 5 min at 1000 g. The supernatant was transferred to ultracentrifuge tubes and the ECV pelleted by centrifugation for 30 min at 100 000 g (24 000 RPM in SW-27 rotor). After decanting off the medium, centrifuge tubes were inverted on a paper towel for a few minutes. The ECV was gently resuspended in a small volume of 0.1 X TE and left overnight. The pellets were then resuspended in 4.5 ml of extraction buffer containing 100 mM Tris-HCL [pH 7.5], 100 mM EDTA, 20 mM KCl, and then digested with 200 μg of proteinase K at 50°C for 1-2 h. Following this, 0.5 ml of 105-Sarkosyl was added and the digestion continued for a further 2 h or left overnight. The DNA was then extracted twice with phenol / chloroform / isoamylalcohol (20:24:1) by mixing the organic and the inorganic phase on a rotating wheel for 10 min gently so as not to shear the large AcMNV DNA. The phases were separated by low speed centrifugation, and the DNA containing aqueous phase carefully transferred to another tube with a wide mouth pipette. The viral DNA was then precipitated by the addition of 10 ml of ice-cold absolute ethanol, mixed well, incubated at -80°C for 10 min, and then pelleted in a low speed centrifuge for 20 min. The DNA pellet was washed with ice-cold 90% (v/v) ethanol, dried, resuspended in 500 μl of 0.1 X TE and incubated at 65°C for 15 min to aid resusupension. The purified DNA was then stored at 4 °C.

2.2.3.3 Transfection of Sf9 cells

Plasmids containing a foreign gene are transferred to the AcMNPV genome by recombination in vivo using a modification of the calcium phosphate precipitation technique as modified for insect cells (Burand et al., 1980), or lipofectin (Gibco-BRL).

2 X 10⁶ Sf9 cells were seeded into a 25 cm² flask and allowed to attach for 1 h, after which the media was carefully aspirated off and replaced with 0.75 ml of Graces medium

supplemented with 1% (v/v)fungizone, 0.1% (v/v) gentamycin, 10% (v/v) FCS, and the flasks left at room temperature. 1 μg of AcMNPV DNA and 2 μg of transfer plasmid DNA containing the foreign gene of interest were mixed in a sterile eppendorf tube, 0.75 ml of transfection buffer (25 mM HEPES [pH 7.1], 140 mM NaCl, 125 mM CaCl₂) was added to the DNA and gently vortexed. The DNA solution was added dropwise to the Graces medium already in the cell culture flasks and calcium phosphate precipitation formed between the calcium chloride in the transfection buffer and the phosphate in the medium. The solution was distributed evenly across the surface of the cells and the flasks incubated for 4 h at 27°C. The medium was then removed and the flasks carefully washed 4 times with IPL4-1 containing 10 FCS, 1% (v/v) fungizone, 0.1% (v/v) gentamycin and then incubated in 5 ml of medium at 27°C for 2.5 - 3 days. The virus was plated on fresh Sf9 monolayers and the recombinant virus identified by either plaque hybridisation or by eye and then purified further by plaque assay as described below.

Lipofectin was used an alternative method of transfection of the recombinant DNA and was especially effective when using linearised baculovirus DNA (see Section 2.2.3.7). The cells were plated as already described for transfection and washed twice in serum-free medium. The lipofectin was diluted 2:1 with sterile water and then mixed with an equal volume of a 1:2 mixture of the linear DNA with the recombinant plasmid. This was left at room temperature for 15 min to allow the liposomes and the DNA to fuse, and then added to the cells in a minumum volume of serum free medium and left overnight. The following morning the transfection medium was replaced with complete medium and the recombinant virus harvested after 4-5 days.

2.2.3.4 Plaque assay

3.5 X10⁶ viable Sf9 cells were seeded onto 60 X 15 mm culture plates in IPL4-1 containing 10% (v/v) FCS. The cells were allowed to attach for approximately 20 min while 10 fold dilutions of virus innoculum in 1 ml was prepared (10⁻¹ to 10⁻⁷ for transfection mixes and 10⁻¹ and 10⁻² for virus-picked plaques). Once the cells had attached to the surface of the dish the media was aspirated off and the 1 ml diluted innoculum was gently added to each plate. The virus was evenly distributed by gentle rocking of the plate and then the plate incubated at 27°C for 1 h. In addition a wild type virus and a uninfected control dish were set up. Meanwhile, a 1.5% (w/v) low melting point agarose (Seaplaque) overlay was prepared. For each 100 ml of final volume overlay required (4 ml per plate), 1.5 g of agarose was resuspended in 50 ml of water and autoclaved for 15-20 min. This was then combined with 50 ml of 2X complete TNM-FH and the mixed solution equilibrated to 39°C in a waterbath until required. Following the 1 h incubation period the virus was aspirated off from the cells and 4 ml of overlay agarose was carefully added to the edge of the dish so as not to disturb the cells. The dishes were then left undisturbed for at least 1 h to allow the

agarose to solidify, after which the dishes were placed into a sandwich box containing damp tissues, the box sealed and incubated for 7 days at 27°C. Recombinant plaques were then first screened for visually and if not identified they were then screened for by hybridisation.

2.2.3.5 Visual Screening

Plaques were examined 7 days after infection using phase contrast-L on a Nikon microscope (807344). The wild-type plaques (occlusion positive) contain polyhedra which are bead-like and very refractile to light, whereas the recombinant plaques do not contain polyhedra and thus are light grey and less refractile (occlusion negative). The location of the occlusion negative plaques was marked on the grid on the bottom of the dish and the area of overlay picked with a pasteur pipette and either stored in 2X TNM-FH or subjected to further plaque purifications. If the number of wild-type plaques were too high to allow visualisation of a recombinant by eye the plaques were hybridised.

2.2.3.6 Plaque hybridisation

This procedure is based on that described by Villareal and Berg (1977). After plaque assay, the plates were allowed to dry overnight on the bench top. The plates were tested for dryness by lifting the edge of the agarose overlay gently with a sterile spatula, if a milky smear of cells formed at the interface of the agarose, then the plates were too wet. If the plates were too dry the agarose was found to crack. Four blottting solutions termed A, B, C and D were prepared as:

- A (Denaturing soln) 0.05 M Tris-HCl [pH 7.4], 0.15 M NaCl
- B. (Neutralising soln) 0.5 M NaOH, 1.5 M NaCl
- C. 1.0 M Tris-HCl [pH 7.4], 1.5 M NaCl
- D. 0.3 M NaCl, 0.03 M sodium citrate

The agarose was marked for orientation using a pasteur pipette for the agarose and a fine point marker for the plate and its edge loosened using a sterile spatula. This allowed the agarose to be lifted from the plate and placed cell side up into another petri dish for storage at 4°C in a closed container. A dry nitrocellulose filter (47 mm) was placed on top of the cells that remained attached to the plate. The filter paper was secured to the plate with the addition of a drop of solution A to the filter. The orientation markings were traced from the plate on the filter paper using a ball point pen and then covered with a piece of 3 MM Whatman saturated with solution A. The filters were pressed down firmly using the lid of a 50 ml falcon tube bringing the filter into contact with the cells and expelling any air pockets. One minute later the filters were pressed again and the Whatman 3MM filter removed. The nitrocellulse filter was then carefully removed and placed cell side up on a Whatman filter paper saturated with solution B for 2-3 min before being dried on paper towels and then transferred to a Whatman filter saturated with solution C for 2-3 mins. The nitrocellulose filters were finally dried again and then gently immersed in a petri dish filled with solution D to remove any cell debris that remained before being baked for 2 h at 80°C under vacuum.

Finally, the filters were hybridised using a suitable probe and exposed to Kodak (XAR 5) film. Positives were marked and aligned to the original agarose layer. A fairly large area of agarose surrounding each putative positive plaque was then picked using a pasteur pipette and then transferred to 1 ml of 2X TMN-FH for a further round of plaque purification.

2.2.3.7 Linear wild type (WT) baculovirus DNA

The major disadvantage of the baculovirus expression system is the labour intensive isolation of recombinant viruses. However, we have recently witnessed the advent of many modified forms of WT DNA which give rise to a lower background of wild type virus, thus making visual plaque purification much easier eg. the "Baculogold baculovirus DNA" (Pharminagen). The Baculogold DNA is linearised wild-type DNA that contains both a lethal deletion and a lac Z gene that is replaced after plasmid rescue by the foreign gene of the plasmid thereby denoting a colourless phenotype to recombinant plaques on Xgal plates. Thus only recombinant circular DNA will give rise to recombinants which can easily be recognised on Xgal plates and unlike other kits marketing linear DNA. The use of this linear AcNVP DNA means that DNA that has not been successfully restricted will not survive due to the presence of the lethal deletion which causes death of any transfected wild type DNA. Since linear DNA is unable to transfect cells, only AcNPV DNA that recombines with the recombinant baculovirus transfer vector containing the foreign DNA is transfected and thus transfection results in almost 100% recombinant plaques.

2.3 THE ANALYSIS OF CELLULAR PROTEINS

2.3.1 Preparation of whole cell lysates

Cells were washed twice with PBS and resuspended in 1 ml of 1X sample buffer (2% (w/v) SDS, 62.5 mM Tris-HCl [pH 7.5], 10% (v/v) glycerol, 5% (w/v) β -mercaptoethanol, 0.01% (w/v) bromophenol blue) previously heated to 95°C and transferred to an eppendorf tube. The pellet was disrupted by repeated passage through a 25-gauge needle and then the sample was heated to 95 °C for 5 min and analysed by SDS-PAGE.

2.3.2 Triton X-100 lysis of cells

Cells were washed twice with PBS and resuspended in 0.5 ml of ice-cold lysis solution (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (w/v) Triton X-100, 500 μ M sodium orthovanadate, 2mM PMSF, and 100 kallikrein inhibitor units of Aprotinin). The lysate was transferred to an eppendorf tube and incubated on ice for 30 min, after which it was centrifuged for 20 min at 4°C to pellet the cell debris. The supernatant was then subjected to further biochemical analyses.

2.3.3 Immunoprecipitation of proteins

Lysates were prepared as described in Section 2.3.2 and then incubated with an appropriate antibody on a rotating wheel for 2 h at 4°C. If a second antibody was required this was added and the incubation continued for a further 1 h. The immune complexes were then collected with the addition of either Protein A- or Protein G-Sepharose beads to the lysate and binding allowed to take place for 1 h at 4°C on a rotating wheel. The immunoprecipitate was collected by low speed centrifugation (2000 g) and washed twice with 1 ml ice-cold lysis buffer after which the sample was washed as required for further analyses or the beads were boiled in 30 μ l 2X sample buffer for 5 min, and the supernatant loaded onto a SDS-PAGE gel.

2.3.4 SDS-PAGE analysis of proteins

SDS-PAGE is performed in two layers, an upper stacking gel and a lower resolving gel. The stacking gel is at a lower percentage of acrylamide [pH 6.8] than the resolving gel and the running buffer [pH 8.8]. This ensures that the proteins run through the stacking gel as tight bands and are only separated when they begin to migrate through the higher pH of the resolving gel. The percentage of acrylamide used depends on the size of the protein of interest. In general 7.5% (w/v) resolving gels and 5% (w/v) stacking gels were used for high molecular weight proteins (80-200 kDa), and 12.5-15% (w/v) resolving gels for low molecular weight proteins (10-40 kDa). The resolving gel was mixed from a stock solution of 30% (w/v) acrylamide (acrylamide:bis 30:0.8) and Tris-HCl [pH 8.8] and SDS were added to a final concentration of 375 mM and 0.1% respectively. Polymerisation was initiated with the addition 0.05% (w/v) APS and 0.005% (w/v) TEMED. The gel was then quickly poured between the plates to approximately 3/4 of the height of the plates and overlayed with water-saturated N-butanol. When the gel was set, the butanol was poured off and the surface of the gel washed well with water. The stacking gel was prepared in the same way but with Tris-HCl [pH 6.8], layered over the resolving gel and the comb inserted. Following polymerisation, the comb was removed, the wells flushed out and the samples applied. Electrophoresis was then performed in running buffer (200 mM glycine, 25 mM Tris-HCl [pH 8.8], 0.1% (w/v) SDS), typically at 40-100 V.

2.3.4.1 Coomassie Blue stain

Following electrophoresis the gel may be stained for the presence of proteins by soaking in Coomassie blue stain for 20 min (45% (v/v) methanol, 10% (v/v) acetic acid, 0.25% (w/v) (60g/10L) Coomassie blue), followed by destaining in 20% (v/v) methanol, 14% (v/v) acetic acid. The gel was then dried under vacuum for 40 min at 80°C.

2.3.4.2 Silver stain

A more sensitive method for the staining of proteins was used if only ng quantities were present. After electrophoresis the proteins were pre-fixed for 1 h in 25% (v/v) isopropan-2-

ol, 10% (v/v) acetic acid, followed by 30 min in 10% (v/v) glutaraldehyde. The gel was then repeatedly washed in large volumes of double distilled water (ddH₂0) for a period of 2 h before being sensitised in 1 mg/200 ml DTT solution, followed by a 30 min incubation in AgNO₃ (0.2 g/200 ml). The gel was washed quickly in ddH₂0 and then a little developer (6 g/200 ml sodium carbonate). Development was allowed to proceed by incubation in this solution containing 100 μ l of stock 37% (v/v) formaldehyde solution. When the desired proteins were visible the reaction was terminated with the addition of 25 ml of 2.3 M sodium citrate solution.

2.3.5 Estimating protein concentration

To estimate protein concentration in solution the Pierce Coomassie Protein Reagent was employed which is based on the absorbance shift from 465 to 595 nm which occurs when Coomassie brilliant blue G-250 binds to proteins in an acidic solution. 0.5 ml of reagent was mixed with protein in 0.5 ml of ddH₂0 and the absorbance monitered at OD595 and compared with a blank. The protein concentration was then determined by comparison with an albumin standard curve.

2.3.6 Transfer-hybridisation of proteins

Proteins separated by SDS-PAGE can be selectively identified by specific antisera using the process known as immunoblotting or Western blotting. The gel was immersed in degassed transfer buffer (1L: 192 mM glycine, 25 mM Tris-HCl [pH 7.5], 0.1% (w/v) SDS, 20% (v/v) methanol) for approx. 5 min. The gel was then placed on top of a piece of nitrocellulose paper presoaked in transfer buffer. The gel/nitrocellulose was sandwiched between several layers of presoaked Whatman 3 MM and placed on the transfer apparatus (Biorad) with the nitrocellulose between the anode and the gel. The proteins were transferred from the gel onto the niotrocellulose by applying a current dependent on the dimensions of the gel for 90 min; mA=gel area cm² X 0.8. The gel was then stained as described to ensure transfer was complete and the nitrocellulose hybridised with the specific antisera.

2.3.6.1 Immunoblotting with $[^{125}I]$ Protein A

The proteins were transferred from the gel to the nitrocellulose as described and the filter immersed in blocking buffer (1X PBS, 5% (w/v) Marvel, 0.05% (w/v) Tween-20) for 2 h at room temperature or overnight at 4°C to block all non-specific sites. The filter was washed twice in blocking buffer and then incubated for 2 h room temperature with the primary antibody in a minimal volume of blocking buffer at a dilution of 1:500. Excess antibody was removed by subjecting the filter to 3x 15 min washes in blocking buffer. The bound antibody was detected using 20 μ Ci [125I] Protein A (specific activity 150 μ Ci/ μ g, Amersham) in blocking buffer for 1 h at room temperature. The filter was washed 2x15 min in blocking buffer and if the background radioactivity remained high 2x 10 min in 1X

PBS, 0.1% (w/v) Tween-20. The membrane was wrapped in Cling film and exposed to Kodak (XAR 5) film at -70°C.

2.3.6.2 Enhanced chemiluminescence (ECL)

This method, developed by Amersham, was subsequently used for all antibody detection because of the speed of the reaction and the exclusion of radioactivity. ECL is a light emmitting non-radioactive method for detection of immobilised specific antigens conjugated with horseradish peroxidase labelled antibodies. The system utilises a chemiluminescent reaction which takes place when the cyclic diacylhydazide luminol is oxidised in the presence of the catalyst hydrogen peroxide (H₂O₂). Following oxidation the luminol is in an excited state which decays to the ground state via a light emitting pathway.

The Western blot was performed in the same way as described above in 2.3.6.1, but after washing to remove excess primary antibody a species specific horseradish peroxidase conjugated second antibody was applied in a minimal volume of blocking buffer for 1 h at room temperature at a dilution of 1/1000. The nitrocellulose filter was then washed 2 X 10 min in PBS, 0.1% (w/v) tween, 5% (w/v) Marvel and 1 X 10 min in PBS alone. Equal volumes of detection solution 1 was mixed with detection solution 2, and added to the filter. The reaction was allowed to proceed for 1 min, the filter blotted dry, wrapped in saran wrap and exposed to Kodak (XAR 5) film for 30 sec, 1 min and 5 min at room temperature.

2.3.6.3 Stripping and reprobing

All immunoblots could be stripped and reprobed, if the blots had been stored wet in Saran wrap at 4°C after immunodetection, using the following protocol. The membrane was immersed in stripping solution (100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl [pH 6.7]) and incubated at 50°C for 30 min with occasional agitation. The membrane was then washed 2 X 10 min in PBS containing 0.1% (w/v) Tween-20 in a large volume of buffer. The membrane was then blocked and immunoblotted as described.

2.3.7 Large scale preparation and purification of proteins

2.3.7.1 Purification of native $p85\alpha$

50x 150 cm² flasks each containing 4 x 10⁷ Sf9 cells were infected with recombinant viruses at a dilution of 1/100. After 2 days of infection cells were harvested by centrifugation at 800 g for 10 min, washed in PBS and then pelleted by centrifugation again. The Sf9 cell pellet was disrupted on ice, using a Dounce homogeniser, in 40 ml of ice-cold extraction buffer A (20 mM β -glycerophosphate [pH 7.5], 10 mM sodium fluoride, 10 mM benzamidine, 2 mM EDTA, 5 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 0.3% (w/v) β -mercaptoethanol, 50 mg/ml phenylmethylsulfonyl-fluoride [PMSF]). After 30 min on ice, the lysate was cleared of particulate material by centrifugation at 15,000 g for 20 min at 4°C. All subsequent procedures were carried out at 4°C. The supernatant was

loaded onto 20 ml Q-Sepharose column (Pharmacia) that had been pre-equilibrated in buffer A. The column was washed with 50 ml of buffer A and bound protein was eluted in 2 ml fractions at 1 ml/min with 80 ml of a linear salt gradient to 1 M NaCl in buffer A. All fractions were assayed by immunoblotting using specific COOH-terminal anti-p85 polyclonal antibodies. Pooled fractions from the Q-Sepharose column (8 ml), containing p85 immunoreactive material, were diluted 5-fold in buffer A and applied to a 2 ml MonoQ column (Pharmacia). After washing in buffer A, protein was eluted in 0.5 ml fractions with a linear NaCl gradient from 0-400 mM. The peak of p85 immunoreactivity was concentrated in a Centriprep concentrator with a molecular weight cut off of 10 kDa (Amicon) and loaded on a Superose 12 column (Pharmacia) equlibrated in buffer A containing 0.2 M NaCl. Eluted fractions containing p85 were pooled and concentrated in a Centriprep concentrator. Purified material was dialysed against 20 mM Hepes (pH 7.0), 150 mM NaCl, 50% (v/v) glycerol (2x 4 h) and then stored at -20°C. Under these conditions p85 proteins remained active, as measured by their ability to associate with tyrosine phosphorylated proteins, for over a year.

2.3.7.2 Preparation of denatured p85 α protein

Insect cells were infected with p85 virus and the cells harvested as described in the preceeding section. Whole cell lysates were prepared as described in section 2.3.1 and loaded onto 1.5 mm SDS-PAGE gels that had been stacked without a comb. The gel was run at constant current (30 mA), stained in 50% (w/v) Coomassie blue until the p85 protein was just visible, destained, and then gel strips of the p85 protein excised. The protein was then electroeluted out of the gel. The band was placed into dialysis tubing containing 2 ml of SDS-PAGE running buffer (section 2.3.4) which was immersed into an an electrophoresis tank filled with SDS-PAGE running buffer. Current was passed through the bag (100 mA overnight), after which the polarity was reversed to release the protein from the wall of the dialysis bag. The gel strips were carefully recovered from the bag, the dialysis bag reclipped and subjected to dialysis in 20% (v/v) methanol to remove any SDS present (4 changes over 48 h). The protein was then aliquoted and stored at -70 °C. (5 X 10⁷ infected cells usually gave rise to 100 μg of purified denatured p85 protein).

2.3.7.3 Preparation of Glutathione S-transferase -fusion proteins

In order to rapidly produce large amounts of pure functional proteins, a procedure essentially as described by Smith and Johnson, (1988) was utilised. Glutathione Stransferase (GST) fusion proteins are expressed in a pGEX vector, which contains a thrombin cleavage site C-terminal to the GST protein. The fusion protein can be readily purified using glutathione-agarose chromotography. Following cleavage of the fusion protein with thrombin, the glutathione S-transferase can be removed from the protein of interest by a second round of chromotography on the glutathione-agarose column.

A single colony of XL-1 Blue transformant grown overnight with shaking at 37°C in 10 ml LB supplemented with (100 µg/ml) Ampicillin was used to innoculate 1 L of LB media containing (100 µg/ml) Ampicillin. This was grown for 3 - 4 h with great aeration at 37°C until $OD_{600} = 0.5$ -0.6. The expression of the recombinant protein was induced by the addition of IPTG to a final concentration of 0.2-0.4 mM and further growth for 4-8 h at 30°C. The bacterial cells were then pelleted by centrifugation for 15 min at 2000 g, washed in PBS twice and lysed in 40 ml of PBST buffer (1 % (w/v) Triton-X100, 2 mM EDTA, 5 mM benzamidine, 0.1% (w/v) 2-mercaptoethanol, 0.2 mM PMSF, 500 µM sodium orthovanadate, and 100 kallikrein inhibitor units of aprotinin) on ice. The cells were subjected to mild sonication and then left on ice for a further 30 min. Cell debris was removed by centrifugation of the lysate at 100 000 g for 20 min at 4°C. The supernatant was incubated with 5 ml of glutathione agarose (Pharmacia) prewashed in PBST buffer for 2 h with rotation at 4°C to allow affinity binding of the recombinant protein. The beads were then washed 4 X with PBST, twice with 50 mM Tris-HCl [pH 8.0], and transferred to a small 10 ml column (Biorad). The protein was eluted off the beads by competition with 20 mM glutathione in 50 ml Tris-HCl [pH 8.0] and collected in 1 ml fractions which were assayed for protein content using Pierce Coomassie Protein Reagent as described in section 2.3.5. The protein containing fractions were combined and dialysed into 20 mM Tris-HCl [pH 7.4], 150 mM NaCl and 0.1 mM 2-mercaptoethanol. These proteins were then used as GST-fusion proteins for biochemical analyses or cleaved free of the glutathione Stransferase by incubating 2 mg of fusion protein with 4 µg of thrombin in the presence of 2.5 mM CaCl₂ at room temperature on a wheel for 20-30 min. The reaction was terminated by the addition of 0.2 mM APMSF on ice and the S-transferase removed by incubation of the solution with 5 ml glutathione agarose beads with rotation for 1 h at 4°C. Purified, cleaved protein was obtained by low speed centrifugation of the beads (2000 g) and the supernatant carefully removed and dialysed as already described in section 2.3.5.

2.3.8 Preparation of $p85\alpha$ antibodies

Polyclonal antibodies were raised against both native and denatured p85 α protein. For antibodies that recognise native protein, 100 µg of purified p85 α protein was mixed with Freunds adjuvant and subcutaneously injected into two rabbits. The animals were boosted every 3 weeks and serum collected 10 days after each injection. Serum was tested after each boost by immunoprecipitation and Western blotting. After the fifth boost the sera were affinity purified using p85 proteins coupled to Actigel-ALD-Superflow resin (Sterogene) which has a monoaldehyde group attached to the matrix that couples to protein on the addition of NaCNBH3. Briefly, to couple the p85 protein to the resin 1 ml of Actigel-ALD-Superflow resin was washed twice with PBS by centrifugation at 1500 g for 2 min, and the beads suspended in 1 ml of PBS containing 2 mg of purified p85 α protein. 300 µl of coupling solution-NaCNBH3 to give a final concentration of 0.1 M was added and the

beads incubated for 2-3 h at room temperature. The supernatant was decanted and excess antibody washed off the resin in 10 vol of 1X PBS containing 0.35 M NaCl. The resin was agitated in 1 ml 0.1 M Tris-HCl [pH 7.0], 300 μ l coupling solution overnight at 4 °C to block all unbound sites and then washed as above. The coupled resin was now ready to be used for affinity purification of the p85 protein or was stored at 4°C in 1X PBS containing 0.02% (w/v) NaN3. For affinity purification 5 ml of sera was added to the affinity matrix and incubated with rotation at 4°C overnight. The sera was removed and the beads washed with several volumes of PBS and transferred to a small column (Biorad). The antibody was eluted under gravity using 0.1 M Glycine [pH 3.0]. 1 ml fractions were collected in eppendorf tubes containing 100 μ l of 1 M Tris-HCl [pH 8.0] to neutralise the glycine upon collection and assayed for protein content using Pierce Coomassie Protein Reagent as described in section 2.3.5. The positive fractions were combined and then dialysed against PBS, 0.02% (w/v) NaN3 immediately for 24 h with 4 changes. The resin was regenerated by washing in 1 M NaCl and was then stored at 4 °C in 1X PBS, 0.02% (w/v) NaN3.

The same approach was used to affinity purify antibodies raised to peptides in p85 and p110. All of these antibodies were then analysed for ability to immunoprecipitate and Western blot specific protein.

2.3.9 Analysis of protein phosphorylation

2.3.9.1 In vitro protein kinase assay

Affinity-purified kinase was washed twice in kinase buffer containing (50 mM HEPES [pH 7.4], 50 mM NaCl, 2% (v/v) glycerol, 12 mM MnCl₂, 2 mM MgCl₂, 0.1% (w/v) Triton X-100) and then resuspended in a final volume of 30 μ l of kinase buffer containing 0.3 μ Ci of [γ^{32} P]ATP. The kinase reaction was allowed to proceed for 10 min at room temperature (unless otherwise stated), after which it was terminated with the addition of 6 μ l of 5X sample buffer. Phosphorylated proteins were then analysed by autoradiography of SDS-PAGE gels.

2.3.9.2 Phosphate labelling

Infections of Sf9 cells were allowed to proceed for 24 h before cells were transferred to phosphate-free Graces insect cell medium (Gibco/BRL) in the absence of serum. ³²P (Amersham) was added at a final concentration of 0.1 mCi/ml and the labelling was allowed to proceed for a further 16 h. One hour prior to labelling SGBAF-1 cells that had been quiesced for 16 h were incubated with phosphate-free DMEM and 10% (v/v) dialysed fetal calf serum. ³²P (Amersham) was added at a final concentration of 2 mCi/ml and the labelling was allowed to proceed for 4 h. At this time cell monolayers were washed twice with ice-cold phosphate-buffered saline and lysed as described above.

2.3.9.3 Biosynthetic labelling of proteins

Twenty four hours post infection Sf9 cells were transferred to methionine-free Graces medium (Gibco/BRL) in the absence of serum. Cells were labelled with 100 μ Ci/ml [35 S]methionine (Amersham) for 15 h.

2.3.9.4 Alkali treatment of gels

As phosphotyrosine is relatively stable to alkaline pH compared with phosphoserine and phosphothreonine, base hydrolysis of proteins on acrylamide gels can be performed to detect tyrosine phosphorylated proteins. If the gel had already been dried the band of interest was cut out and the gel and backing paper were hydrated by submersion in water, or the gel itself was immersed in 500 ml 1 M KOH and incubated at 55°C for 2 h. The hot KOH was replaced with 10% (v/v) acetic acid, 10% (v/v) propan-2-ol (destaining solution) until the gel shrunk back to its original size and then rinsed in water, dried and exposed to film.

2.3.9.5 Antiphosphotyrosine blot

Another sensitive procedure for identification of tyrosine phosphorylated proteins was by immunoblotting with anti-phosphotyrosine antibodies according to the method of Kamps and Sefton (1988). The proteins were immunoblotted as before, but with the addition of 7.5% (w/v) sodium orthovanadate to the transfer buffer. The blot was then blocked for 2 h at room temperature (or overnight at 4°C) in rinse buffer (10 mM Tris-HCl [pH7.4], 0.9% NaCl, 0.01% (w/v) NaN3, 5% (w/v) BSA [fraction V], 1% (w/v) orthovanadate). The blot was incubated with a 1:200 dilution of the antisera for 2 h at room temperature in rinse buffer. Unbound antibody was removed by 2 X 10 min washes in rinse buffer, 1 X 10 min in rinse buffer, 0.05% (w/v) NP40, and a further 2 X 5 min washes in rinse buffer alone. The blot was incubated for 1 h with 20 μ Ci [125 I] Protein A (specific activity 150 μ Ci/ μ g, Amersham) in rinse buffer and then washed as described before. The blot was then exposed to Kodak (XAR 5) film.

2.3.9.6 Phosphoamino acid Analysis

This method determines the relative content of phospho-serine, -threonine, -tyrosine in a phosphoprotein and was performed essentially as described in Cooper, Sefton and Hunter (1983). Radioactively-labelled phosphoproteins were separated on a conventional SDS-PAGE gel, which was then stained/destained, dried and exposed to film. The resulting autoradiograph was aligned with the gel and the phosphorylated band of interest marked on the gel. This band was excised, Cerenkov-counted and hydrated for 5 min in 0.5 ml freshly prepared 50 mM NH4HCO3. The swollen gel piece was then homogenised with a small teflon pestle (Kontes), which was rinsed with a further 0,5 ml NH4HCO3. To this was added 50 µl of 2-mercaptoethanol and 10 µl 10% (w/v) SDS, followed by boiling and vortexing of the slurry. Protein was eluted by rotation for 3-16 h and the supernatant removed after centrifugation in a microfuge for 2 min. A further wash with the 2-mercaptoethanol/SDS/NH4HCO3 mixture (boil,vortex,rotate 3 h and centrifuge) extracted

residual protein to give a total volume of 1.2 ml supernatant. This was reclarified by microcentrifugation for 10 min. The supernatant was carefully removed and cooled on ice. To this 20 μ g RNAse was added as a carrier, followed by 250 μ l ice-cold 100% (v/v) trichloroacetic acid. The protein was precipitated for at least 1 h on ice, after which it was pelleted by microcentrifugation for 10 min at 4°C. The pellet was carefully washed with cold absolute ethanol and air-dried. Cerenkov counting usually confirmed a yield of about 70%. The isolated protein was then hydrolysed with constant-boiling HCl [5.7 M]. 100 μ l HCl was added directly to the TCA-precipitated protein eluted from a gel. The tube was sealed and incubated at 100°C for 60 min. The reaction was stopped immediately by the addition of 400 μ l cold water and freezing, to prevent further hydrolysis leading to the removal of phosphate from the phosphoamino acids. After rotary evaporation with a SpeedVac equipped with an acid-trap, the sample was resuspended in 10-20 μ l [pH 1.9] running buffer 88% (v/v) formic acid, acetic acid and ddH2O in a ratio 0.025:0.078:0.897 and 70 μ g/ml of each cold phospho-serine, -threonine,-tyrosine as standards.

Samples containing ~100 - 1000 cpm were loaded onto precoated cellulose plates (Merck), and the spot was focussed to about 2 mm diameter by wetting the plate with running buffer by using Whatman 3MM paper with a hole cut at the origin. The wet plate was then electrophoresed in a HTLE-7000 apparatus (CBS Scientific, CA, USA) in the presence of marker dye (5 mg/ml DNP-lysine + 1 mg/ml xylene cyanol FF) in running buffer [pH 1.9] in the first dimension for 20 min at 1500 V. The plate was then air-dried focussed along the first dimension in running buffer [pH3.4] containing acetic acid, pyridine and ddH20 at a ratio of 0.05:0.005:0.945 before being electrophoresed in the second dimension at 1300 V for 20 min in running buffer [pH3.5]. After drying the plates were sprayed with 0.5% (w/v) ninhydrin in acetone and heated to 65°C for 20 min to visualise the phosphoamino acid standards. Plates were exposed directly to Kodak (XAR 5) film at -70°C with intensifying screens.

2.3.9.7 Phosphopeptide mapping

For phosphopeptide mapping, gel excised p85 α was washed twice for 20 min in 1 ml of water, once for 12 min in 10 mM Tris-HCl [pH 8.0], 50% (v/v) acetonitrile and finally a 5 min wash in 100 mM Tris-HCl [pH 8.0]. The gel slice was then crushed and incubated for 24 h at 30°C in 100 mM Tris-HCl [pH 8.0] containing 1:100 by weight ratio trypsin to protein, followed by a further addition of the same amount of trypsin for another 24 h. The supernatant was collected and the gel bits were washed 3x 20 min in 10 mM Tris-HCl [pH 8.0], 50% (v/v) acetonitrile. The combined supernatants were then filtered through a Millipore 0.22 μ m filter unit and analysed by reversed-phase H.P.L.C with assistance from G. Panayotou. A 100 x 2.1 mm Aquapore OD 300 column was used on a Hewlett Packard HP1090 HPLC system and equilibrated in buffer A (0.08% (v/v) Trifluoroacetic acid [TFA], 1% (v/v) acetonitrile) at a flow rate of 0.2 ml/min. A gradient with buffer B (0.08%

(v/v) TFA, 90% (v/v) acetonitrile) was applied as follows: 0-60% buffer B in 60 min, 60-100% in 5 min, 100% for 5 min and 100-0% for 5 min. Fractions were collected every 0.5 min and their radioactivity measured by Cherenkov counting.

2.3.9.8 Phosphatase treatment

Proteins can be dephosphorylated by incubating in the presence of phosphatases. Protein phosphatase 2A is specific for phosphoserine residues and alkaline phosphatase (Pharmacia) is a non-specific phosphatase. These phosphatases were used to examine the phosphorylation state of the PI 3-kinase phosphorylated in vitro.

PI 3-kinase was immunoprecipitated using anti-p110 antibodies from lysates of Sf9 cells. Immunocomplexes were washed twice in lysis buffer, twice in protein kinase buffer and then phosphorylated for 30 min at 22°C in the presence of [γ^{32} P]ATP and 15 μ M ATP. Radiolabelled proteins were washed extensively with lysis buffer and then washed twice in either alkaline phosphatase buffer (50 mM NaCl, 10 mM Tris HCl [pH 7.4], 10 mM MgCl₂, 1 mM DTT), or protein-serine phosphatase buffer (10 mM MgCl₂, 1 mM MnCl₂, 20 mM HEPES [pH 7.4], 1 mM DTT). Samples were incubated for 20 min at 22°C with either 10 units or 50 units of protein phosphatase 2A. After several washes samples were subjected to PI 3-kinase assays or visualised on SDS-PAGE gels.

2.3.9.9 PI 3-kinase assay

PI 3-kinase assays were carried out essentially as described in Whitman et al., (1987) in 50 μ l containing 50 mM HEPES [pH 7.4], 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 5 mM MgCl₂, 100 μ M ATP (plus 0.5 μ Ci of [γ -32P]ATP/assay), 200 μ g/ml PI plus immobilised bovine brain PI 3-kinase. Incubation was for 5 min at room temperature. The reaction was terminated by the addition of 100 μ l of 0.1 M HCl and 200 μ l of chloroform/methanol (1:1). The mixture was vortexed and then discarded and the lower organic phase washed with 80 μ l of methanol 1 M HCl (1:1). After centrifugation the upper phase was again discarded and the lower phase evaporated to dryness. Reaction products were spotted on thin layer Silica Gel-60 plates (pretreated with 1% (w/v) oxalic acid, 1 mM EDTA in water/methanol [6:4]) and developed in chloroform, methanol, 4 M ammonia (9:7:4).

2.3.9.10 Analysis of the products of PI kinase assays

To establish the lipid products of the PI 3-kinase assay deacylation and HPLC analysis was performed on PI 3-kinase assays run as described above. Lipid products of the PI 3-kinase assays were deacylated essentially as described in Clarke and Dawson (1981). The region of the TLC plate corresponding to PIP was dampened with water and the silica scrapped into a eppendorf tube. A 0.75 ml aliquot of monomethylamine (33% (v/v) in industrial methylated spirits):methanol:n-butanol (50:15:5) was added to the silica scrappings and the mixture was incubated at 53 °C for 50 min. Reactions were stopped on ice and the samples dried under vacuum prior to the addition of 0.75 ml water and 0.9 ml n-butanol:light petroleum:ethyl formate (20:40:1). The lower phase was washed with a further 1.2 ml of

the butanol mixture prior to the addition of $100 \mu l$ 50 mM mannitol and freeze drying under vacuum.

HPLC was performed on a Beckman System Gold apparatus essentially as described by Auger et al., (1989) with assistance from M. Fry. Glycero-Inositol phosphates were eluted from a Partisphere SAX column on a gradient of (NH4)2HPO4. Pump A {water}, pump B {1 M (NH4)2HPO4 taken to pH 3.8 with orthophosphoric acid}: time 0-10 min 0% B, followed by a gradient to 25% B over 50 min, then to 100% B over 60 min at a flow rate of 0.5 ml/min. Tritiated standards of GroPI(4)P and GroPI(4,5)P2 were prepared from Ptd[3H](4)P and Ptd[3H](4,5)P2 respectively and run similtaneously with the ³²P-labelled deacylated product resulting from the phosphorylation of PI by PI 3-kinase. The eluted fractions were counted for radioactivity.

2.3.9.10 Substrate analysis

Proteins for substrate analysis were analysed by incubation of the substrate ie. histone, casein or enolase with the protein-kinase in kinase buffer and then the proteins subjected to in vitro kinase assay in the presence of $[\gamma^{32}P]ATP$ as described in section 2.3.9.1. The phosphorylated products of the kinase reaction were then analysed by autoradiography of SDS-PAGE gels. If the substrate was too small to be analysed by SDS-PAGE as for substrate-peptides such as poly-serine phosphopeptides the following procedure was followed. The immunoprecipitated kinase was washed twice in lysis buffer and twice in kinase buffer (as described in section X) and incubated with 2 μ g peptide and 2 μ Ci γ^{32} P[ATP] at room temperature for 5 min. The beads were removed by low speed centrifugation and kept for analytical SDS-PAGE and an equal volume of 5% (v/v) trichloroacetic acid and 30 μ g of BSA was added to the supernatant, followed by spinning in a microfuge for 15 min at 4°C. The supernatant was then spotted in duplicate on phosphocellulose paper and then washed 4 X 10 min with 30% (v/v) acetic acid and pyrophosphate with stirring. The filters were dried and then Cherenkov counted.

2.3.10 Analysis of protein interactions

2.3.10.1 Inhibition of kinase activity by 5,5'-Dithio-bis[2-Nitrobenzoic acid]

Two days post infection Sf9 cells were lysed and immunoprecipitated using anti-p110 affinity-purified, polyclonal antibodies as described (Hiles et al., 1992). These immunoprecipitates were washed twice with PI 3-kinase buffer (20 mM Tris-HCl pH[7.5], 100 mM NaCl, 0.5 mM EGTA) and then either left untreated, or incubated in the presence of 0.3 mM 5,5'-Dithio-bis[2-Nitrobenzoic acid] (Nbs2-Sigma) for 15 min at 22°C. Excess reagent was removed by washing the complexes three times with PI 3-kinase buffer and the immunoprecipitate was then incubated for 15 min at 22°C with increasing concentrations of

dithiothreitol (DTT). A control sample was incubated with DTT alone at a final concentration of 300 mM. Finally, the remaining DTT was removed by washing with lysis buffer and the immunoprecipitates were subjected to PI 3-kinase assays or to in vitro protein kinase assays as described.

2.3.10.2 Elastase treatment

The immunoprecipitated EGF receptor was washed twice in elastase buffer (Tris-HCl [pH 8.0]) resuspended in a final volume of $50 \mu l$ and than incubated on ice in the presence of $10 \mu l$ mg/ml elastase for the appropriate time period. The reaction was terminated with the addition of PMSF to a final concentration of $200 \mu M$.

2.3.10.3 Association Assays

In vivo association; To examine the interaction of tyrosine kinases with SH2 domain-containing proteins, coinfected cells were harvested and lysed 3 days after infection. Three days after infection the cells were either lysed and boiled in 2X SDS-PAGE sample buffer and then subjected to Western blot analysis with an affinity purified anti-phosphotyrosine antibody as performed according to Kamps and Sefton (1988), or lysed in lysis buffer and lysates incubated with antibodies specific for the appropriate protein-tyrosine kinase. After collecting the immune complexes on Protein A-Sepharose beads and extensive washing (twice with lysis buffer, twice with kinase buffer) the immune complexes were phosphorylated in the presence of 5 μ Ci [γ -32P]ATP and phosphorylated proteins analysed by SDS-PAGE.

For p85 and p110 coinfected cells were normally harvested 2 days post-infection. Lysates were then either immunoprecipitated using appropriate antibodies or were applied to a phosphopeptide affinity column derived from sequence surrounding tyrosine 751 of the human PDGF β -receptor to which PI 3-kinase has been shown to bind (Otsu et al., 1991; Fry et al., 1992). Peptide affinity columns were washed twice with lysis buffer and twice with 1 M NaCl. Enzyme complexes were either analysed by 7.5% (w/v) SDS-PAGE gels or subjected to in vitro kinase assays as described above.

In vitro association assay; In vitro complex formation of the p85 α or p85 β subunit with either wild type p110 or with the mutant p110-R916P was examined by immunoprecipitating p85 α from insect cells using an affinity-purified, polyclonal antibody. The immunoprecipitate was collected on Protein A-Sepharose beads and washed three times in lysis buffer. This immune complex was then incubated for 1 h at 4°C with cell lysates prepared from Sf9 cells infected with viruses expressing p110 or the mutant p110-R916P. The resultant enzyme complexes were washed extensively and either analysed on 7.5% (w/v) SDS-PAGE gels or assayed directly for associated PI 3-kinase and protein kinase activities. In vitro binding of p110 to p85 α domains was performed in essentially the same manner. GST fusion proteins were immobilised on Glutathione-Sepharose beads (Pharmacia) as described previously (Smith and Johnson, 1988). These affinity columns were then incubated for 1 h at 4°C with cell lysates prepared from Sf9 cells infected with a

baculovirus expressing p110. After extensive washing with lysis buffer, bound proteins were subjected to kinase assays.

For association of the GST-p110 constructs with p85 α and p85 β , cell lysates of Sf9 cells infected with baculoviruses expressing either p85 α , p85 β or wild type proteins were added to immobilised GST-p110 fusion proteins, and binding was allowed to proceed for 2 h at 4°C. The beads were then washed four times with lysis buffer and resuspended in 100 ml 2X Laemmli sample buffer. Samples were boiled for 5 min and proteins resolved by SDS-PAGE on 10% gels.

2.3.10.4 In vitro activation of protein-tyrosine kinases and substrate association

This assay was performed essentially as described by Kazlauskas and Cooper (1990). Sf9 cells were infected as already described and lysed 48 h after infection as described in section 2.3.2. The tyrosine kinase was immunoprecipitated from insect cells and collected on Protein A-Sepharose beads. The immunocomplex was then subjected to extensive washing with lysis buffer, followed by twice with kinase buffer (section 2.3.9.1). The kinase was then phosphorylated in vitro in the presence of 20 μ M ATP at room temperature for 20 min. The precipitates were washed with lysis buffer again to remove free ATP and incubated for 2 h at 4 °C with the protein being analysed. The immune complexes were washed again with lysis buffer and subjected to in vitro kinase assays (section 2.3.9.1) and/or assayed for associated PI 3-kinase activity (section 2.3.9.9).

Chapter 3: Expression and characterisation of the p85 subunit of the PI 3-kinase complex

3.1 Introduction

Elucidation of the primary structure of the PI 3-kinase and its relation to function has been critical in understanding its role in signal transducing processes. PI 3-kinase was originally identified as a 85 kDa phosphoprotein on SDS-PAGE gels, whose presence correlated with the detection of PI 3-kinase activity in immunoprecipitates of mT mutants and the PDGF receptor (Courtneidge and Heber, 1987; Kaplan et al., 1987). PI 3-kinase purified from the bovine brain (Morgan et al., 1990; Fry et al., 1992), bovine thymus (Shibasaki et al., 1991), rat liver (Carpenter et al., 1990) and Balb/c 3T3 lysates (Escobedo et al., 1991) has been shown to be a heterodimer of 85 and 110 kDa subunits.

The cDNAs for the p85 proteins were subsequently cloned using three different approaches: Firstly, by utilising amino acid sequence of tryptic peptides, obtained from the purified bovine brain preparation, to generate oligonucleotides which were used to isolate clones from bovine brain cDNA libraries (Otsu et al., 1991). Secondly, by using a phosphorylated, immobilised PDGF receptor as an affinity reagent to purify PI 3-kinase from BALB/c 3T3 cell lysates. Partial amino acid sequence was then used to clone a cDNA from a BALB/c 3T3 library (Escobedo et al., 1991). Finally, an 85 kDa protein was identified from a screen of a human $\lambda gt11$ expression library using the phosphorylated tail of the EGF receptor as a high affinity probe (Skolnik et al., 1991). Two highly homologous forms of the 85 kDa protein, termed p85 α and p85 β , have been identified in the bovine brain (Otsu et al., 1991). Analysis of the primary sequence of both p85 proteins reveals a common multidomain structure, which contains a number of non-catalytic domains. In particular a N-terminal SH3 domain followed by a Bcr domain and two SH2 domains. As both p85 proteins lack sequence motifs common to any known kinases (Hanks et al., 1988), the p110 protein was assumed to contain the catalytic domain of the enzyme.

3.1.1 Aim

In order to study in detail the biological functions and biochemical properties of $p85\alpha$ and $p85\beta$, it was necessary to express them in systems which would produce large amounts of p85 proteins which were correctly post-translationally modified, as carried out by higher eukaryotes, and allow in vivo and in vitro biochemical analyses of the recombinant proteins. The baculovirus expression system has proved to be a suitable

system which allows accurate proteolytic cleavage, phosphorylation and glycosylation of the exogenous protein (Reviewed in Summers and Smith., 1988). The p85 proteins were subcloned into baculovirus transfer vectors and transfected into insect Sf9 cells. The expressed proteins were then purified and used to generate specific antisera which allowed analysis of the distribution of PI 3-kinase activity associated with either the p85 α or the p85 β protein. The simultaneous expression of protein-tyrosine kinases in Sf9 cells permitted study of the properties inherent to the endogenous p85 proteins which mediate interactions with receptors.

3.2 Results

3.2.1 Expression of the p85 proteins.

3.2.1.1 Construction of the p85 α baculovirus transfer vector.

For high level expression of proteins in baculovirus, the coding region of a cDNA is placed under the control of the powerful polyhedrin promotor. As any leader sequences 5' to the initiating ATG are thought to be inhibitory for expression, the cDNA for p85 α was subcloned into the pAcC4 baculovirus transfer vector so that the initiating methionine residue of the p85 α coding sequence was introduced directly downstream of the polyhedrin promotor.

p85α cDNA does not contain a NcoI site at the ATG, so the following procedure was used to clone this sequence into pAcC4. Initially, using the unique KpnI site 18 bp after the ATG, a 2.22 kb KpnI-EcoRI fragment for p85α was subcloned into pAcC4. Two overlaping oligonucleotides, containing a KpnI cloning site on one side and a sticky end for NcoI on the other (CATGAGTGCCGAGGGGTAC and CCCTCGGCATC), were generated, annealed together and ligated to NcoI-KpnI digested pAcC4, containing 2.22 kb KpnI-EcoRI fragment for p85α. Several positive pAcC4-p85α clones were identified by colony hybridisation using the 19 mer oligonucleotide which had been end-labelled. Clones were sequenced around the ATG and two of these were subsequently used for transfection of Sf9 cells. The subcloned fragments were sequenced by the dideoxy chain termination methods using ³⁵S-dATP.

3.2.1.2 Expression of p85 α and p85 β in insect cells.

Wild type baculovirus Autographa californica nuclear polyhedrosis Virus (AcMNPV) was cotransfected with recombinant baculovirus transfer vectors encoding pAcC4-p85 α and pAcC4-p85 β (made by I. Gout) into Sf9 cells and extracellular virions were

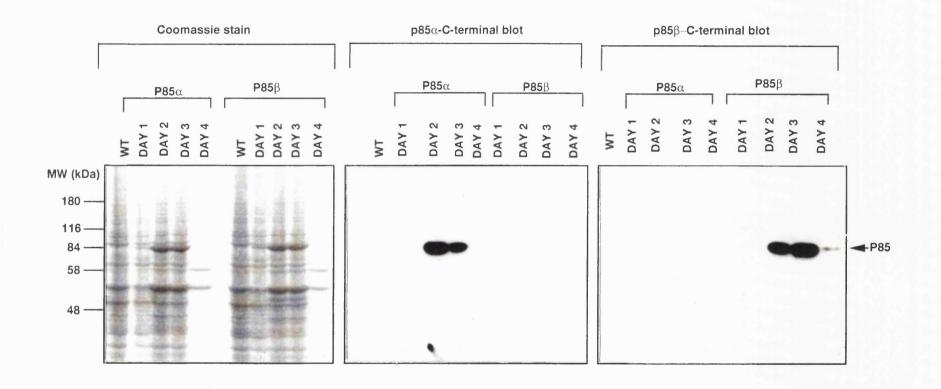


Figure 3.1. Expression of p85 α and p85 β in Sf9 cells.

Sf9 cells were infected with either wildtype (WT) or recombinant baculovirus vectors expressing p85 α or p85 β as indicatedlegends. WT baculovirus infected cells were harvested after 2 days and cells infected with recombinant viruses were harvested at the times indicated at the top of each panel. Lysates were subjected to SDS-PAGE in 10% gels and then analysed as follows: Left panel: Coomassie blue stain of lysates from Sf9 cells infected with recombinant baculovirus vectors. Middle panel: Immunoblot of Sf9 cell lysates probed with COOH-terminal p85 α specific antiserum. Right panel: Immunoblot of Sf9 cell lysates with probed COOH-terminal p85 β specific antiserum.

harvested 2.5 days later. Recombinant viruses were identified by plaque hybridisation with p85α or p85β cDNA fragments, ³²P-labelled by the random priming method (Feinberg and Vogelstein, 1983), or by identifying polyhedrin-negative plaques under the light microscope. These were then amplified to make virus stocks which were titred for optimal protein production. The levels of p85 protein expression were monitored by analysing whole cell lysates using SDS-PAGE at various times post infection. Figure 3.1 (left panel) shows results which indicate that within two days, high levels of both p85 α and p85\beta proteins begin to accumulate inside the Sf9 cells. Maximal expression was observed between day two and three after infection, at which point p85 proteins accounted for up to 10% of total cell protein, a level which decreased as the virus entered into its lytic phase. Probing with specific, affinity purified COOH-terminal peptide antibodies in Western blot assays further confirmed the identity of the proteins (Figure 3.1, middle (p85 α) and right (p85 β) panels). These antibodies were specifically reacting only with the p85 produced in cells infected with the p85 α (Figure 3.1, middle panel) or p85\(\text{B}\) (Figure 3.1, right panel) recombinant viruses respectively. Cells infected with wild type AcMNPV alone showed no detectable immunoreactive protein two days postinfection.

3.2.1.3 Purification of recombinant p85 α and p85 β proteins from insect cells.

Both forms of the p85 proteins could be isolated using identical purification schemes reflecting the high degree of structural similarity between them. Briefly, the purification protocol adopted was as follows. Stocks of recombinant virus were used to infect approximately 5 x 10⁹ Sf9 cells grown in monolayer cultures in fifty 225 cm² flasks. Two days post-infection, cells were harvested by centrifugation and washed with PBS. The solubility of the two proteins during the cell extraction process was tested. Cells were either homogenised using a Dounce homogeniser in the absence of detergents or lysed in the presence of 1% Triton X-100. After centrifugation the clarified supernatants and the insoluble pellets were analysed by SDS-PAGE and the p85 proteins identified by Coomassie blue staining. Both p85 proteins were found to be readily extractable from cells in the absence of detergents. This is in agreement with earlier reports which have suggested that in unstimulated cells the bulk of p85 is cytosolic (Cohen et al., 1990). The soluble cell lysate was applied to a 20 ml Q-Sepharose (anion-exchange) column and elution of bound p85 was achieved at a NaCl concentration of 250-300 mM using a linear gradient (Figure 3.2, top left panel). Further purification and concentration was achieved by applying the pooled peak fractions to a MonoQ FPLC column (Figure 3.2, middle left panel). Both p85 proteins eluted from this column at approximately 250 mM salt. This material was then subjected to gel exclusion chromatography using a Superose 12 FPLC column (Figure 3.2, bottom left panel). Analysis of a typical purification procedure for p85α is shown in Figure 3.2. Both p85 proteins exhibited identical chromatographic

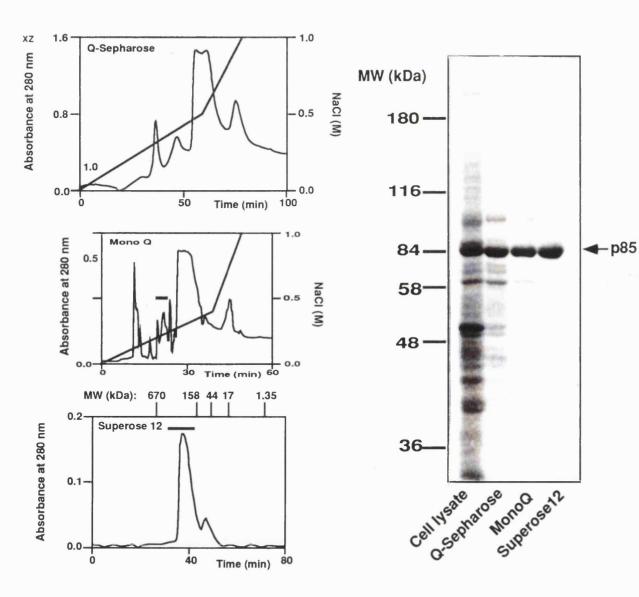


Figure 3.2. Purification of p85 proteins. Typical column profiles from a representative purification of p85α. The bars above each trace show the fractions containing p85 proteins eluting from each column which were then pooled.

Top left panel: Profile of Q-Sepharose column. Middle left panel: Profile of MonoQ FPLC column. Bottom left panel: Profile of Superose 12 FPLC column.

Right panel: Coomassie stained gel of total Sf9 cell lysates and aliquots from pooled peak fractions throughout a typical purification of p85α.

behavior on these columns. The p85 α and β proteins eluted from the Superose 12 column at a position suggesting that they had an apparent molecular weight of approximately 210 kDa. It is unclear at the moment whether this is due to oligomerisation. Figure 3.2 (right panel) shows SDS-PAGE analysis of the material applied to the various columns and of the purified product. No significant further purification could be achieved using a highly specific affinity purification step (Otsu et al., 1991; Fry et al., 1992) based on the interaction of p85 with a tyrosine-phosphorylated synthetic peptide corresponding to the kinase insert region of the PDGF receptor. The overall yield of pure p85 was approximately 50% resulting in approximately 10 mg of protein purified from 5 x 10^9 infected Sf9 cells.

Due to the exceptional high expression levels of both the p85 proteins, slab-gel SDS-PAGE proved to be a rapid procedure for the production of large amounts of purified p85 protein which could subsequently be used to generate polyclonal antibodies in rabbits. Insect cells were infected with p85 virus and the cells harvested as described above. Whole cell lysates were prepared and subjected to SDS-PAGE. The gel was then stained in 50% Coomassie blue until the p85 protein was just visible. Gel strips of the p85 protein were carefully excised, placed into dialysis bags and subjected to electroelution. The gel strips were then carefully removed from the bag, the dialysis bag reclipped, and subjected to dialysis to reduce the level of residual SDS. The protein was aliquoted and stored at -70 °C.

3.2.1.4 Preparation and characterisation of antisera against p85.

The purified native and denatured p85 proteins were injected separately into rabbits to generate polyclonal antibodies. By immunoprecipitation analysis, the antibodies obtained predominantly recognised the p85 species against which they were raised (Figure 3.3). The two antisera were affinity purified using p85 α and p85 β Actigel columns and their ability to specifically immunoprecipitate recombinant p85 proteins from Sf9 cell lysates was tested. As can be seen in Figure 3.3 despite the high degree of homology between the two p85 proteins (Otsu et al., 1991) these antibodies were specific in their ability to immunoprecipitate the appropriate p85 protein.

These antisera were then used to immunoprecipitate PI 3-kinase activity from the partially purified bovine brain preparation. As can be seen in Figure 3.4 despite the high titre of both of these antisera only the $p85\alpha$ antibody immunoprecipitated significant amounts of bovine brain PI 3-kinase activity, although a small amount of PI 3-kinase activity was seen to be immunoprecipitated with the $p85\beta$ antibody. To further characterise these antisera the ability of increasing concentrations of baculovirus



Figure 3.3 Characterisation of the polyclonal antibodies raised against the p85 α and p85 β protein.

The specificity of the affinity purified p85 α and p85 β antibodies to the proteins to which they were raised was examined. The antibodies were incubated with lysates of Sf9 cells expressing p85 α and p85 β proteins and the immunocomplexes then subjected to SDS-PAGE. Lane 1: Crude lysate of Sf9 cells expressing p85 β protein, Lane 2: p85 β expressing Sf9 cells immunoprecipitated with p85 β antibody, Lane 3: p85 β expressing Sf9 cells immunoprecipitated with p85 α , Lane 4: MW standards (kDa); 97, 68, 44, 30, 21, Lane 5: Crude lysate of Sf9 cells expressing p85 α protein, Lane 6: p85 α expressing Sf9 cells immunoprecipitated with p85 α antibody, p85 α expressing Sf9 cells immunoprecipitated with p85 α antibody, p85 α expressing Sf9 cells immunoprecipitated with p85 α , Lane 8: crude lysate of WT infected Sf9 cells.

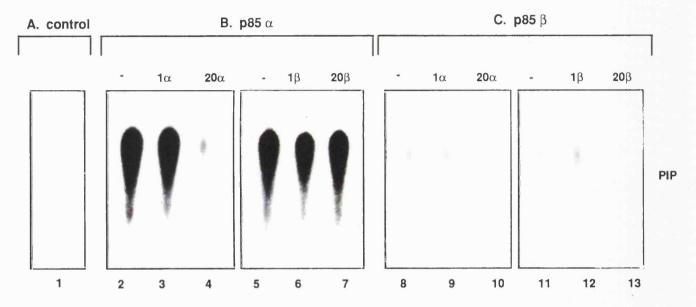


Figure 3.4 Immunoprecipitation of PI 3-kinase activity from the partially purified bovine brain preparation using affinity purified polyclonal p85 antibodies.

Polyclonal p85 antibodies were incubated with partially purified bovine brain preparation diluted in EB buffer (+) or (-) baculovirus purified p85 proteins. These immunocomplexes were then used in a PI 3-kinase assay. Lanes 2, 5, 8 and 11: PI 3-kinase in EB buffer. Lanes 3 and 9: PI 3-kinase in EB buffer +1 μ g p85 α . Lanes 4 and 10: PI 3-kinase in EB buffer +20 μ g p85 α . Lanes 5 and 11: PI 3-kinase in EB buffer +1 μ g p85 α . Lanes 7 and 13: PI 3-kinase in EB buffer +20 μ g p85 α . Lanes 2 to 7 were immunoprecipitated with anti p85 α serum. Lanes 8 to 13 were immunoprecipitated with anti p85 α serum. Lanes 8 to 13 were immunoprecipitated with anti p85 α serum. Lanes 8 to 13 were alone. PIP markers indicate the positions of PI 3-phosphate.

expressed, recombinant p85 proteins to compete with the bovine brain PI 3-kinase in an immunoprecipitation assay with the p85 α specific antisera was examined. Purified baculovirus p85 α protein was observed to block the immunoprecipitation of PI 3-kinase activity from bovine brain, but no competition was observed even at the highest concentrations (20 µg) of added purified baculovirus p85 β protein (Figure 3.4B). It should be noted that the small amount of PI 3-kinase activity immunoprecipitated with the p85 β antiserum was also specifically competed with purified baculovirus p85 β , but not p85 α protein (Figure 3.4C). This result strengthens the suggestion that there is a small population, of ~1%, of p85 β -associated PI 3-kinase activity present in the bovine brain purified PI 3-kinase.

3.2.1.5 Examination of the cellular distribution of p85 α – and p85 β – associated PI 3-kinase activity.

The cellular distribution of $p85\alpha$ - and $p85\beta$ -associated PI 3-kinase activity was next examined. An 80-90% confluent dish of the appropriate cell types were lysed and p85 immunoprecipitated with antibodies that recognise either the $p85\alpha$ or the $p85\beta$ protein. The immunocomplexes were collected on Protein A-Sepharose beads and washed several times in lysis buffer. Complexed proteins were then assayed for associated PI 3-kinase activity. The affinity-purified $p85\alpha$ antibody was found to immunoprecipitate PI 3-kinase activity from a wide range of species including human (A431), bovine (BARC), simian (COS-1), murine (NIH 3T3), and porcine (ZNR) cells (Figure 3.5). However little or no PI 3-kinase activity was detected in any of these cell lines after immunoprecipitation with the affinity-purified $p85\beta$ antibody (Figure 3.5). Thus, $p85\alpha$ would appear to be more ubquitously expressed than the $p85\beta$ isoform.

3.2.2 Association of protein-tyrosine kinases with the p85 proteins

PI 3-kinase activity was first identified in immunoprecipitates of the oncoproteins pp60^{v-src} and v-ros (Sugimoto et al., 1984; Macara et al., 1984) and was subsequently found in association with a number of receptor protein-tyrosine kinases, including the PDGF (Kaplan et al., 1987) and CSF-1 (Varticovoski et al., 1989) receptors. To examine whether the p85 proteins displayed similar properties to the cellular PI 3-kinase, their ability to interact with protein tyrosine kinases was examined using the baculovirus system.

3.2.2.1 Construction of the CSF-1 receptor baculovirus transfer vector and expression in insect cells.

The entire coding region of the human CSF-1 receptor was obtained in the Bluescript vector (Stratagene, gift of C. Sherr) where it was inserted as a BamH1 fragment (BS-CSF-1R). In order to clone the fragment expressing the receptor into the baculovirus

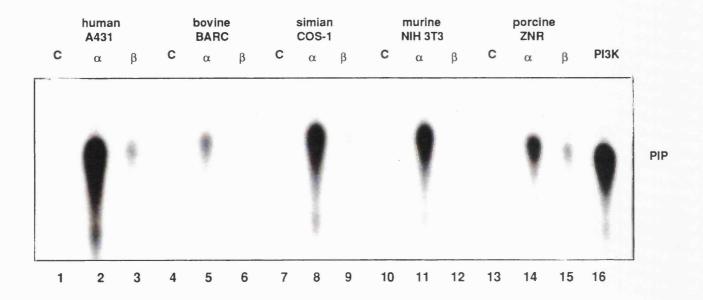


Figure 3.5. Immunoprecipitation of PI3 kinase activity from cell lines.

Confluent A431, BARC, COS-1, NIH 3T3, and ZNR cells were lysed and immunoprecipitated with affinity purified anti-p85 α (α), p85 β (β) antibodies or Protein A-Sepharose alone (C). PI 3-kinase assays were then performed on the samples. Lanes 1, 4, 7, 10 and 13 were precipitated with Protein A-Sepharose alone. Lanes 2, 5, 8, 11 and 14 were immunoprecipitated with anti-p85 α serum. Lanes 3, 6, 9, 12 and 15 were immunoprecipitated with anti-p85 β serum. Lane 16: PI 3-kinase activity in 0.5 μ l of partially purified bovine brain preparation. PIP markers indicate the migration of PI 3-phosphate.

transfer vector pAcC4, the BS-CSF-1R vector was subjected to two double digests. First, with Nco1-Hind111 and second, with Hind111-EcoR1. Two fragments of molecular size 1.048 kb and 2.036 kb were gel purified and ligated to pAcC4 restricted with the Nco1-EcoR1 in a triple ligation. Positives were isolated by a diagnostic restriction enzyme digest which cut at the unique EcoR1 site in the baculovirus transfer vector causing it to linearise. To transfer the CSF-1 receptor human cDNA into the AcMNPV baculoviral genome, Sf9 cells were cotransfected with pAcC4-CSF-1 receptor and wild-type AcMNPV DNA. Recombinant virus was initially identified by DNA hybridisation analysis of infected cells using CSF-1 receptor cDNA fragments ³²P-labelled by the random priming method (Feinberg and Vogelstein, 1983), and later visually by selecting for occlusion negative plaques, ie. those lacking the polyhedrin protein. These viruses were then amplified to make virus stocks which were titred for optimal protein production for infection of Sf9 cells.

3.2.2.2 Characterisation of the CSF-1 receptor in insect cells.

The properties of the recombinant CSF-1 receptor were analysed using structural and functional criteria based upon the use of antibodies to recognize epitopes in the human receptor and second by demonstration of ligand stimulatable protein-tyrosine kinase activity.

- 1. Two recombinant CSF-1 receptor expressing viruses were first checked for activity by immunoprecipitating lysates of CSF-1 receptor virus-infected insect cells, with a monoclonal antibody that recognised the extracellular domain of the receptor. The immunoprecipitates were collected on Protein A-Sepharose beads and then washed several times with lysis buffer and twice with kinase buffer. The receptor was allowed to autophosphorylate in the presence of $[\gamma^{32}P]ATP$ before analysis by autoradiography of SDS-PAGE gels. Wild type AcMNPV-infected cells were used as a control. The results in Figure 3.6A show that both recombinant viruses expressed CSF-1 receptors which were highly phosphophorylated and considered functional (lanes 2 and 3). The antibody did not recognise any endogenous insect proteins of this molecular size from control infected cells (lane 1).
- 2. The levels of CSF-1 receptor protein expression were monitored by immunoprecipitating lysates of CSF-1 receptor virus-infected insect cells at various days following infection. Figure 3.6B shows a Coomassie blue stain of SDS-PAGE gel of these immunoprecipitations, which indicates that within two days post-infection high levels of the CSF-1 receptor protein has begun to accumulate in Sf9 cells. Maximal expression was observed between day two and three post-infection, after which the protein could not be detected by Coomassie blue staining. The level of tyrosine phosphorylation of the CSF-1 receptor was similtaneously analysed in these cells by immunoblotting 1/5 of these immunoprecipitations with an affinity purified anti-

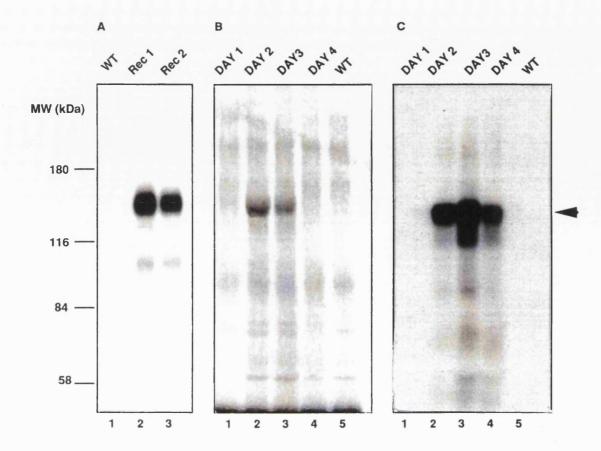


Figure 3.6. Characterisation of the recombinant CSF-1 receptor expressed in baculovirus.

- (A). SF9 cells were infected with wild-type [WT] (lane 1), or two independently isolated CSF-1 expressing baculoviruses (lanes 2 and 3). Lysates prepared from these cells were incubated with a monoclonal antibody recognising the extracellular domain of the CSF-1 receptor. These immune complexes were subjected to protein kinase assays and analysed by SDS-PAGE. Active receptor was detected by autoradiography and is indicated by an arrow.
- (B). Sf9 cells were infected with recombinant baculovirus expressing the CSF-1 receptor were harvested at the times indicated at the top of each panel. WT baculovirus infected cells were harvested 2 days post-infection. Lysates from these cells were then immunoprecipitated with excess anti-CSF-1 antibody. Immune complexes were analysed by SDS-PAGE and proteins detected by Coomassie Blue staining.
- (C). Anti CSF-1 immunoprecipitates prepared and treated as described in (B) were immunoblotted with antiphosphotyrosine antibody and proteins detected by autoradiography.

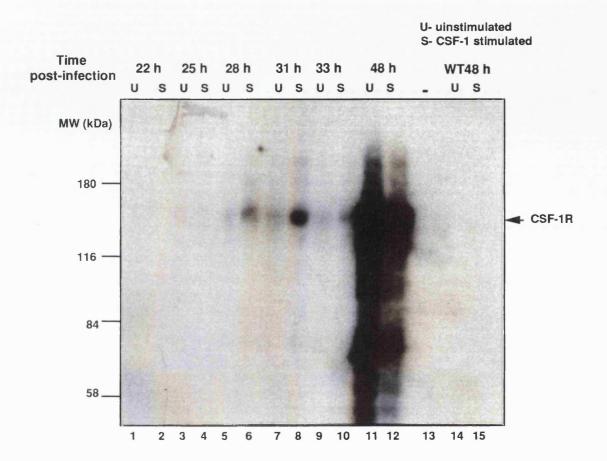


Figure 3. 7. Ligand stimulation of recombinant CSF-1 receptor expressed in baculovirus.

Sf9 cells growing in serum-free media were infected with recombinant baculovirus expressing CSF-1 receptor and stimulated with CSF-1 at times indicated on the Figure (Lanes 1-12). Wild-type (WT) baculovirus infected cells (lanes 14-15) were stimulated with CSF-1 48 h post-infection. Lysates were subjected to SDS-PAGE and Western-blot analysis, probed with antiphosphotyrosine antibody.

phosphotyrosine antibody. The results in Figure 3.6C show that the CSF-1 receptor is highly phosphorylated in insect cells, and the level of tyrosine phosphorylation is found to increase over the time course of infection. In particular, it can clearly be seen that at four days post-infection, immunoprecipitated CSF-1 receptor protein cannot be detected by Coomassie blue staining using SDS-PAGE (Figure 3.6B, lane 4), however a high level of tyrosine phosphorylation of the receptor is still observed (Figure 3.6C, lane 4). It should be noted that the anti-CSF-1 receptor and the anti-phosphotyrosine antibody did not recognise any endogenous insect cell proteins of this molecular weight (Figure 3.6A, lane 1; 3.6B and C, lane 5).

3. As the level of tyrosine phosphorylation observed on the CSF-1 receptor was found to increase with time post-infection, irrespective of the level of receptor protein, the effects of ligand stimulation were also analysed. 4.5 x 10⁶ insect cells were cultured in Excel media, which is a high-protein insect cell medium that does not require the addition of FCS for cell growth. Cells were infected with recombinant CSF-1 receptor expressing virus at different time points so that the cells could be stimulated simultaneously with 10 ng of human CSF-1 (obtained from the Genetics Institute). Whole cell lysates were subjected to anti-phosphotyrosine western blot analysis. The results in Figure 3.7 show that CSF-1 receptor can be ligand activated up until 28 h post-infection (lane 5 and 6), but is constitutively activated 48 h after infection with recombinant virus (lanes 11 and 12).

3.2.2.3 Recombinant p85 proteins are substrates for protein-tyrosine kinases.

In order to examine the interaction between the expressed p85 α and p85 β proteins and a variety of protein tyrosine kinase growth factor receptors, these molecules were coexpressed in insect cells using recombinant baculoviruses. The receptor molecules employed were the EGF receptor (Greenfield et al., 1988), the CSF-1 receptor, and the c-erbB2 protein (L. Wilson, unpublished data). The non-receptor protein-tyrosine kinase pp59c-fyn (Courtneidge et al., 1993) was also used in these assays. The protein-tyrosine kinases were expressed in insect cells either alone or together with p85 α or p85 β . Cell lysates from infected cells were subjected to SDS-PAGE and immunoblotting with an affinity-purified, anti-phosphotyrosine antibody. As can be seen in Figure 3.8, both p85 α and p85 β were highly phosphorylated on tyrosine residues when coexpressed with any of the protein-tyrosine kinases, but not when expressed on their own.

3.2.2.4 A tight complex forms between the p85 proteins and protein-tyrosine kinases.

The results described above demonstrate that both p85 proteins are in vivo substrates for a variety of protein-tyrosine kinases. However, it was still not known whether complex formation between the p85 protein and the protein-tyrosine kinase was actually taking place, or whether the p85 proteins were simply acting as exogenous substrates. To

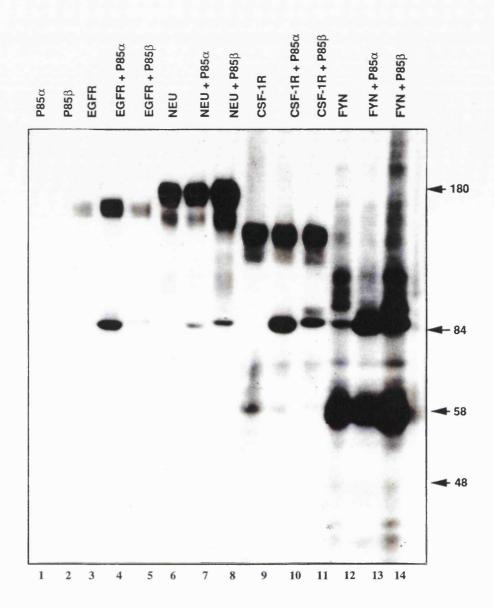


Figure 3.8. In vivo phosphorylation of p85 proteins by protein-tyrosine kinases.

Co-expression of p85 proteins together with protein-tyrosine kinases in Sf9 cells analysed by western blot analysis with an affinity purified anti-phosphotyrosine antibody. Lanes 1 and 2: Crude lysate of Sf9 cells expressing p85 α and p85 β proteins alone. Lanes 3 to 5: Crude lysates of Sf9 cells expressing EGF receptor alone or with p85 α or p85 β proteins respectively. Lanes 6 to 8: Crude lysates of Sf9 cells expressing NEU alone or with the p85 α or p85 β proteins respectively. Lanes 9 to 11: Crude lysates of Sf9 cells expressing CSF-1 receptor alone or with p85 α or p85 β proteins respectively. Lanes 12 to 15: Crude lysates of Sf9 cells expressing p59c-fyn kinase alone or with p85 α or p85 β proteins respectively.

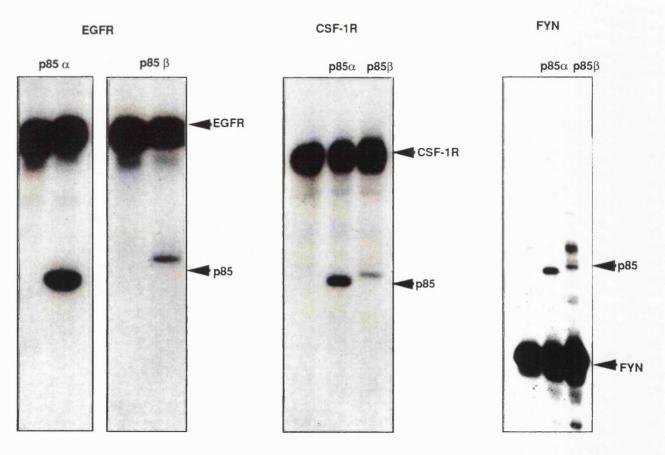


Figure 3.9. Association with and in vitro phosphorylation of p85 proteins by protein-tyrosine kinases. Lysates prepared from Sf9 cells infected with recombinant baculovirus virus expressing EGF receptor, CSF-1 receptor, or p59c-fyn protein-tyrosine kinase alone, or with one of the p85 proteins, as indicated, were incubated with appropriate protein kinase-specific antibodies and the immune complexes then radiolabelled in the presence of $[\gamma$ -32P]ATP, resolved on 7.5% SDS-PAGE gels, and finally subjected to autoradigraphy. Migration of protein-tyrosine kinases are marked by arrow heads.

investigate this, lysates of cells, coinfected with two recombinant baculovirus expressing a protein-tyrosine kinase and one of the p85 species, were incubated with antibodies specific for the appropriate protein-tyrosine kinase and the immunocomplexes were collected on Protein A-Sepharose beads. After stringent washing in lysis buffer, the beads were incubated with a kinase assay mixture containing radiolabelled ATP and phosphorylated proteins were analysed by SDS-PAGE and autoradiography. Figure 3.9 shows that both p85 α and p85 β formed tight complexes in Sf9 cells with, and were phosphorylated by, the protein-tyrosine kinase receptors for EGF and CSF-1, and by the src-family protein-tyrosine kinase, pp59c-fyn.

3.2.2.5 Phosphorylation of the protein-tyrosine kinase is required for efficient binding of the p85 proteins.

PI 3-kinase activty has only been found in association with activated protein-tyrosine kinases (Kaplan et al., 1987; Courtneidge and Heber, 1987). To examine whether the p85 proteins exhibited similar characteristics, the ability of p85 α and p85 β to associate with the EGF and CSF-1 receptors and the non-receptor tyrosine kinases pp60^{c-src} and pp59^{c-}Fyn was examined using an in vitro association assay described by Kazlauskas and Cooper (1990).

The protein-tyrosine kinases were immunoprecipitated from lysates of insect cells 1.5 days post-infection to keep tyrosine phosphorylation of the receptor/non-receptor kinase to a minimal level (see section 3.2.2.2). The immunoprecipitated kinase was collected on Protein A-Sepharose beads and subjected to several washes with lysis buffer. The immune complexes were washed twice with kinase buffer and then phosphorylated in vitro in the presence of non-radiolabelled ATP. Excess ATP was removed by several washes in lysis buffer and the phosphorylated protein-tyrosine kinase was incubated with Sf9 cells that had been infected with either of the p85 proteins. The resultant receptor complexes were then subjected to in vitro kinase assay and analysis by Coomassie blue staining and autoradiography of SDS-PAGE gels. The results in Figure 3.10A show a Coomassie blue stain of the EGFR immunoprecipitated from insect cells (lane 1). In vitro phosphorylation of the receptor was observed to cause a shift in mobility due to increased tyrosine phosphorylation. Equal amounts of both the p85 α and p85 β proteins were found to associate with the EGFR but only if it had been phosphorylated in vitro (lanes 2 and 4 respectively). If the receptor was not first phosphorylated in vitro the p85 proteins were not seen to bind (lanes 3 and 5). The stoichiometry of receptor/p85 binding appears to be ~1:1 from Coomassie blue staining of SDS-PAGE gels (lanes 2 and 4). Results in Figure 3.10B show an autoradiograph of the Coomassie blue stained SDS-PAGE gel. Both the p85 proteins were found to associate tightly with, and to be phosphorylated by, the EGF receptor in vitro.

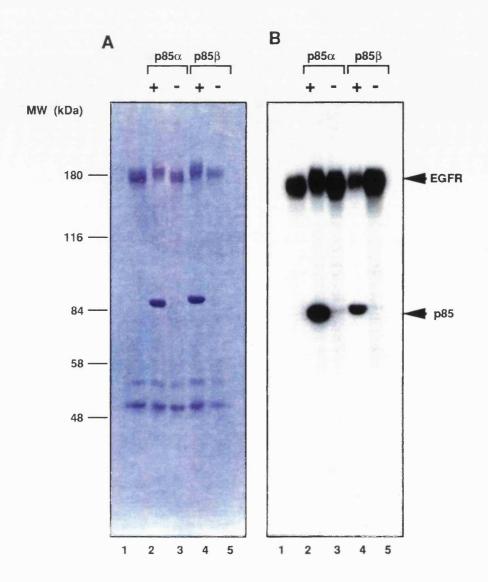


Figure 3.10. In vitro association of p85 α and p85 β with the EGF receptor. (A) EGF receptor was immunoprecipitated using the R1 monoclonal antibody from baculovirus infected Sf9 cells. To form in vitro receptor complexes, the EGF receptor was incubated with nonlabelled ATP (lanes 2 and 4) or lysis buffer (lanes 1, 3 and 5) and then exposed to lysates of insect cells that had been infected with p85 α (lanes 2 and 3) or p85 β (lanes 4 and 5) for 2 hr. The protein complexes were washed with lysis buffer and subsequently with kinase buffer. The samples were then subjected to protein kinase assays and analysed by SDS-PAGE. The associated proteins were detected by Coomassie Blue staining (A) and autoradiography (B).

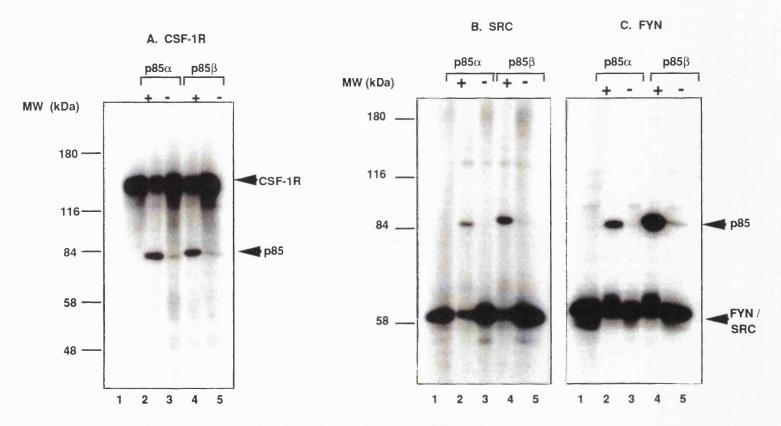


Figure 3.11. Association of protein tyrosine kinases with the p85 α and p85 β proteins.

Insect cells were infected with recombinant baculoviruses expressing the CSF-1 receptor (A), Src non-receptor protein tyrosine kinase (B) and Fyn non-receptor protein tyrosine kinase (C). Lysates were immunoprecipitated using PTK-specific antibodies. To form in vitro receptor complexes, the protein tyrosine kinase receptor was incubated with nonlabelled ATP (Panals A, B and C, lanes 2 and 4) or lysis buffer (Panals A, B and C, lanes 1, 3 and 5) and then exposed to lysates of insect cells that had been infected with p85 α (Panals A, B and C, lanes 2 and 3) or p85 β (Panals A, B and C, lanes 4 and 5) for 2 hr. The protein complexes were subjected to protein kinase assays and associated proteins were detected by autoradiography of SDS-PAGE gels.

The CSF-1 receptor and the non-receptor tyrosine kinases, pp60^{c-src} and pp59^{c-Fyn}, immunoprecipitated from insect cells were then used in essentially the same assay with similar results. Both the p85α and the p85β proteins were found to be associated with, and phosphorylated by the phosphorylated forms of CSF-1 receptor, pp60^{c-src} or pp59^{c-Fyn} (Figure 3.11, Panels A, B and C, lanes 2 and 4). If the protein-tyrosine kinase was not phosphorylated prior to incubation with the p85 proteins, then both the p85 proteins were not seen to bind (Figure 11 Panals A, B and C, lanes 3 and 5).

3.2.2.6 Phosphoamino acid analysis of in vitro associated p85.

The phosphorylation state of the p85 α and the p85 β protein when in association with activated protein-tyrosine kinases was then examined by performing phosphoamino acid analysis of p85 phosphorylated in vitro. Essentially, the same experiment as described above (section 3.2.2.5) was performed. The radiolabelled p85 phosphoproteins were then excised from the gel, the proteins isolated and subjected to acid hydrolysis as described in Methods. The sample was electrophoresed in two dimensions and phosphoamino acids identified by autoradiography. As can be seen in Figure 3.12, both the p85 α and the p85 β proteins are phosphorylated solely on tyrosine following association with, and phosphorylation by either the EGF, CSF-1 receptor or pp59c-Fyn non-receptor kinase, in vitro (Panals A-C).

3.2.2.7 SH2 domains mediate the binding of the p85 proteins with activated receptor-tyrosine kinases.

The data so far stress the importance of phosphotyrosine for mediating the tight association between the p85 proteins and protein tyrosine kinases. This phosphotyrosine-dependency suggests that the mechanism of p85 binding protein-kinase is probably mediated by the SH2 domains of p85. The p85 protein contains two SH2 domains; the basis of the specificity of binding to receptors was next investigated.

Two forms of the N-terminal SH2 domain were used in this study, the first was constructed exactly to the boundaries described by direct amino acid alignment with other SH2 domains (residues 329-416), and was termed sNSH2. The boundaries of the second N-terminal SH2 domain were defined by limited proteolysis of recombinant p85α using V8 protease. This produced a fragment which covered residues 301-439 and was termed NSH2. Structural analyses have revealed that the NSH2 domain was correctly folded (Panayotou et al., 1992; Booker et al., 1993). The C-terminal SH2 (CSH2) included analogous residues to that of the functional NSH2. Both SH2 domains were constructed by I. Gout, expressed in bacteria as Glutathione S-transferase fusion proteins and used as such.

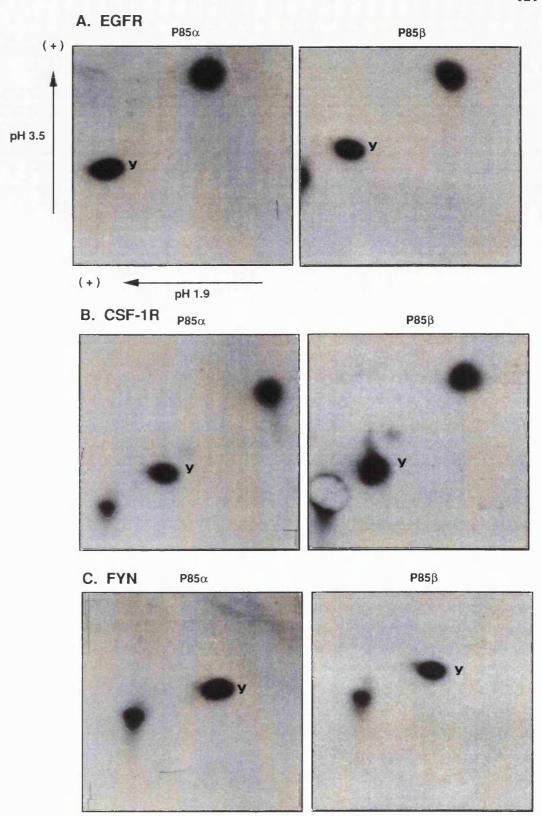


Figure 3.12. Phosphoamino acid analysis of PI 3-kinase phosphorylated in vitro. The p85 proteins were phosphorylated in vitro and analysed by autoradiography of SDS-PAGE. Phosphoamino acid analysis was then performed on both p85 α and p85 β following excision from the gel.

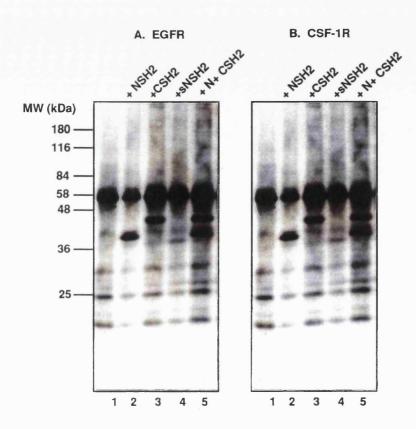


Figure 3. 13. Both the N- and the C-terminal SH2 form a tight association with the EGF and the CSF-1 Receptors.

Lysates prepared from SF9 cells infected with recombinant baculovirus expressing EGF receptor and CSF-1 receptor were immunoprecipitated with appropriate receptor specific antibodies. The receptors were then prephosphorylated by incubation with non-labelled ATP (Panels A and B, lanes 2-5) and then incubated with lysis buffer containing 1 μ g of GST-NSH2 domain (Panels A and B, lane 2), 1 μ g of GST-CSH2 (Panels A and B, lane 3), 1 μ g of the shorter NSH2 (sNSH2) domain (Panels A and B, lane 4) or with lysis buffer containing 1 μ g of both the NSH2 and the CSH2 domain (Panel A and B, lane 5) for 2 h. Lane 1 of panels A and B show control untreated receptor immunoprecipitated from Sf9 cells. Protein complexes were subjected to Western blot, probed with GST-specific polyclonal antiserum.

To determine whether the N-, the C- terminal and the shorter N-terminal (sN) SH2 domains of $p85\alpha$ were able to form a tight association with the EGF and the CSF-1 receptor, both protein-tyrosine kinases were immunoprecipitated from lysates of insect cells that had been infected with the appropriate recombinant baculovirus and collected on Protein A-Sepharose beads. These immunoprecipitates were incubated for 1 h with equal amounts of GST-fusion proteins of the N-, sN- and the C-terminal SH2 domains diluted in lysis buffer, after which the immune complexes were subjected to several washes with lysis buffer. The associated GST-fusion proteins were analysed by immunoblotting of SDS-PAGE gels with an anti-GST polyclonal antibody. The results in Figure 3.13 show that both the N- (Panels A and B, lane 2) and the C- (panal A and B, lane 3) terminal SH2 domain of p85 α were able to form a tight complex with the EGF and the CSF-1 receptors. The sNSH2 domain was seen not to bind significantly to either the EGF or the CSF-1 receptor (panels A and B respectively, lane 4). This demonstrates the importance of correct folding of the SH2 domain for association with receptor tyrosine kinases. Both the N- and the C-terminal SH2 domains were able to bind the receptor simultaneously (panels A and B, lane 5), although this does not necessarily indicate that both SH2 domains are bound to a single receptor molecule.

3.2.2.8 The N- and the C-terminal SH2 domains are substrates for both the EGF and the CSF-1 receptors.

SH2 domains have been demonstrated to interact specifically with polypeptides containing phosphotyrosine (Kazlauskas and Cooper, 1989; Mayer et al., 1991). However, it remained unclear whether they would behave as substrates for the receptor-tyrosine kinases once associated. Thus, the EGF and the CSF-1 receptor were immunoprecipitated from insect cells and incubated with GST-fusion proteins of either the N- or the C-terminal SH2 domains of p85 α . Following binding, the immune complexes were washed several times in lysis buffer and subjected to in vitro kinase assay. Phosphoproteins were analysed by autoradiography of SDS-PAGE gels. The results in Figure 3.14 show that both the N- and the C-terminal SH2 domains of p85 α can act as direct substrates for the EGF and the CSF-1 receptor (Panels A and B, lanes 2 and 3).

3.2.2.9 Multienzyme complex formation

A wealth of biochemical and structural data has now been accumulated on the proteins which are found associated in signal transduction complexes upon activation of protein-tyrosine kinases, (reviewed in Pawson and Schlessinger, 1993). Several enzymes or regulatory proteins have been detected in these multienzyme complexes, for example, the PDGF β -receptor has been shown to associate with the GTPase-activating protein (RasGAP) for Ras (Kazlauskas et al., 1990), phospholipase C γ (PLC γ) (Meisenhelder et

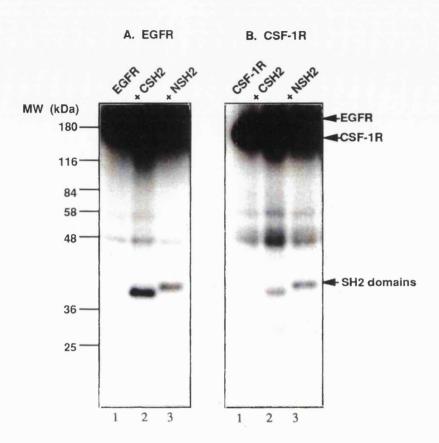


Figure 3. 14. Both the N- and C-terminal SH2 domains are substrates for the EGFR and the CSF-1R.

Lysates prepared from SF9 cells infected with recombinant baculovirus expressing EGF receptor and CSF-1 receptor were incubated with appropriate receptor specific antibodies. The receptors were then prephosphorylated by incubation with non-labelled ATP (Panels A and B, lanes 2 and 3) and then incubated with $1\mu g$ of GST-CSH2 domain (Panels A and B, lane 2) or $1~\mu g$ of GST-NSH2 (Panels A and B, lane 3) for 2 h. Protein complexes were subjected to in vitro protein kinase assays and then detected by autoradiography of SDS-PAGE gels. Lane 1 of panels A and B show control untreated receptor immunoprecipitated from Sf9 cells.

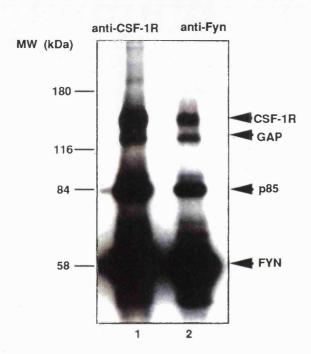


Figure 3.15. Multienzyme complex formation. Lysates of SF9 cells that had been infected with recombinant baculoviruses expressing the CSF-1R, Fyn, p85 and GAP proteins were immunoprecipitated with antibodies directed to the CSF- 1R (lane 1) and the Fyn kinase (lane 2). These immune complexes were then subjected to protein kinase assays and analysis by autoradiography of SDS-PAGE.

al., 1989), src-family protein tyrosine kinases (Mori et al., 1993), the protein tyrosine phosphatase Syp/SHPTP2 (Feng et al., 1993; Vogel et al., 1993), the Shc gene product (Pelicci et al., 1992), Grb2 (Lowenstein et al., 1992); Nck (Nishimura et al., 1993) and the phosphatidylinositol (PI) 3-kinase (reviewed in Cantley et al., 1991).

The association of receptors with individual signalling molecules is well documented, however, whether several of these second messengers were able to bind an activated receptor simultaneously had not been studied in such detail. The ability of the CSF-1 receptor, which has been shown to bind a subset of these molecules (Cantley et al., 1991), to participate in multienzyme complex formation was examined. Sf9 cells were coinfected with recombinant viruses expressing the CSF-1 receptor, pp59c-Fyn, p85a Three days post-infection the cells were harvested, lysed and immunoprecipitated with antibodies that either recognise the CSF-1 receptor or pp59^c-Fyn. The immune complexes were collected on Protein A-Sepharose beads, washed several times with lysis buffer, twice with kinase buffer and subjected to in vitro kinase assays. The phosphorylated proteins were resolved on SDS-PAGE gels and detected by autoradiography. The results in Figure 3.15 show a multienzyme complex, consisting of the CSF-1 receptor, pp59c-Fyn, p85α and RasGAP being precipitated by antibodies raised to the CSF-1 receptor (lane 1) and the non-receptor pp59c-Fyn (lane 2). It is difficult to assess the stoichiometry of phosphorylation of these molecules due to the differences in expression of the proteins in the insect cells and the non-quantitative nature of the immunoprecipitating antibodies used in these experiments. However, qualitative assessment suggests that although the CSF-1 receptor, p85 α and pp59c-Fyn appear to be present in approximately a 1:1:1 ratio, the level of the RasGAP protein present is much lower (Figure 3.15).

3.3 Discussion

3.3.1 Expression of the p85 proteins

In this study the p85 α protein was expressed in insect cells using recombinant baculovirus transfer vectors in order to obtain a large amount of protein that would have correct post-translational modifications for definitive study of the biological functions and biochemical properties of the p85 subunit of the PI 3-kinase. Both p85 α and p85 β were found to be expressed to high levels and readily extracted from the cell in the absence of detergent. This allowed the development of a simple and efficient purification protocol which led to the isolation of milligram quantities of the two p85 proteins. The purified protein was used to generate antibodies that further facilitated the characterisation of the PI 3-kinase complex, for analytical studies which may eventually

lead to a 2-D and 3-D structural analysis of the proteins, and for kinetic analyses on the BIAcore which measures protein-protein interactions in real time (Panayotou et al., 1993a; Felder et al., 1993).

Both the p85 α and the p85 β polyclonal antibodies were found to immunoprecipitate PI 3kinase activity from the bovine brain preparation, although the predominant species present was the p85α-associated PI 3-kinase activity (Otsu et al., 1991; Gout et al., 1992; Fry et al., 1992). This also reinforced the fact that p85 is a multidomain structural protein that is found associated with a catalytic subunit which contains enzymatic activity. Polyclonal antibodies raised to the bovine p85 proteins were found to recognise PI 3kinase activity from a variety of species including human, bovine, simian, murine and porcine, suggesting a structural conservation of the protein across a number of mammalian species. Similar results were observed with an antibody raised to the human p85 protein (Skolnik et al., 1991). The conservation of this protein in mammalian species is also reflected in the high level of sequence identity observed between the bovine, human and murine p85α proteins (Otsu et al., 1991; Escobedo et al., 1991; Skolnik et al., 1991). It was also apparent that while p85 α associated PI 3-kinase activity appears to be ubiquitously expressed, p85β is much more restrictive. This was also observed in Chinese Hamster Overy (CHO) cells, where the predominant form of PI 3-kinase activity is found to immunoprecipitate with anti-p85α antibodies (Yonezawa et al., 1992b). Consistent with this observation p85 α was found to be ubiquitously expressed in tissues (Skolnik et al., 1991) and p85\(\beta\) was expressed primarily in the brain (Unpublished data from Fry et al., 1992). Indeed, a high level of p85\beta activity has been specifically immunoprecipitated from oligodendrocytes (I. Gout. unpublished data), moreover, this is the only cell line examined to date that contains a p85\beta associated PI 3-kinase activity but no p85 α associated PI 3-kinase activity. p85 α - and p85 β - associated PI 3-kinase activity has also been found in T-cells (Reif et al., 1992), and the two proteins were shown to be subjected to differential regulation during T-cell activation. Southern blotting analyses suggest the presence of at least four species of the p85 proteins (Otsu et al., 1991). Indeed, almost complete sequence data exists for a p85y clone (M. Otsu and I. Hiles, unpublished data). This suggests that PI 3-kinase may exist in a number of distinct isoforms in mammalian cells as do PLC and PKC. A more extensive examination is required to determine whether the different isoforms of PI 3-kinase are subject to differential tissue expression or function in different biochemical pathways. Indeed, the Ber domain, which shows the lowest sequence homology between the α and β isoform, may define a region where different proteins interact with each isoform to regulate or be regulated by the PI 3-kinase. In addition, p85β contains a much longer proline rich region than p85 α which may provide another element of variability.

3.3.2 Receptor and non-receptor interactions.

As PI 3-kinase activity was first discovered in association with a number of protein-tyrosine kinases (Kaplan et al., 1987; Courtneidge and Heber., 1987), the potential range of p85 proteins and receptor/non-receptor kinase interactions was an important issue. The EGF, ErbB-2 and CSF-1 receptors and the non-receptor kinases pp60^{c-src} and pp59^{c-Fyn} were found to be substrates for both the p85α and the p85β proteins when coexpressed in insect cells. Moreover, both p85α and p85β formed high affinity associations with these protein-tyrosine kinases with complex formation being inherent to the presence of phosphorylated receptor or non-receptor kinase. Similarly, the p85α protein has been observed to bind the phosphorylated HGF, PDGF and EGF receptors both in vivo and in vitro (Bardelli et al., 1992; Otsu et al., 1991; Escobedo et al., 1991a; Klippel et al., 1992; Hu et al., 1992). Further, a radiolabelled PDGF receptor or its kinase-insert region, was shown to bind directly the p85α subunit of PI 3-kinase from cell lysates of unstimulated cells that had been separated electrophoretically and transferred to nitrocellulose (Escobedo et al., 1991a; Ruiz-Larrea et al., 1993).

It should be noted that in vivo complex formation between the p85 proteins and protein-tyrosine kinases could be achieved in Sf9 cells in the absense of ligand-stimulation, only because both receptor and non-receptor kinases are constitutively active in Sf9 cells. This effect has also been observed with other receptors expressed in the baculovirus system (Morrison et al., 1989; Bardelli et al., 1992). The antiphosphotyrosine blot of stimulated and unstimulated Sf9 cells infected with recombinant virus expressing the CSF-1R clearly shows this phenomenum, where no activation of the CSF-1R was seen upon ligand stimulation 48 h post-infection. The reason for this remains unknown but presumably reflects oligomerisation induced by overexpression (Ullrich and Schlessinger, 1990). Thus, for phosphotyrosine-dependent complex formation, insect cells that had been coinfected with the p85 protein and a protein-tyrosine kinase were harvested 72 h after infection, and if unphosphorylated protein-tyrosine kinase was required, insect cells were harvested 36 h post-infection.

3.3.3 SH2 domains mediate the interaction of p85 with protein tyrosine kinases.

The phosphotyrosine dependency for p85 binding to receptors and non-receptor tyrosine kinases implicates the SH2 domains as mediators of this event. Analyses have revealed that SH2 domains bind with high afffinity to phosphotyrosine residues within a specific peptide sequence, thus, mediating the formation of signalling complexes in response to activation of receptor protein tyrosine kinases (Pawson and Gish, 1992; Pawson and Schlessinger, 1993). Receptor autophosphorylation may therefore act as a switch to elicit high affinity binding of SH2 domain-containing signalling proteins. The SH2 domains of p85 have been shown to associate tightly with tyrosine phosphorylated receptors, again

reinforcing the role of the p85 protein as an adapter protein which recruits the catalytic domain of the PI 3-kinase to activated protein-tyrosine kinases. The p85 protein contains two SH2 domains, both of which were shown to bind the EGF and the CSF-1 receptor, thus demonstrating no particular specificity of association as determined by this biochemical assay. Similar results have been obtained by Hu et al. (1992) and McGlade et al. (1992), where binding assays with GST-fusion proteins demonstrated that either SH2 domain of the p85α protein was sufficient for binding to the EGF, PDGFβ, CSF-1 or the c-kit receptor. Direct phosphorylation of the SH2 domain upon binding has been demonstrated in vitro and consistent with this Y368 in the N-terminal SH2 domain was recently mapped as an in vivo phosphorylation site for the p85α protein (Hayashi et al., 1993). Phosphorylation may also represent a mechanism for dissociation of the PI 3kinase by promoting the folding of the p85 α into a conformation with altered activity or affinity. Kavanaugh et al, (1992) have shown that tyrosine phosphorylated p85 α was unable to bind the activated PDGF receptor. In particular, phosphorylation of the Nterminal SH2 domain was shown to modulate the binding of p85α to the activated receptor.

3.3.4 Multienzyme complex formation.

A number of structurally diverse polypeptide growth factors act through high affinity protein-tyrosine kinase receptors inducing receptor autophosphorylation and phosphorylation of a variety of intracellular targets. The CSF-1 receptor has been shown to bind p85\alpha at Y721, (Reedijk et al., 1992), pp59c-Fyn on Y809 (Courtneidge et al., 1993; Mori et al., 1993), Grb2 at Y697 (Van der Greer and Hunter, 1993), RasGAP (Reedijk et al., 1990) and activate the Raf-1 serine/threonine kinase (Choudhury et al., 1990; Baccarini et al., 1990). In this study the CSF-1 receptor, pp59c-Fyn, p85 α and RasGAP were found to be precipitated using different antibodies, as part of a signalling complex. However, although these results show that multienzyme complex formation is possible in vivo with the CSF-1 receptor, they do not specify that an individual receptor molecule is binding the three second messenger molecules concurrently. Activated receptors are known to exist as dimers in cells (Schlessinger and Ullrich, 1990), and smaller complexes containing different combinations of signalling molecules may also exist. Whether a multienzyme complex as this exists in mammalian cells is presently unknown although several studies suggest that it may be possible. Using a similar approach Kaplan et al (1990) showed the PDGF receptor simultaneously complexes with Raf-1, PLCy, PI 3-kinase and RasGAP. Indeed, the PDGF receptor has been shown to have distinct substrate binding sites, where binding of any one signal transducing molecule does not affect the interaction of others (Fantl et al., 1992; Klippel et al., 1992). Similar multienzymic complexes have been observed with the activated neu protein associated with PI 3-kinase, PLCy, and the RasGAP protein (Peles et al., 1991; Fazioli et

al., 1991; Scott etal., 1991) and the NGF receptor has been found to complex both p85 α and PLC γ simultaneously in vitro (Obermeier et al., 1993). The receptor may be the core of the complex, and it is also possible that the specific activities of the individual transducing molecules may be altered when they are part of the complex. Since the molecules studied here have been implicated in mediating the reponses of cells to CSF-1, it is possible that the complex may play a important role in signal transduction.

3.3.5 Conclusion

The results presented here show that the two recombinant p85 proteins possess most of the observed properties of the endogenous cellular p85. They are good in vivo and in vitro substrates for all protein-tyrosine kinases thus far examined including members of both the receptor and non-receptor classes. They can also associate directly with these protein-tyrosine kinases when co-expressed and this association can be reconstituted in vitro in a manner dependent on tyrosine phosphorylation of the protein-tyrosine kinase. These observations further demonstrate the importance of SH2 domains in mediating these interactions and their role in coupling protein-tyrosine kinases to important intracellular second messenger systems such as the PI 3-kinase. Both p85 α and p85 β appear to bind undifferentially to every protein-tyrosine kinase examined. However, this may be an in vitro artefact and not a true reflection of the properties associated with the p85 protein when in complex with its catalytic subunit. The cloning and expression of the p110 subunit will subsequently allow analysis of this which is discussed in the following Chapter.

Chapter 4: Structure and Expression of the 110 kDa subunit

Introduction

PI 3-kinase affinity purified from the partially purified bovine brain preparation was shown to contain two distinct polypeptides (Morgan et al., 1990; Fry et al., 1992) and purification studies by other groups have also suggested similar findings (Carpenter et al., 1990; Escobedo et al., 1991; Shibasaki et al., 1991). Through the analysis of cDNA clones from bovine brain libraries, the cDNA for a bovine p110 was isolated (Hiles et al., 1992).

4.1.1 Aim

In order to study the biological functions inherent to the p110 protein and carry out biochemical analyses analogous to the p85 protein interactions with protein-tyrosine kinases, I subcloned the p110 cDNA into baculovirus transfer vectors and expressed the protein in Sf9 cells. In this study I have shown that the expressed protein is catalytically active when expressed alone in insect cells and could be reconstituted with p85 to form an active complex in vivo. The reconstituted complex had properties similar to those inherent to the PI 3-kinase partially purified from the bovine brain. Mutagenesis is used to define p110 as the catalytic subunit and I have used further biochemical analyses to show that the reconstituted p85/p110 interacts with protein tyrosine kinases.

4.2 Results

4.2.1 Expression of the p110 protein.

4.2.1.1 Construction of the p110 baculovirus transfer vector and expression in Sf9 cells.

To clone the p110-coding region into the baculovirus transfer vector p36C (Page, 1989) a Sau3A1 site (GGATCA) present 10 nucleotides upstream of the initiation codon was changed to a BamHI (GGATCC) site by PCR-mediated mutagenesis. In brief, a sense oligonucleotide substituting C for A at position 6 of the Sau3A1 site was used in a PCR reaction with an antisense primer comprising nucleotides (102-124) of the p110 sequence

employing Vent polymerase (New England Biolabs). Template DNA was random-primed and first strand cDNA prepared from SGBAF-1 cell mRNA (PCR conditions: 94°C for 1 min, and 50°C for 1 min, and 72°C for 2 min; 35 cycles). The PCR product was digested with BamHI-EcoN1 and a 118 bpr fragment isolated from a low melting point agarose gel. This BamH1-EcoN1 fragment was cloned into p110 in the Bluescript vector digested with BamH1 and EcoN1 (nucleotide 108) giving plasmid p110-(BamH1). The BamH1-EcoN1 fragment of p110-(BamH1) was sequenced and found to agree with that previously determined. A 3.4 kb BamH1-Kpn1 (Kpn1 present in the the baculovirus transfer vector) fragment was isolated from p110-(BamH1) and ligated into p36C baculovirus transfer vector previously digested with the same enzymes.

Wild type AcMNPV was coinfected with a recombinant baculovirus transfer vector encoding p110, p36C-p110, and recombinant virions harvested 2.5 days later. Recombinant viruses were identified visually and amplified to make a virus stock that was subsequently titred for optimal protein production.

4.2.1.2 p110 alone has catalytic activity when expressed in insect cells.

Analysis of amino acid sequence of the p85 proteins revealed no kinase motifs and the proteins lacked any intrinsic PI 3-kinase activity. However, inspection of the amino acid sequence of the p110 cDNA revealed the presense of sequence motifs previously found in many known protein-kinases (Hank et al., 1988). In this thesis I have used the baculovirus-expressed p110 to examine whether p110 alone possessed catalytic activity or if a p85-p110 complex was required for activation of PI 3-kinase activity.

Insect cells were infected with a recombinant baculovirus that expressed either p85 α or p110 or as a control wild type AcMNPV. Lysates of these cells were then immunoprecipitated with affinity purified polyclonal antibodies that recognised either the p85 α protein or the p110 protein. Immunocomplexes were subjected to analysis by SDS-PAGE using Coomassie Blue staining or assayed for associated PI 3-kinase activity. The results in Figure 4.1A show the immunoprecipitated p85 α protein alone (lane 3) and the p110 protein expressed alone migrating at molecular weights of 85 000 Da and 110 00 Da respectively (lane 4). The anti-p85 α and the anti-p110 affinity purified polyclonal antibodies used here failed to recognise any endogenous insect proteins of this molecular size from control infected cells (lanes 1 and 2). Through PI 3-kinase assay of these immunoprecipitations p85 α was shown not to possess any intrinsic PI 3-kinase activity (Figure 4.1B, lane 3), however, p110 was found to be active when expressed alone in insect cells (Figure 4.1B, lane 4). Antibodies that recognised either subunit of the PI 3-

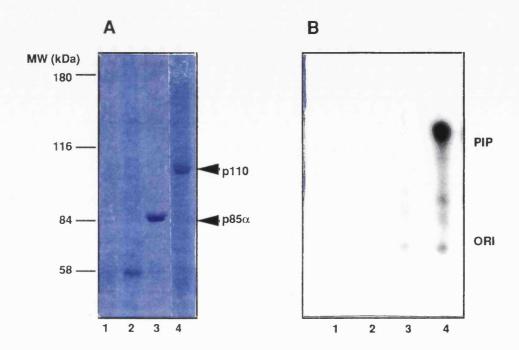


Figure 4.1. p110 is active when expressed in Sf9 cells.

(A). Sf9 cells were infected with wild-type virus (lanes 1 and 2), p85 alone (lane 3) or p110 alone (lane 4). Lysates of these cells were immunoprecipitated with anti-p85 antibodies (lanes 1 and 3) or with anti-p110 antibodies (lanes 2 and 4). Immunocomplexes were analysed by Coomassie Blue staining of SDS-PAGE gels.

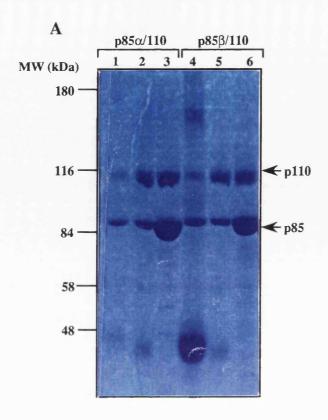
(B). Immune complexes treated as described in (A) were assayed for associated PI 3-kinase activity

kinase complex did not immunoprecipitate any endogenous insect PI 3-kinase activity from control infected cells (Figure 4.1B, lanes 1 and 2). These results clearly demonstrated that the p110 subunit of PI 3-kinase alone has catalytic activity.

4.2.2 Reconstitution of a heterodimeric PI 3-kinase complex.

4.2.2.1 110 can reconstitute with either p85 α or p85 β to form active PI 3-kinase complexes in vivo.

Since PI 3-kinase purified from bovine brain is a heterodimeric complex made up of p85α and p110 subunits the capacity for p85α and p110 expressed in insect cells to reconstitute into an enzymatically active complex was examined. Since p85 β is highly homologous to p85 α , the p85 β protein was also used in these analyses. Insect cells were coinfected with either p85 α and p110 or p85 β and p110 recombinant baculoviruses, lysates were affinity purified or immunoprecipitated with antibodies specific to either subunit and then PI 3-kinase activity was measured. In the double infections using baculoviruses expressing p85 α and p110, both subunit proteins were detected in either anti-p85\alpha or anti-p110 immunoprecipitates, or when the enzyme was bound to Y751 phosphopeptide affinity beads (Figure 4.2A, lanes 1, 2, 3). All affinity and immune precipitates were found to possess PI 3-kinase activity (Figure 4.2B, lanes 1, 2, 3). Similar results were seen with viruses expressing the β-form of p85 and p110. Infected cells immunoprecipitated with antibodies directed to the p85 β or the p110 subunit, or affinity purified by binding to Y751 phosphopeptide affinity beads, were found to contain both p85β and p110 subunit proteins when analysed by SDS-PAGE (Figure 4.2A, lanes 4, 5, 6). Examination of associated PI 3-kinase activity in the immunoprecipitates and affinity purified proteins showed the p85β/p110 complex to be enzymatically active (Figure 4.2B, lanes 4, 5, 6). The levels of Coomassie Blue staining protein precipitated in the p85\beta/p110 complex are essentially the same as those seen precipitated in the p85α/p110 complex using identical amounts of both p110 antibody or Y751 phosphopeptide affinity beads. Since both p85 α and p85 β are expressed to approximately the same levels in insect cells (Chapter 2, section 3.2.1.2; Gout et al., 1992) this would suggest that formation of the p85 β /p110 complex in Sf9 cells is as efficient as reconstitution of a p85α/p110 complex. Indeed, as comparable amounts of PI 3-kinase activity were precipitated with either the p85 α /p110 complex or the p85 β /p110 complex this suggests that p110 is able to form a stable, active PI 3-kinase complex with either of the p85 isoforms in insect cells.



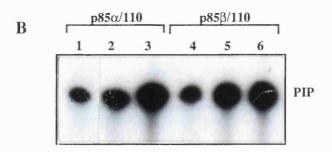


Figure 4.2: Both p85 α and p85 β are able to form stable active complexes when coinfected with p110 in insect cells in vivo

(A) Sf9 cells were coinfected with p85 α and p110 viruses (lanes 1-3), or with p85 β and p110 viruses (lanes 4-6). Lysates of these cells were precipitated using polyclonal affinity purified antibodies raised against p85 α (lane 1); monoclonal antibodies raised against p85 β (lane 4); polyclonal affinity purified antibodies raised against p110 (lanes 2 and 5) or bound to immobilised Y751 phosphopeptide affinity beads (lanes 3 and 6). Samples were analysed by Coomasssie Blue staining of SDS-PAGE gels.

(B) PI 3-kinase assays were performed on immunoprecipitates from Sf9 cells that had been infected and treated as described in (A).

4.2.2.2 p85/p110 complex formation can take place in vitro.

To ascertain whether the two subunits of the PI 3-kinase complex were able to associate post-translationally in vitro, one subunit of the PI 3-kinase complex was first immobilised onto an appropriate matrix, and then allowed to bind the other subunit in solution. First, the p85 proteins immunoprecipitated from infected insect cells using isoform-specific monoclonal antibodies were collected on Protein A-Sepharose beads. These immunocomplexes were then incubated with lysates of insect cells that had been infected with p110 viruses, after which they were washed and subjected to analysis by either SDS-PAGE or by PI 3-kinase assays. Alternatively, p110 was immunoprecipitated from infected insect cells, incubated with p85 α expressing lysates, and then treated as described above. Both p85 α and p85 β were seen to bind p110 as revealed by Coomassie Blue staining following SDS-PAGE (Figure 4.3A lanes 1 and 2) or through the assay for associated PI 3-kinase activity (Figure 4.3B lanes 1 and 2). Similar results were obtained when p110 was immobilised and used as an affinity matrix; the bound p85 α protein was detected as a Coomassie Blue stained protein on SDS-PAGE following binding in vitro (Figure 4.3C, lane 2).

4.2.2.3 Time course of expression of the recombinant PI 3-kinase complex in insect cells.

The levels of p85α/p110 complex expression was monitored at various days following infection. Lysates of insect cells that had been coinfected with p85α and p110 recombinant baculoviruses were immunoprecipitated using a polyclonal antibody that recognised the p110 protein. Figure 4.4A shows a Coomassie blue stain of an SDS-PAGE gel which indicates that within 2 days, high levels of the PI 3-kinase complex begins to accumulate inside the insect cells. Maximal expression was observed between day two and three post-infection, after which the protein could no longer be detected by Coomassie blue staining. The level of PI 3-kinase activity was similtaneously analysed in these cells. The results in Figure 4.4B show that equivalent amounts of PI 3-kinase activity were immunoprecipitated on days 2 and 3 following infection and that no activity was detectable on day 4 once the virus had passed into the lytic phase of the infection cycle.

4.2.2.4 Comparison of the purified bovine brain PI 3-kinase with the recombinant PI 3-kinase.

The recombinant PI 3-kinase complex was next compared with material from peak 1 (Fry et al., 1992; Ruiz-Larrea., 1993) of the partially purified bovine brain PI 3-kinase preparation using SDS-PAGE analysis. Lysates of insect cells infected with recombinant

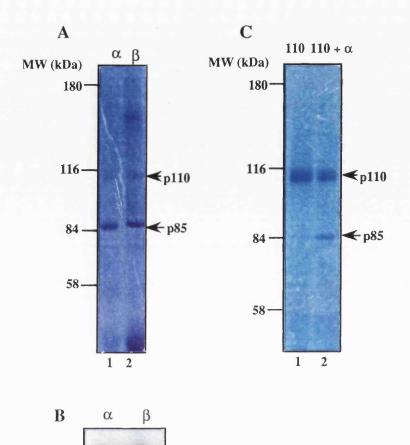


Figure 4.3: Both p85 α and p85 β are able to form stable active complexes with p110 in vitro.

PIP

(A) p85 α and p85 β were immunoprecipitated from insect cells and then incubated with lysates of Sf9 cells that had been infected with p110 viruses (lanes 1 and 2). Complexed proteins were resolved on SDS-PAGE and identified by staining with Coomassie Blue.

(B PI 3-kinase assays were performed on immunoprecipitates from Sf9 cells that had been infected and treated as described in (A)

(C) Lysates of insect cells that had been infected with p110 viruses were immunoprecipitated and then either left untreated (lane 1) or incubated with p85 α containing Sf9 cells (lane 2). Samples were washed in lysis buffer, resolved on SDS-PAGE and identified by Coomassie Blue staining.

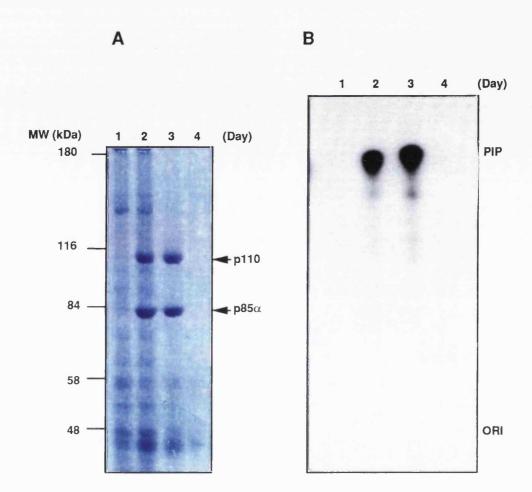


Figure 4.4. Time course of expression of the recombinant PI 3-kinase complex in insect cells.

(A). Sf9 cells were coinfected with p85 α and p110 expressing baculovirus and cells harvested at the times indicated at the top of each panel (lanes 1-4). Lysates prepared from these cells were immunoprecipitated with anti-p110 antibodies and then visualised by Coomassie Blue staining of SDS-PAGE gels.

(B). Sf9 cells that had been infected and treated as described in (A) were assayed for associated PI 3-kinase activity.

baculoviruses expressing p85α and p110 and a sample of the partially purified bovine brain PI 3-kinase were immunoprecipitated with antibodies directed to the p110 component of PI 3-kinase. Immune complexes were washed several times with lysis buffer and then analysed by Coomassie blue staining of SDS-PAGE gels. As can be seen in Figure 4.5 the two subunits of this bovine purified and the recombinant PI 3-kinase complex comigrated on SDS-PAGE (lanes 1 and 2 respectively).

4.2.2.5 HPLC analysis of the lipid products of p110 alone and p85 α /p110 complex.

HPLC analysis was employed to ensure that the lipid products generated by the isolated p110 and p110 in complex with p85α were phosphorylated in the 3-position. PI 3-kinase assays, utilising PI as the substrate, were performed on p110 and p110/p85α immunoprecipitated from lysates of appropriately infected Sf9 cells. The phosphorylated lipids were detected by autoradiography after TLC and then extracted from the silica TLC plate. The lipid products were deacylated essentially as described in Clarke and Dawson (1981) and then analysed using HPLC essentially as described by Auger et al. (1989). Tritiated standards of GroPI(4)P were run simultaneously with the ³²P-labelled deacylated product which was synthesised by phosphorylation of PI by PI 3-kinase. The results in Figure 4.6 show that for both p110 alone and p85α/p110 complex, a prominent peak of ³²P-labelled activity migrated with an identical retention time to a 32PGroPI(3)P standard prepared using purified bovine brain PI 3-kinase. PI(4)P was not detected in immunoprecipitates of either p110 alone or p85α/p110 from lysates of infected insect cells.

4.2.2.6 The binding of the p85 α subunit does not modify the enzymatic activity of the p110 protein in vitro.

Shibasaki et al (1991) have described the purification of two forms of PI 3-kinase from the bovine brain thymus. In addition to a heterodimeric p85/p110 complex, they report a catalytically active monomeric p110 species of PI 3-kinase. The free p110 protein alone was shown to have a somewhat higher specific activity than the hetreodimeric p85/p110 complex. Utilising individual recombinant components of the bovine PI 3-kinase I was able to investigate whether the recombinant p110 protein had a different activity to that of p110 in complex with the p85 subunit. In the absence of a purified p110 protein, the catalytic subunit was isolated by immunoprecipitation with antibodies directed to the C-terminus of this protein. Two approaches were utilised to compare the specific activity of the p110 with that of the p85α/p110 complex. First, the activity of the free p110 protein

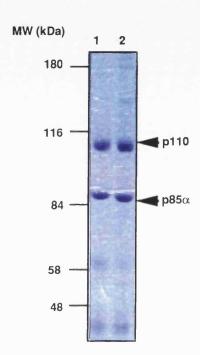


Figure 4.5 Comparison of the purified bovine brain PI 3-kinase with the recombinant PI 3-kinase on SDS-PAGE. Partially purified bovine brain PI 3-kinase diluted in lysis buffer (lane 1) and lysates of Sf9 cells infected with p85 α and p110 expressing baculovirus (lane 2) were immunoprecipitated with anti-p110 antibody. Immune complexes were analysed by Coomassie Blue staining of SDS-PAGE gels.

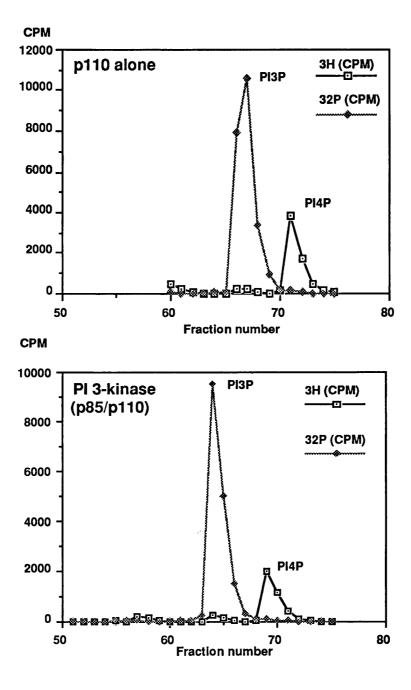


Figure 4.6. HPLC analysis of the lipid products of recombinant PI 3-kinase.

Immunoprecipitated p110 and p85/p110 complex were used to phosphorylate PI and the phosphorylated lipids were separated by TLC. 32P-labelled phospholipids were extracted from the TLC plates, deacylated and analysed by anion exchange HPLC. [3H]PI(4)P was used as an internal standard

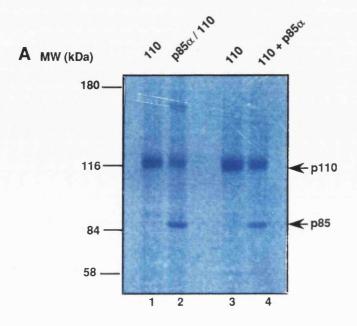
alone was compared to that of a heteromeric complex consisting of p85 and p110 formed in vitro by immunoprecipitating p110 from lysates of infected cells and then incubating it with lysates of insect cells that had been infected with p85 α or with lysis buffer. Second, PI 3-kinase activity associated with p110 was compared to that in a complex formed in vivo by immunoprecipitation using antibodies that recognised the catalytic subunit from lysates infected with p110 alone or those coinfected with p85 α and p110 recombinant viruses. The immune complexes were either assayed for associated PI 3-kinase activity, or fractionated by SDS-PAGE and then the proteins visualised by Coomassie Blue staining. The results in Figure 4.7A showed essentially the same amounts (~1 μ g) of p110 to be immunoprecipitated from Sf9 cells infected with p110 alone or if coinfected with p85 (lanes 1-4). Analysis of the specific activity of the p110 alone and of p110 coupled to p85 α in vitro, revealed no significant differences in PI 3-kinase activity of the free p110 with the heteromeric p85 α /p110 (Figure 4.7B, lanes 3 and 4). Essentially the same results were observed between p110 and p85 α associated with p110 in vivo (Figure 4.7B, lanes 1 and 2).

4.2.3 The C-terminal region of p110 contains the catalytic domain of the PI 3-kinase.

The D916RHNSN and the D933FG sequence motifs are known to be essential for binding the nucleotide phosphate moieties and for phosphotransferase activity in classical protein kinases (Taylor et al., 1992). These sequence motifs are present in the C-terminal region of the bovine p110 and equivalent residues are present in p110β, VPS34P, Tor2/DRR2, and the yeast PI 4-kinase (Hiles et al., 1992; Hu et al., 1993; Kunz et al., 1993; Cafferkey et al., 1993; Flanagan et al., 1993). To determine whether the same kinase motifs were also important for the enzymatic transfer of phosphate from ATP to 3-position on PI these sequences were mutated by Ian Hiles and I then subcloned the mutated regions into baculovirus transfer vectors and generated recombinant viruses.

4.2.3.1 Construction of mutant-p110 baculovirus transfer vectors.

Arginine 916 of p110 was changed to a proline residue by oligonucleotide mediated site-directed mutagenesis. Briefly, the oligonucleotide 5'-TGGGAATTGGGGATCCTCACAATAGTA-3' was synthesised (Genosys Biotechnologies Inc., Cambridge UK) and used to incorporate the R916P mutation into p110-Bam HI (Hiles et al., 1992) using the Stratagene "Double Take" mutagenesis kit. In addition to the R916P mutation, this oligonucleotide was designed to similtaneously introduce a novel BamHI site by means of silent codon changes. The sequence of a 802 bp PstI-HindIII cartridge containing the R916P mutation was verified by DNA sequence



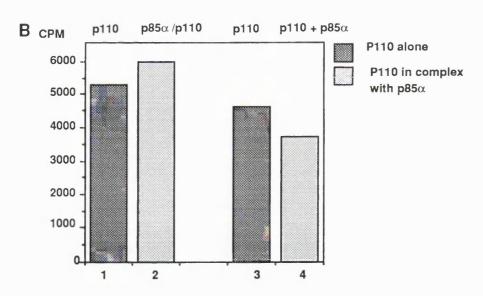


Figure 4.7. The p85 subunit does not modify the enzymatic activity of the p110 protein.

(A) Coomassie Blue stain of a 7.5% SDS-PAGE of insect cell lysates immunoprecipitated with p110 antibody which had been infected and treated as follows: p110 virus alone (lanes 1 and 3); coinfected with p85 α /p110 viruses (lane 2), p110 immunoprecipitate incubated with p85 α containing Sf9 cell lysate in vitro (lane 4).

(B) PI 3-kinase assays were performed on immunoprecipitates from Sf9 cells infected and treated as described in panel (A).

analysis. For expression in Sf9 cells, a 903 bp PstI-KpnI cartridge from the baculovirus transfer vector, p36C-P110 (See section 4.2.1.1) was replaced with the corresponding cartridge from the p110-Bam HI plasmid containing the R916P mutation. The presence of the R916P mutation was confirmed by restriction mapping with Bam HI. For mutation of the DFG sequence, phenylalanine 934 was converted to a serine residue by oligonucleotide mediated site-directed mutagenesis. Briefly, the oligonucleotide 5' CTGTTTCATATCGATTCTGGACACTT3' (altered bases underlined) was synthesised as described above and used to incorporate the F934S mutation into p110-BamHI (Hiles et al., 1992). In addition to the F394S mutation this oligonucleotide also introduces a novel Cla1 site by means of silent codon changes. The sequence of a 802 bp Pst-Hind111 cartridge containing the F394S mutation was verified by DNA sequence analysis. For expression in Sf9 cells, a 903 bp PstI-KpnI cartridge from the baculovirus transfer vector, p36C-P110 (See section 4.2.1.1) was replaced with the corresponding cartridge from the p110-Bam HI plasmid containing the F394S mutation.

4.3.2.1 Expression of p110-R916P and p110-F933S mutants in Sf9 cells.

Baculovirus transfer vectors p110-R916P and p110-F933S were then transfected into Sf9 cells using linearised AcNVP DNA (BaculoGold, Pharmingen). Recombinant plaques were isolated visually by plaque assay and used to generate stocks of virus which were then titred for optimal protein production.

The p110 subunit has been shown by itself to express PI 3-kinase activity (Section 4.2.1.2). To definitively establish that the C-terminus of p110 protein contains the catalytic domain the effects of the p110-R916P and p110-F933S mutations on the intrinsic phosphoinositide 3-kinase activity were assessed. Insect cells were infected with p85 α and p110 viruses either separately or together. Immunoprecipitated p85 α and p110 could then be visualised as Coomassie Blue stained proteins following resolution on SDS-PAGE gels (Figure 4.8A, lanes 1 and 2). The p110 protein was found to form a stable complex with p85\alpha if the two proteins were allowed to associate in vitro or alternatively, if they were coexpressed they associated 'in vivo' in insect cells, this interaction takes place as can be seen in the SDS-PAGE analysis (Figure 4.8A, lanes 3 and 4). PI 3-kinase assays of these samples showed that the wild type p110 protein alone was active (Figure 4.8B, lane 2) and in addition, an active complex was formed when p110 was bound to p85 α either in vitro or in vivo (Figure 4.8B, lanes 3 and 4). Immunoprecipitated p110-F933S and p110-R916P was also visualised by Coomassie Blue staining of SDS-PAGE gels (Figure 4.8A, lanes 6 and 10 respectively) and was seen to comigrate with the wild type p110 protein (Figure 4.8A, lane 2). A stable complex

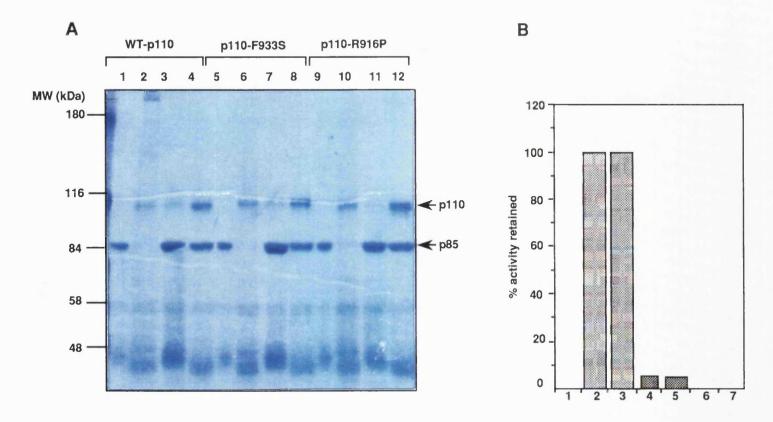


Figure 4.8. Mutagenesis defines p110 as the catalytic subunit of the PI 3-kinase

(A) Coomassie Blue stain of a 7.5% SDS-PAGE of lysates of infected insect cells immunoprecipitated with the described antibody and treated as follows: anti-p85α (lane 1); anti-p110 (lane 2); anti-p85α immunoprecipitate, incubated with p110 containing Sf9 cell lysate in vitro (lane 3); anti-p110 immunoprecipitate of insect cells coinfected with p85α/p110 viruses (lane 4); anti-p85α (lane 5); anti-110 immunoprecipitate of insect cells infected with p110-F933S (lane 6); anti-p85α immunoprecipitate incubated with mutant p110-F933S viruses (lane 8); anti-p85α (lane 9); anti-110 immunoprecipitate of insect cells infected with p110-R916P (lane 10); anti-p85α immunoprecipitate incubated with mutant p110-R916P containing Sf9 cells in vitro (lane 11); Anti-p110 immunoprecipitate of insect cells that had been coinfected with p85α/mutant p110-R916P viruses (lane 12).

(B) PI 3-kinase assays were performed on anti-p110 immunoprecipitates of Sf9 cells infected with baculoviruses expressing: WT p110 (lane 2); p85 and WT p110 (lane 3); mutant p110-F933S (lane 4); mutant p110-F933S and p85 (lane 5); mutant p110-R916P (lane 6); mutant p110-R916P and p85 (lane 7); lane 1 shows a control of an anti-85 immunoprecipitate of p85 virus infected cells.

was also recovered when insect cells coinfected with either p110-F933S or p110-R916P and p85α were analysed by immunoprecipitation using anti-p110 antibodies (Figure 4.8A, lanes 8 and 12 respectively), or when immunopurified p110-F933S or p110-R916P were allowed to associate with p85α in vitro (Figure 4.8A, lanes 7 and 11 respectively). These immunoprecipitates, which contained the mutant p110-R916P, were all found to lack PI 3-kinase activity (Figure 4.8B, lanes 4 and 5) while those immunoprecipitates which contained the mutant p110-F933S were found to retain 3% PI 3-kinase activity (Figure 4.8B, lanes 6 and 7). As the expression levels and p85 binding capabilities of the mutants were commensurate with those of wild type p110, this data suggests that these mutations have disrupted the catalytic site of the protein without completely disrupting the structure of the p110 protein.

4.3 Discussion

The cDNA cloning of the p110 subunit has allowed a more definitive characterisation of the PI 3-kinase complex and was a fundamental step towards increasing understanding of the regulation and function of this enzyme in mammalian cells. In the present study the p110 protein was expressed in insect cells in an effort to carry out biochemical analyses analogous to those which were conducted with the p85 proteins to yield a better understanding of the biological functions of the catalytic subunit.

4.3.1 p110 alone is catalytically active

The p110 subunit expressed in insect cells was shown to be active alone, and does not require the presence of the p85 subunit for activation. However, the bovine p110 was inactive when it was expressed alone in COS-1 cells and the activity of a p110 isoform, p110β was significantly reduced when expressed alone in 293 kidney fibroblasts (Hu et al., 1993). The reason for the observed negative regulation of p110 activity seen when it was expressed alone in mammalian cells is unclear. Indeed, it is not clear if pools of free p110 exist in a physiological situation. Purification schemes based on the assay of PI 3-kinase activity (Carpenter et al., 1990; Morgan et al., 1990; Otsu et al., 1991; Escobedo et al., 1991b, Shibasaki et al., 1991), have in the main resulted in the isolation of PI 3-kinases as heterodimeric complexes of p85 and p110 subunits. Antibodies that recognised free p85α but do not recognise the PI 3-kinase complex, (presumably as they recognise a region of p85α involved in binding to p110), do not immunoprecipitate free p85α from Chinese Hamster Overy (CHO) cells (Yonezawa et al., 1992). This indicates that in CHO cells, p85 exists predominantly as a heterodimer with p110. However, p85

transiently overexpressed in cells is able to bind PI 3-kinase activity (Hu et al., 1992; Klippel et al., 1992; Dhand et al., 1994). This allows one to speculate that the heterodimeric PI 3-kinase exists in a dynamic equilibrium between tightly complexed and uncomplexed forms, where overexpression of either subunit may perturb this equilibrium, allowing the exchange of subunits.

A catalytically active monomeric PI 3-kinase in the form of p110 alone has been purified from bovine thymus (Shibasaki et al., 1991), in addition to an active p85/p110 heterodimeric PI 3-kinase complex. Both forms of PI 3-kinase were found to exhibit similar biochemical properties when assayed for pH optima, cation requirement and quercetin inhibition (Shibasaki et al., 1991; 1992). However, the free p110 protein was shown to have a five-fold higher specific activity than the p85/p110 complex. In this study, recombinant free p110 and the heteromeric p85/p110 complex were found to have the same specific activity. It is unclear whether the bovine thymus p110 (Shibasaki et al., 1991) had been activated during purification eg, by protein phosphatase action, or whether the heterodimeric complex is modified and thus inactivated (Discussed in Chapter 7). The origin of these results will await more detailed studies of the recombinant bovine thymus PI 3-kinase.

4.3.2 A family of PI 3-kinases?

The p110 subunit is able to form an active complex with the p85 α protein which migrates in a similar manner to the bovine brain purified PI 3-kinase complex on SDS-PAGE. Stable complex formation has been shown here to take place both in vivo and in vitro, suggesting that the two proteins do not need to be cotranslated in order to associate. The only fully characterised PI 3-kinase has been purified from the bovine brain, sequenced at the protein level, cloned at the DNA level and is found to contain the α form of p85 (Otsu et al., 1991; Hiles et al., 1992). However, it is clear that the p85 proteins are encoded by a gene family of at least three members α , β and γ (Otsu et al., 1991; Volinia et al., 1992). Analysis of the amino acid sequence of p110 revealed significant sequence homology with the yeast PI 3-kinase VPS34P (Herman and Emr. 1990, Hiles et al., 1992; Schu et al., 1993), which was isolated from a screen for yeast mutants deficient in the targeting of proteins to the yeast vacuole. Indeed, there is evidence for a VPS34P homologue in humans and Drosophilia (Unpublished data L. MacDougall; S. Volinia). The bovine and human isoforms of p110 also share significant homology with another putative yeast PI(3)-kinase termed TOR2/DRR2 (Kunz et al., 1993; Cafferkey et al., 1993; Helliwell et al., 1994), yet to be ascribed with a catalytic activity. This protein is sensitive to the immunosuppressant rampamycin and is suggested to be a component of a signal

transduction pathway that acts on Gi cyclin and is required for cell cycle activation. In situ hybridisation and PCR analysis confirm the presence of three related sequences of p110 cDNA in Drosophilia (unpublished data, L. MacDougall) and five related sequences of human p110 cDNA (S. Violinia et al). Indeed, one of these human sequences was found to encode a human p110β homologue with 62% sequence homology (Hu et al., 1993). This suggests the evolutionary conservation of a family of PI 3-kinases. It is thus not surprising that p85β is also found to form a stable and active complex with p110, both in vivo and in vitro. Indeed, PI 3-kinase activity specifically precipitated with the p85 β subunit has been found in several cell lines (Gout et al., 1992; Yonezawa et al., 1992b; Reif et al., 1993). However, whether these PI 3-kinase activities are the same as that of the well characterised bovine p110 (Hiles et al., 1992; Hu et al., 1993) remains to be determined. These data suggest the existence of a family of lipid kinases, which are perhaps subject to differential regulation and expression. Indeed, the yeast VPS34P and the bovine p110 have been expressed in S. pombe where they show distinct substrate specificities (Kodaki et al., 1994). The bovine p110 utilised PI, PI(4)P and PI(4,5)P2 as substrates, however, the VPS34 protein preferentially utilised only PI as a substrate.

4.3.3 Definition of a lipid kinase domain

The affinity purified bovine brain PI 3-kinase has been shown to label with 5'-pflourosulphonylbenzoyladenosine (FSBA) suggesting that p110 contains an ATP binding site and conferring catalytic activity to the complex (Ruiz-Larrea et al., 1993). More direct evidence that the p110 is the catalytic subunit of the PI 3-kinase comes from the observation that baculovirus expressed p110 alone has catalytic activity and HPLC analysis confirms that this activity phosphorylated PI at the 3 position to produce PI3P. Mutagenesis was used to unequivocally establish that the catalytic domain of PI 3-kinase resides in the p110 subunit. Amino acid sequence analysis of p110 has revealed significant homology (57%) of its C-terminal region to the C-terminal of the yeast PI 3kinase, VPS34P (Hiles et al., 1992; Herman and Emr, 1990; Schu et al., 1993). This region is found to contain amino acids conserved in the active sites of kinases in particular, G842, K863, D916, N921 and the DFG triplet at residues 933-935 that might be homologous to G₅₂, K₇₂, D₁₆₆, N₁₇₁ and the DFG triplet at residues 184-186 in cAMP-dependent protein kinase (Knighton et al., 1991a, 1991b). Equivalent residues are present in p110β, VPS34P Tor2/DRR2, and the yeast PI 4-kinase (Hiles et al., 1992; Hu et al., 1993; Kunz et al., 1993; Cafferkey et al., 1993; Flanagan et al., 1993). The mutations D916P and D933S in the p110 catalytic domain render the p110 protein inactive. Lack of the glycine-rich P-loop in the bovine p110, p110\beta, VPS34P, Tor2/DRR2 and PI 4-kinase, which is resident in most other kinases (Hanks et al., 1988) suggests that although similarities between lipid kinases and protein kinases do exist, lipid kinase catalytic domains probably represent a distantly related domain with its own specific hallmarks.

4.3.3 Conclusion

This study has shown that the p110 protein is active alone and mutagenesis is used to unequivocally establish that the catalytic domain of PI 3-kinase resides in this subunit. The p110 protein is shown to interact in a non-covalent manner with the p85 subunit and this complex reconstitutes efficiently both in vitro and in vivo in insect cells. In addition both forms of PI 3-kinase were found to have the same specific activity. Knowledge of the sites of interaction between the p85 and p110 subunits may shed more light on the mechanism of activation of PI 3-kinase and this is examined in the following Chapter.

Chapter 5: Structural analysis of intersubunit interaction.

5.1 Introduction

The modular structure of p85 has facilitated both structural and functional studies on this protein and has led to rapid advances in our understanding of its role in the PI 3-kinase complex. At the N-terminus lies an SH3 domain, the function of which is at unpresent unclear although it has been suggested that SH3 domains might regulate the function of G-proteins (Discussed in Chapter 1). Indeed, the SH3 domain of p85 was recently shown to stimulate the intrinsic activity of the GTPase dynamin (Gout et al., 1993). A domain with sequence similarity to the product of the breakpoint cluster region gene Bcr has also been identified adjacent and C-terminal to the SH3 domain (Otsu et al., 1991). This region of the Bcr protein also shows homology to regions in RhoGAP (Diekmann et al., 1991), n-chimaerin (Hall et al., 1993) and p190 (Settleman et al., 1992). Further, the Bcr domains of these proteins have been shown to stimulate the GTPase activity of Rac and Rho. It is not clear whether this domain of p85 possesses any GAP activity but PI 3kinase has been found to function in a p21rho-dependent pathway in platelets (Zhang et al., 1993) and CHO cells (Kumagai et al., 1993). Two proline rich stretches flank the Bcr domain. These regions have been shown to be involved in the binding of p85 to the SH3 domains of pp60c-Src and pp59c-Fyn (Plieman et al., 1993; Liu et al, 1993; Prasad et al., 1993; Kapeller et al., 1994), concomitant with activation of the PI 3-kinase (Pleiman et al., 1994). Indeed, it is postulated that the SH3 domain of p85 might be bound in an intramolecular manner to this proline rich region (Gout et al., 1993). The C-terminus of p85 contains two SH2 domains, both of which have been shown to bind with high affinity to a phosphotyrosine residue contained within a YXXM sequence motif (Cantley et al., 1991; Fantl et al., 1992). The region between the SH2 domains shows no homology with domains having a recognised binding function.

By comparison, very little is known as yet about the structure of the p110 protein. Amino acid sequence comparison with other PI 3-kinases and mutagenesis studies have defined a catalytic domain within the C-terminal region of p110 (Hiles et al., 1992; Kunz et al., 1993; Cafferkey et al., 1993; Flannagan et al., 1993; Chapter 4). The N-terminal half of p110 contains a calcium and phospholipid binding (CaLB) domain (Stephens et al., 1993b; Clarke et al., 1991). This region has homology to a sequence found in a number of phospholipid binding/membrane-associating proteins including yeast TOR2/DRR2, human VPS34P, p110 β , PKC α , β , γ , PLC $\beta\gamma$, δ , rasGAP and NF1 (Reviewed in Stephens et

al., 1993b). The CaLB domain is implicated in calcium-dependent binding of proteins to membranes (Clarke et al., 1991) which may have important implications for the mechanism of catalysis and activation of PI 3-kinase. The N-terminus of P110 also contains a second region of homology, termed the PIK domain which it shares with the yeast PI 4-kinase, VPS34, p110β, and Tor2/DRR2 (Flannagan et al., 1993).

PI 3-kinase may be regulated by a series of events, such as translocation of PI 3-kinase to cell membranes with consequent access to substrate, and the subsequent association with, and phosphorylation by, protein-tyrosine kinases (reviewed in Panayotou and Waterfield. 1992). The binding of PI 3-kinase through its SH2 domains to phosphotyrosine-containing sequences on protein tyrosine kinases can activate the enzyme. Tyrosine phosphorylated peptides corresponding to potential PI 3-kinase sites on the PDGF receptor have also been shown to activate PI 3-kinase in vitro (Carpenter et al., 1993). Further, it has been shown that upon binding the Y751-phosphopeptide, the p85 subunit of the PI 3-kinase and its N-terminal SH2 domain in particular, undergo a conformational change (Panayotou et al., 1992; Shoelson et al., 1993). This induced change in conformation of the p85 protein may be transmitted to the associated p110 subunit and thus contribute to its enzymatic activation.

5.1.1 Aim

An understanding of the nature of the interaction between the regulatory p85 subunit and its catalytic domain would shed more light on the mechanism of activation of PI 3-kinase. Although there is a wealth of biochemical and structural data on the association of PI 3-kinase with other proteins, little is known of the interaction between the p85 and p110 subunits. In this study both the p85 α and the p85 β proteins have been used in complementary studies to define the regions responsible for stable subunit association with the p110 protein both in vivo and in vitro. The possible significance of the intersubunit interaction with respect to activation of the enzyme is discussed.

5.2 Results

5.2.1 The inter-SH2 region of p85 α is required for binding to p110

The specific region of p85 α responsible for binding to p110 was defined by analysis of the individual domains of p85 which may be involved in the interaction. GST fusion proteins of the various subdomains of p85 α , which were constructed by I. Gout in the lab, were immobilised on Glutathione-Sepharose beads (Figure 5.1A) and used for affinity analysis with cell lysates prepared from Sf9 cells infected with a recombinant baculovirus expressing p110, after which the resultant complexes were washed and subjected to PI 3-

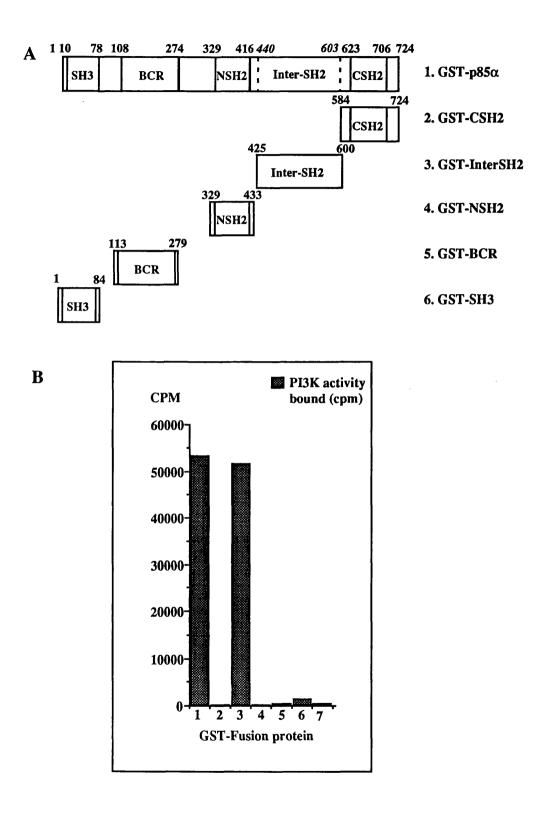


Figure 5.1. The inter-SH2 region of p85 α is required for binding p110. (A) Full-length GST-p85 α (1) and GST-domains of p85 α (2-6).

⁽B) Domains 2-6 of p85 α as described in (A), and GST alone (lane 7) were bound to Glutathione-Sepharose beads and then incubated with lysates of insect cells that had been infected with p110 virus. After washing with lysis buffer, proteins were subjected to PI 3-kinase assays.

kinase assay. GST alone, and GSTp85 α bound to Glutathione-Sepharose beads incubated with wild-type lysate were used as negative controls. Figure 5.2B shows that PI 3-kinase activity only associated with either the full-length GSTp85 α protein (lane 1) or with the inter-SH2 region of the p85 α (lane 3). PI 3-kinase activity did not bind to GST alone (lane 7), and no endogenous insect cell PI 3-kinase activity bound to the full-length GSTp85 α protein when incubated with control lysates (data not shown).

To study the interaction between the inter-SH2 region of p85 α with p110 in the context of the entire p85 protein monoclonal antibodies whose epitopes lie within the inter-SH2 region were used to block intersubunit associations. p85 α from insect cells was immunoprecipitated, either using two monoclonal antibodies which bound epitopes located in the inter-SH2 region of p85 α , or with two control antibodies that recognise the Bcr domain (End et al., 1993). These immunocomplexes were washed stringently and incubated with lysates of insect cells that had been infected with p110 virus. Bound proteins were then subjected to analysis by SDS-PAGE and PI 3-kinase assays. Similar quantities of p85 α were found to be immunoprecipitated by all four antibodies as determined by Coomassie Blue staining (Figure 5.2A, lanes 1-4). However, the amount of PI 3-kinase activity that bound to p85 α immunoprecipitated with the two antibodies that recognize the inter-SH2 region was significantly less (Figure 5.2B, lanes 3 and 4), than the activity which bound to p85 α immunoprecipitated with antibodies to the Bcr domain (Figure 5.2B, lanes 1 and 2).

5.2.2 Refined mapping of the site in the inter-SH2 region, required for the interaction with p110

To define which component of the inter-SH2 region is involved in directly binding the p110 subunit, deletions were introduced by I. Gout from both ends of the inter-SH2 region of p85α, reducing the size of this region from 175 amino acids (425-600) down to 104 residues (451-555) (Figure 5.3A). The truncated inter-SH2 regions expressed as GST fusion proteins were bound to Glutathione-Sepharose beads which were then incubated with lysates of insect cells that had been infected with p110 and associated PI 3-kinase activity measured. The results in Figure 5.3B (lanes 2-6) show that the deletion of amino acids from either end of the inter-SH2 region gradually reduced the amount of bound PI 3-kinase activity. This may be explained if disrupting the inter-SH2 structure is destabilising a putative domain thus preventing protein-protein interactions. In addition, these results identified a structural element of 104 amino acids, between residues 451-555 of the inter-SH2 domain of p85α, that was able to directly bind the p110 protein and associated PI 3-kinase activity (Figure 5.3B, lane 6).

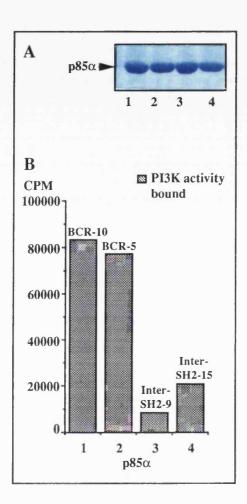
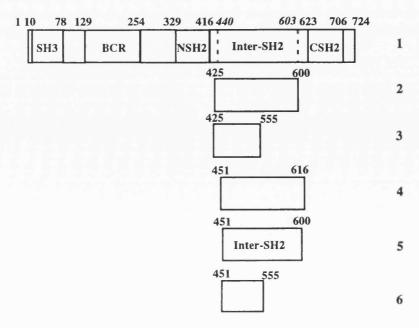


Figure 5.2: Antibodies with epitopes in the inter-SH2 region of $p85\alpha$ inhibit the binding of the p110 protein.

(A) p85 α was immunoprecipitated from infected insect cells with monoclonal antibodies which have epitopes in either the Bcr domain (lanes 1 and 2); or with epitopes in the inter-SH2 region (lanes 3 and 4) and incubated with p110 containing lysates. Immunoprecipitated p85 proteins were visualised by Coomassie Blue stain of SDS-PAGE gels.

(B) PI 3-kinase assays were performed on immunoprecipitates infected and treated as described in (A)

A p85α





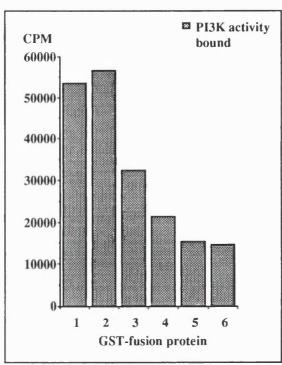


Figure 5. 3 Identification of the region of the inter-SH2 region that directly binds P110.

(A) Full-length GST-p85 α (1); and varying sizes of the inter-SH2 region (2-6).

(B) The different sized GST-inter-SH2 regions were bound to Glutathione-Sepharose beads and incubated with p110 containing Sf9 cell lysates (lanes 1-6). Samples were washed and assayed for associated PI 3-kinase activity.

A series of nested deletions of GSTp85 β fusion proteins from the 3' end of the N-terminal SH2 domain to the 5' end of the C-terminal SH2 domain were then constructed in collaboration with K. Hara to refine the binding site within the inter-SH2 region (Figure 5.4A). The interaction of these GST fusion proteins with p110 was then measured as described above. Full-length GSTp85 β (1-724) and GSTp85 β (446-724), which contains an almost complete inter-SH2 region, were clearly able to bind PI 3-kinase activity (Figure 5.4B, lanes 1 and 2). However, mutants with deletions that advanced further into the inter-SH2 region such as those in GSTp85 β (486-724) and in GSTp85 β (516-724) were unable to bind PI 3-kinase activity (Figure 5.4B, lanes 3 and 4). These results suggest that 71 amino acids (residues 445-516) in p85 β were critical for the interaction between the p85 β protein and p110.

In order to locate more definitively, the binding site in p85 β , mutants that contained deletions in this region such as GSTp85 β Δ 446-516, GSTp85 β Δ 445-485 and GSTp85 β Δ 445-469 were constructed (Figure 5.4A, mutants 5, 6 and 7). The function of these mutants was then assayed as described above. Following binding, PI 3-kinase assays revealed that both GSTp85 β Δ 486-516 and GSTp85 β Δ 445-469 were still able to bind approximately 65% of the PI 3-kinase activity (Figure 5.4B, lanes 5 and 7), in comparison to wild type GSTp85 β . However, the deletion mutant GSTp85 β Δ 445-485 (analogous to residues 452-492 in p85 α) was unable to bind any detectable PI 3-kinase activity (Figure 5.4B, lane 6), thereby defining 40 amino acid residues which were sufficient to abolish the binding of PI 3-kinase activity to p85 β .

To assess whether a similar region of p85 α was involved in binding to the p110 protein, an analogous deletion to that made in p85 β was constructed (Figure 5.5A, mutant 3) and assayed for its ability to bind PI 3-kinase activity as described above. This deletion mutant, GSTp85 α NC Δ 478-513, (analogous to amino acid residues 471-504 in p85 β) was unable to bind PI 3-kinase activity (Figure 5.5B, lane 3) in comparison to a similar construct, GSTp85 α NC, lacking the deletion (Figure 5.5B, lane 2). These results define a 35 amino acid region in p85 α that is necessary to mediate binding of the p110 catalytic subunit.

5.2.3 Mapping the site for p85 binding on p110

To map the regions of p110 which could interact with p85 α and p85 β a collaborative study was undertaken with Ian Hiles in our lab. GST-p110 fusion proteins encompassing various regions of p110 were made (Figure 5.6A) and their ability to bind p85 expressed in an insect cell expression system assessed. Results in Figure 5.6B show that of the six fusion proteins p110.1-p110.6 only p110.1 was able to bind to p85 α (lane 16) and p85 β

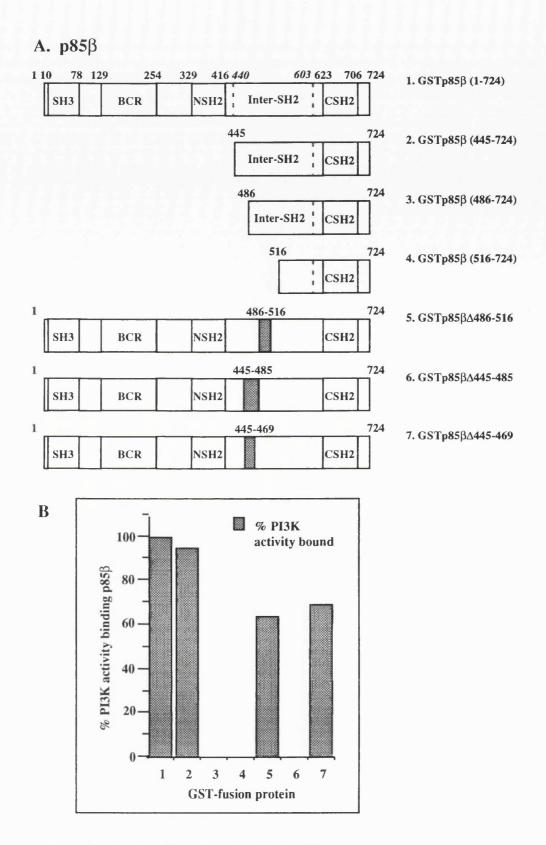
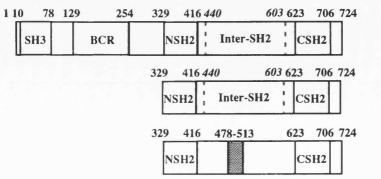


Figure 5.4. Amino acid residues 445-485 of p85 β are neccessary for the binding of p110 (A) Full-length p85 β (1); 5' nested deletions of p85 β (2-4), deletion mutants of p85 β (5-7) (B) GST fusion proteins of p85 β were bound to Glutathione-Sepharose beads and then incubated with p110 containing Sf9 cell lysates (lanes 1-7). Samples were then washed and subjected to PI 3-kinase assays.

A. p85α



- 1. GSTp85α (1-724)
- 2. GSTp85αNC
- 3. GSTp85 α NC Δ 478-513

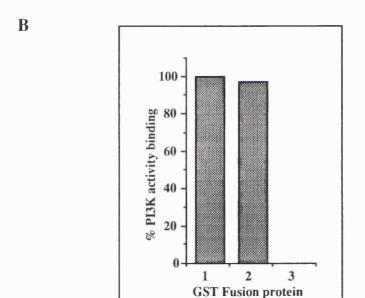


Figure 5.5: Amino acid residues 478-513 of p85 α and are neccessary for the binding of p110

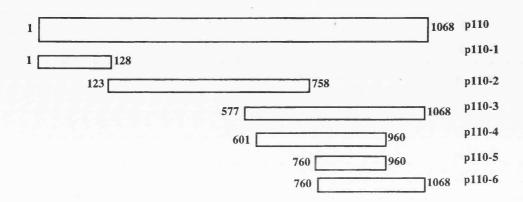
- (A) Full-length p85 α (8); p85 α NC (9), and deletion mutant of p85 α NC (10).
- (B) GST fusion proteins of p85 α were bound to Glutathione-Sepharose beads and then incubated with p110 containing Sf9 cell lysates (lanes 1-3). Samples were then washed and subjected to PI 3-kinase assays.

(lane 17) with high affinity. Binding was not detected in the control Sf9 cell lysate and binding to either subunit was not seen in experiments performed with any of the other constructs. This indicates that residues 1-128 of p110 contain a binding site for the p85 proteins. To investigate further the sequences required for binding to p85, seven GST-fusion proteins (p110-1.1-p110-1.7) which encompassed the first 128 amino acids of p110 were constructed (See Figure 5.7A). Of these, p110-1.3 and p110-1.5 bound both p85 α and p85 β (Figure 8, lanes 20, 21 and 5, 6 respectively). None of the other constructs showed any ability to bind either p85 subunit. Together these results are able to define amino acid residues 20-108 of p110 as a minimum structural element which is required for binding the p85 subunit. It should be noted that the GSTp110 fusion proteins appear to be very susceptible to degradation which explains the presence of numerous proteins on SDS-PAGE gels even after affinity binding to Glutathione-Sepharose beads.

5.2.4 The inter-SH2 region of p85 is predicted to be a coiled-coil of two anti-parallel alpha-helices

The demonstration that the inter-SH2 domain mediated intersubunit association led to further evaluation of the possible structure of this region of the p85 protein. Previously this region had been predicted to be largely α -helical in character (Panayotou et al., 1992) and it was suggested that it might adopt the structure of a four helix bundle. To extend this work the sequence of the inter-SH2 region of p85α was used to search the aminoacid sequences in the Owl 19.0 database (Proterin Engineering Club, Leeds University, U. K.). This work was carried out in collaboration with B. Bax at Birkbeck College. Twelve of the top twenty hits were with sequences from the proteins myosin or paramyosin sequences which have coiled-coil regions, the top five hits being with isoforms of p85. Although the percentage sequence identity between the aligned amino acid sequences was relatively low, heptad repeats (see below) were often found to be aligned. The amino acid sequences of proteins in the Brookhaven databank of protein structures (Bernstein et al., 1977) was also searched with the sequence of the inter-SH2 region. Here two of the top three hits were with tropomyosin, a two stranded α -helical coiled-coil muscle protein whose structure has been solved to 15Å resolution (Philips et al., 1979; Philips et al., 1986). Although the amino acid sequence identity with tropomyosin was quite low (20% over 175 amino acids), the heptad repeats in the inter-SH2 region and that of tropomyosin were in register for a considerable part of the alignment. Sequence analysis of tropomyosin (Hodges et al., 1972; Parry, 1975, McLachlan and Stewart, 1975) had shown an unbroken series of heptad repeats (a,b,c,d,e,f,g)n, where residues a and d are conserved as hydrophobic residues, which suggested that the structure would be a coiled-coil of α -helices. Our earlier sequence analysis of the helical domain of p85 α and p85 β (Panayotou et al., 1992) showed the

A



B

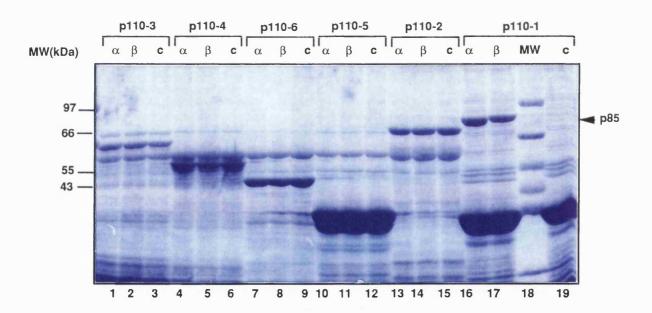
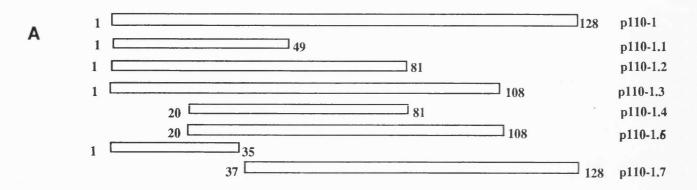


Figure 5.6. Identification of a region of p110 which binds p85.

(A) Diagramatic view of the regions of p110 expressed as Glutathione transferase fusion proteins. Numbers correspond to the amino acid residues at the extremes of the expressed regions of p110.

(B) The different GST-p110 fusion proteins were bound to Glutathione-Sepharose beads and incubated with Sf9 cell lysate containing p85 α , (α) p85 β (β) or control (C) wild type proteins. Samples were washed and bound proteins resolved by SDS-PAGE on a 10% gel. The arrow indicates the position of the p85 proteins.



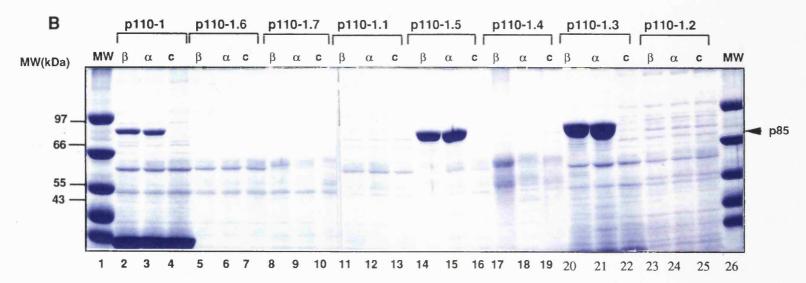


Figure 5.7. Fine scale mapping of a region of p110 capable of binding p85.

- (A) Diagramatic view of the regions of p110-1 expressed as glutathione transferase fusion proteins. Numbers correspond to the amino acid residues at the extremes of the expressed regions of p110.
- (B) The different GST-p110-1 fusion proteins were bound to Glutathione-Sepharose beads and incubated with Sf9 cell lysate containing p85α (α), p85β (β) or control wild type (C) proteins. Samples were washed and bound proteins resolved by SDS-PAGE on a 10% gel.
- The arrow indicates the position of the p85 proteins and lanes containing molecular weight markers indicated (MW).

presence of two long unbroken heptad repeats of the form (a,b,c,d,e,f,g)n where residues a and d are hydrophobic.

The heptad repeats in the inter-SH2 region of p85 and the similarity of its amino acid sequence with sequences of coiled-coil proteins such as tropomyosin, paramyosin and myosin suggested that the domain might consist of a coiled-coil of α -helices. Analysis of the sequence of the inter-SH2 region showed that its sequence is consistent with the structure being an anti-parallel coiled-coil of two seventy-residue α -helices (residues 441-512 and 518-588 in p85 α - see Figures 5.8 and 5.9). The proposed register of the helices was that which gave the maximum number of possible ion pairs. A single proline residue (568 in p85 α) which occurs on the outside of the second predicted α -helix should be tolerated (Blundell et al., 1983). Sequence comparison with the crystal structure of tropomyosin (Philips et al., 1979; Philips et al., 1986) suggests the anti-parallel coiledcoil region of p85 is expected to have a length of about 110Å, and contain three-quarters of a turn of coiled-coil (see Figure 5.9A). A large number of charged residues are present at positions e and g on the two α -helices, as is commonly found in α -helical coiled-coil proteins (Conway and Parry, 1990) and the amino acid residues found at the seven positions in the heptad repeat are fairly typical of those found in two-stranded coiled-coils (Conway and Parry, 1990). Six ion pairs are predicted to help to stabilize the coiled-coil region, giving a favourable 0.6 ionic interactions per heptad repeat. The coiled-coil has two faces, one having residues B,E,A, a, e, b and the other residues C, G, D, d, g, c; we shall call these the AEB face and the DGC face (Figure 5.8). In this model the first helix starts at residue 441, a G position, and it may be that preceding residues (431-440) pack against the DGC face at the bottom of the coiled-coil. The second helix is predicted to end at residue 588, an e position on the second helix, and it may be that residues Cterminal to the second helix (residues 589-618) pack against the AEB face at the bottom of the coiled-coil. Possibly some of the seven acidic residues between 604 and 615 could interact with the exposed basic residues Arg 577 (a), Arg 574 (e), and Lys 567 (e), some 20-30Å from the bottom of the coiled-coil on the BAE face. These results provide a possible structural basis for consideration of the intersubunit binding and enzyme regulation.

5.3 Discussion

PI-3 kinase has two distinct subunits; p85, which can serve to link the enzyme to activated protein-tyrosine kinase receptors and other proteins (Gout et al., 1992; Otsu et al., 1991), and p110, which has an intrinsic catalytic activity (Hiles et al., 1992). The regulation of enzyme activity and the complex protein interactions which may be

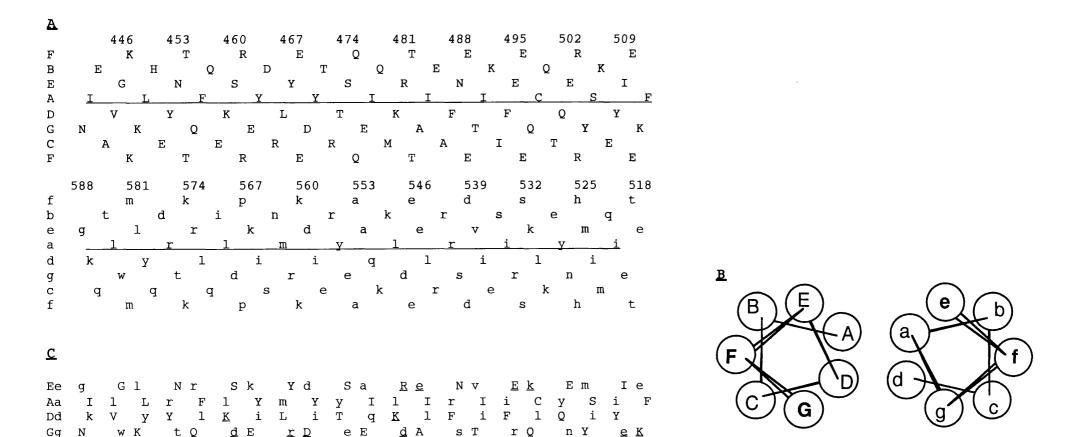


Figure 5.8. Prediction analysis of the helical nature of the inter-SH2 region of p85.

(A) The amino acid sequences of the two helices have been drawn on a helical net. Note that for helix-2, the sequence has been drawn backwards, so that the two helices are in register. The solid line indicates the line of intersection of the two helices. Hydrophobic residues at positions A/a and D/d of the two helices are expected to interlock.

(B) A pair of anti-parallel α -helices interacting. Heptad repeats from two anti-parallel helices are shown, residues at A/a and D/d positions are hydrophobic. Note the register (height) of the residues in the two helices:

ABCDEFGA dcbagfed

(See Crick, 1953 for further details on how helices interact to form coiled-coils).

(C) Residues on the two α -helices in p85 α that are expected to interact are shown superposed. Residues from HELIX 1 are in CAPITALS, while those from helix 2 are in lower case. Residues which could form ion pairs between the two helices are underlined. Figure courtesy of B. Bax.

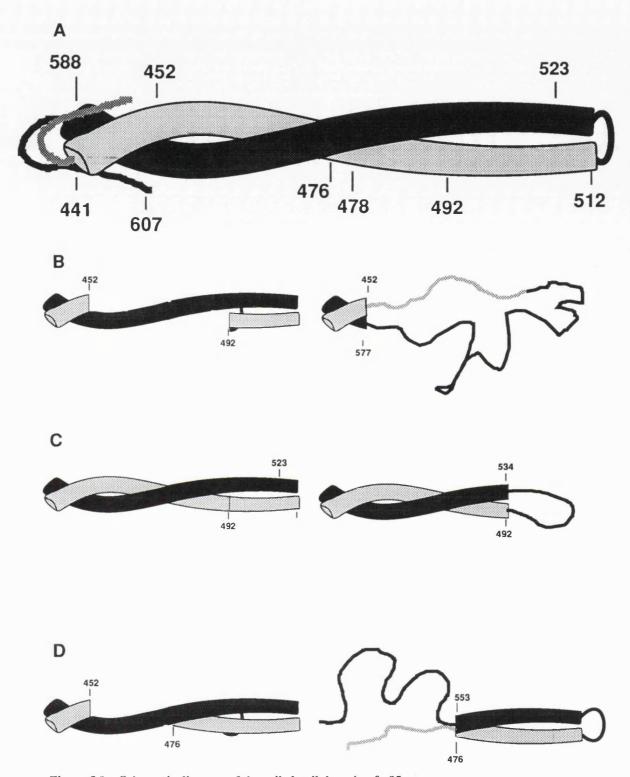


Figure 5.9: Schematic diagram of the coiled-coil domain of p85.

A. Numbers are residues according to p85α numbering. The first helix (h

A. Numbers are residues according to p85 α numbering. The first helix (helix-1; residues 441-512) is depicted with light shading, the second, (helix-2, residues 518-588) is shaded more darkly. Note that the deletion mutants in p85 β correspond to residues 452-476 (445-469 p85 β numbering), 452-492 (445-485 p85 β numbering) and 493-523 (486-523 p85 β numbering).

- B. The deletion 452-492 abolishes binding and may completely disrupt the structure.
- C. The deletion 493-523 will remove the lower quarter of the coiled-coil
- **D**. The deletion 452-476 will remove the top half of the coiled-coil.

involved in the function of PI 3-kinase seem to require the presence of both subunits. The most obvious example of intersubunit regulation is the ability of phosphotyrosine-containing proteins or peptides to cause activation of the PI 3-kinase activity (Backer et al., 1992; Carpenter et al., 1993; Giorgetti et al., 1993). The basis for activation could lie in phosphopeptide induced conformational changes which have been seen in the N-terminal SH2 domain of p85 α and the p85 α protein itself (Panayotou et al., 1992; Shoelson et al., 1993), although the structural implications of this on p110 remain unknown. Here the basis for the intersubunit interactions is determined so that the function of this enzyme both in vitro and and in vivo may be understood.

5.3.1 The inter-SH2 region of p85 binds the N-terminal region of p110.

To examine whether the same specific region of p85 α and p85 β interacts with p110, domains of p85 were expressed as GST fusion proteins. The results clearly show that the inter-SH2 region of p85 is required for the association with p110, and blocking of the inter-SH2 region on the intact p85 protein with monoclonal antibodies that have epitopes in this region prevents p110 binding in vitro. Similar results were obtained using the yeast two-hybrid system and GST fusion proteins of the various domains of p85 (Klippel et al., 1993; Hu et al., 1993; Holt et al., 1994). These data preclude SH2 domains as binding PI 3-kinase activity in vivo as was shown by Cooper and Kazlauskas (1993).

A more detailed analysis of the inter-SH2 region of p85 α using varying sizes of this domain defined 104 amino acids, that is residues 451-555, that are able to bind directly the p110 protein and PI 3-kinase activity. Deletion mutants of the p85 proteins, identified a short stretch of approximately 35 amino acids in both p85 proteins, which includes residues 478-513 in p85 α and 445-485 in p85 β necessary for binding p110 in vitro. Expression of the full-length p85 α Δ 478-513 in mouse-L cells has showed that it was also unable to associate with PI 3-kinase activity, in contrast to wild type p85 α , thus verifying that the deleted region of p85 α defined in this study is the binding site for the p110 protein in vivo (K. Hara, unpublished data). In addition, the ability of the full length p85 α and this deletion mutant p85 α Δ 478-513 to bind PI 3-kinase activity and the insulin receptor substrate IRS-1 in vivo has been examined using stable cell lines overexpressing these constructs and the insulin receptor (Hara et al., 1994). Insulin stimulation of cells expressing wild-type p85α showed that it associated with IRS-1 and PI 3-kinase activity, moreover, an accumulation in the levels of PI(3,4)P₂ and PI(3,4,5)P₃ was observed. However, insulin stimulation of cells expressing p85 α Δ 478-513 showed that although p85α was still able to associate with IRS-1 it was unable to bind PI 3-kinase activity and no change in the levels of PI(3,4)P2 and PI(3,4,5)P3 was seen. These data would suggest that these constructs may be used in further studies as dominant-negative mutants in the analysis of mitogenic signalling pathways. The mapping of the p85 binding site on p110 has shown that amino acids 20-108 are capable of binding to the p85 proteins and no other expressed fragments (P110.2-p110.6) show any detectable binding to either subunit. Thus the inter-SH2 region of p85 and the N-terminal region of p110, as independently folded modules, are responsible for mediating the interaction of the two subunits of PI 3-kinase (Figure 5.10). However it cannot be ignored that other regions of both the p85 and the p110 proteins may also play some role in the interaction between the subunits but have been overlooked in this study due to the inability of a particular domain to fold correctly.

5.3.2 The inter-SH2 region of p85 is predicted to be a domain which adopts a helical conformation.

To search for a structural basis for the region of p85 which binds p110 the predictive structural studies of Panayotou et al (1992) were extended. Sequence analysis suggests the inter-SH2 region to be an anti-parallel coiled-coil of two α -helices that we have termed helix-1 and helix-2 (Figure 5.9A). Evidence supporting the concept that this region may be a discrete domain is seen using various size GST fusion proteins of the inter-SH2 region. It is demonstrated that as the boundaries of the inter-SH2 region are decreased the amount of PI3-kinase activity seen to bind is also decreased. This could be accounted for if disrupting the inter-SH2 structure is destabilising the putative domain, and thus preventing protein-protein interactions. When deletions in the p85 α protein and those in the p85\beta protein are compared and considered with the corresponding loss of activity, the third quarter of helix-1 of the coiled-coil, residues 478-492 in p85 α , is identified as the common region between the two p85 proteins that is absent in deletion mutants that are unable to bind PI 3-kinase activity (Figure 5.9A). Deletions which remove either the top half of helix-1, residues 452-476 on p85α (Figure 5.9D) or the lower quarter of helix-1, residues 492-523 on p85α (Figure 5.9C) destabilise the coiled structure and thus disrupt the association with the p110 protein but do not completely inhibit its binding. Only when the residues 478-492 are removed, which are contained in the mutation which deletes residues 452-492 in p85 α , is a complete loss of function observed (Figure 5.9B) and the p110 protein is seen not to bind to p85. Sequence analysis of both the p85 proteins reveals that residues 478-492 of p85 α are contained in three heptad repeats (residues 470-497 in p85 α) which are identical between the two proteins at the amino acid level except for a single conservative amino acid change (Figure 5.8A). A peptide corresponding to residues 470-497 of p85 α was synthesised and chemically coupled to Sepharose beads, however, it was unable to bind to p110 and PI 3-kinase activity (data not shown). Analysis of the p110 binding characteristics of the p85 deletion mutants suggests that while helix-1 of the inter-SH2 region is primarily responsible for the interaction between the two subunits of the PI3-

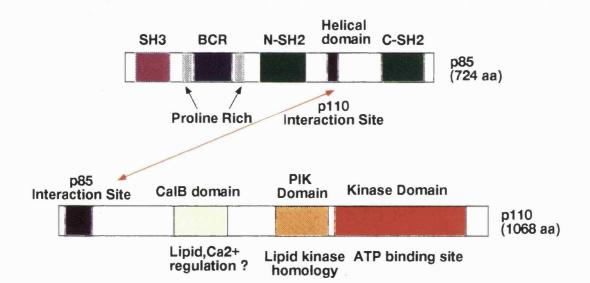


Figure 5.10. Schematic of the PI 3-kinase showing domain homologies with other proteins.

kinase complex, helix-2 probably donates a structural element to the folding of helix-1. Thus, in order to produce a stable structure, regions corresponding to 470-497 on helix-1 of p85 α and the adjacent region on helix-2 are probably required for the interaction between the p85 and the p110 subunit to take place.

The interaction between the p85 and the p110 proteins can take place both in vivo and in vitro, suggesting that the two proteins do not need to be co-translated in order to associate. The binding between the p85 subunit and the p110 subunit is of very high affinity, and no suitable conditions have been established which result in dissociation of an active PI 3-kinase complex once formed (Fry et al., 1992; Ruiz et al., 1993). In addition, it is known that the interaction between the two subunits is not dependent on the presence of phosphotyrosine (See Chapters 4 and 6) and there are no proline-rich motifs present in this region of p110 which may provide a binding concensus for the SH3 domain of the p85 protein. Secondary structure analysis using the Leeds Prediction Package (Eliopoulos, 1989), predicts that some 60% of the N-terminal 120 residues, that have been shown to directly bind the p85 proteins, will adopt a α-helical conformation and some 20% a β-sheet conformation. Thus, the N-terminal region of p110 will consist of a α/β region, where the α -helices might form interactions with the inter-SH2 region of p85. Since the major binding site on p85 α is considered to be between residues 478-492 on Helix-1, it is of interest that there is a small hydrophobic pocket in this region formed by Met-479 (C position on Figure 5.8A), Ala-483 (G position on Figure 5.8A) and Ala-486 (C position on Figure 5.8A). As the N-terminus of p110 is approximately 48% hydrophobic in character, this pocket may be important in forming a high affinity interaction with the p110 protein.

5.3.3 Regulation of the PI 3-kinase

It is intriguing that a lipid binding site has also been identified in the inter-SH2 region of the p85 protein. Two monoclonal antibodies that specifically recognize this region have been shown to inhibit the binding of phospholipids, in particular PI(4,5)P2 to the p85 subunit (End et al., 1993). Amino acid sequence analysis reveals the presence of a short basic motif in the inter-SH2 region of the p85 α protein, similar to that found in profillin and gelsolin and demonstrated to confer PI(4,5)P2 binding properties to these proteins (Janmey et al., 1992; Yu et al., 1992). Moreover, analogous residues are well-conserved in p85 β , which suggests that it might be important as a regulatory site for these proteins. The function of this lipid binding site is presently unknown, but there are several possible implications. As the p85 protein has been shown to bind p110 via the inter-SH2 region, the lipid binding site may form a part of the substrate binding pocket for PI 3-kinase, specific for PI(4,5)P2. Indeed, at the membrane where PI is the most abundant substrate, this region may be important in the selection of PI(4,5)P2, thus increasing the affinity for

this phospholipid. Evidence from a number of cell lines suggests that PI(3,4,5)P3 is the key signalling lipid generated by the PI 3-kinase (Hawkins et al., 1992; Stephens et al., 1991), thus implicating PI(4,5)P2 as the critical substrate required for activation of the PI 3-kinase. It could be speculated that the conformational change induced in p85 upon peptide binding, may cause a sterically masked PI(4,5)P2 lipid binding site to become more accessible to the substrate binding pocket of PI 3-kinase, thus resulting in its activation. The substrate specificity of purified monomeric p110 and comparison with purified p85α/p110 complex and mutagenesis of critical residues in the lipid binding site of p85 will be required to resolve this issue.

5.3.4 Conclusion

This study has localised the regions of $p85\alpha$ and p110 involved in interacting with each other which will in turn permit the use of dominant negative mutants that may define a more precise role for PI 3-kinase in mitogenic signalling pathways. As the inter-SH2 domain region has been defined as the site of interaction for the catalytic subunit, it will be interesting to examine if the p85/p110 complex binds to receptors with the same specificity and affinity as the SH2 domains and the p85 proteins were shown to in Chapter 3. This will thus be examined in the next Chapter.

Chapter 6: Receptor interactions

6.1 Introduction

The isolated p85 proteins were shown to bind every activated protein-tyrosine kinase thus far examined (Chapter 3). In particular both p85 α and p85 β bound with high affinity the receptors for EGF and CSF-1 and the non-receptor tyrosine kinases pp60^{c-Src} and pp59^{Fyn} (Gout et al., 1992). Further analyses have defined the inter-SH2 domain of p85 as the region that mediates the interaction with a region in the N-terminus of p110 (Chapter 5). In this Chapter the earlier studies are extended to investigate whether the association of p85 with the catalytic subunit confers specificity to the binding properties of the PI 3-kinase complex. In addition, the properties of the p85 β /p110 complex are examined to probe the function of p85 β .

6.2 Results

6.2.1 Recombinant p85 α /p110 complex associates with activated CSF-1 and EGF receptors but not with non-receptor PTKs

To study the specificity of binding of recombinant p85α/p110 (αPI 3-kinase) with PTKs the in vitro association of CSF-1, EGF and pp59c-Fyn PTKs with the αPI 3-kinase complex was examined employing methods based on those previously described. Briefly, PTKs were immunoprecipitated from lysates of insect cells that had been infected with the appropriate virus 1.5 days previously, and collected on Protein A-Sepharose beads. Following several washes with lysis buffer and two washes with kinase buffer, half of the immunoprecipitated receptor was phosphorylated in the presence of cold ATP, while the other half was incubated with kinase buffer lacking ATP. Excess ATP was removed using several washes in lysis buffer and the activated PTKs were then incubated with lysates of insect cells expressing p85α alone, p110 alone, control AcMNPV infected cell lysates alone, or lysates that had been coinfected with p85α/p110 viruses. The complexed proteins were then subjected to an in vitro kinase assay and analysed by autoradiography of SDS-PAGE gels or were assayed for associated PI 3-kinase activity.

The results of the experiments in Figure 6.1 show that αPI 3-kinase activity was only found to associate with the EGF and the CSF-1 receptors providing both receptors were prephosphorylated in vitro (Panels A and B, lane 2). The p110 protein alone was unable

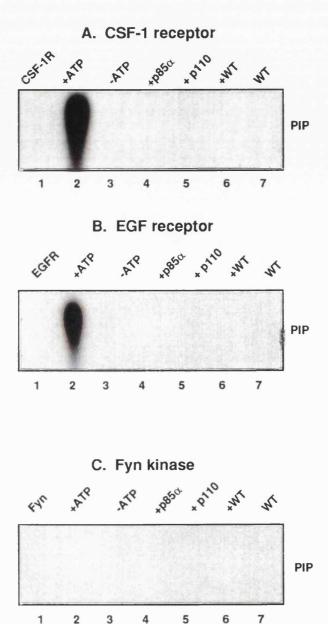


Figure 6.1. In vitro association of PI 3-kinase activity with protein tyrosine kinases.

PI 3-kinase assay was performed on anti-PTK immunocomplexes prepared from Sf9 cells infected with an appropriately infected baculovirus and treated as follows: anti-PTK immunoprecipitate, untreated (lane 1); anti-PTK immunoprecipitate pretreated with ATP and incubated with a p85 α /p110 containing Sf9 cell lysate (lane 2); anti-PTK immunoprecipitate, treated in the absence of ATP and incubated with a p85 α /p110 containing cell lysate (lane 3); anti-PTK immunoprecipitate pretreated with ATP and incubated with a p85 α containing Sf9 cell lysate (lane 4); anti-PTK immunoprecipitate pretreated with ATP and incubated with a p110-containing Sf9 cell lysate (lane 5); anti-PTK immunoprecipitate pretreated with ATP and incubated with a cell lysate infected with wild-type virus (lane 6); anti-PTK immunoprecipitate of wild-type infected SF9 cells (lane 7).

to associate with the phosphorylated receptors (Panel A and B, lane 5), substantiating the notion that the p85 subunit is an adapter protein that serves to link the catalytic subunit of the PI 3-kinase with activated phosphorylated receptors in vivo. Neither receptor was seen to coimmunoprecipitate with any endogenous insect PI 3-kinase activity (panels A and B, lane 1), or to bind any endogenous insect PI 3-kinase activity if it had been prephosphorylated and then incubated with a control AcMNPV lysate (panels A and B, Antibodies that recognised the receptors were also found not to immunoprecipitate any insect PI 3-kinase activity (Panels A and B, lane 7). It should be noted that although there was greater amount of EGF receptor present in these immunoprecipitates than CSF-1 receptor, due to a difference in the level of receptor expression in insect cells (See Chapter 3), significantly less PI 3-kinase activity was seen to bind the activated EGF receptor than to the CSF-1 receptor (Figure 6.1 compare panels A and B, lane 2). Figure 6.2B (panel A and B) shows the autoradiographical analysis of the in vitro kinase assays performed on these immunoprecipitations. The p85 protein was visible as a phosphoprotein of 85 kDa, however, the p110 was not phosphorylated by either the CSF-1 or EGF receptors (panels A and B, lanes 2 and 4).

Identical analyses with the non-receptor kinase pp59c-Fyn revealed contrasting results. Immunoprecipitated pp59c-Fyn prephosphorylated in vitro, failed to bind recombinant α PI 3-kinase, when incubated with lysates of insect cells that had been infected with p85 α /p110 (Figure 6.1C, lane 2), although p85 α alone was still seen to associate with, and be phosphorylated by pp59c-Fyn (Figure 6.2C, lane 2). (It should be noted that p85 is expressed in excess of p110 in insect cells [described in Chapter 4] and thus the free p85 subunit as opposed to PI 3-kinase is observed to bind to Fyn in Figure 6.2C, lane 2). These results suggest that the associated p110 protein is able to confer a change in the specificty of binding on the p85 α protein.

6.2.2 Comparison of receptor interactions with the partially purified bovine brain PI 3-kinase

Identical analyses to those described above were carried out using the partially purified bovine brain enzyme. The results in Figure 6.3 show that the partially purified bovine PI 3-kinase bound to the CSF-1 receptor (panel A, lane 3) but not the phosphorylated pp59^{c-} Fyn non-receptor kinase (Panel B, lane 6) as was observed with the recombinant PI 3-kinase. However, the activated EGF receptor was also unable to bind the partially purified bovine PI 3-kinase (Panel B, lane 2). This will be examined in greater detail in Section 6.2.8.

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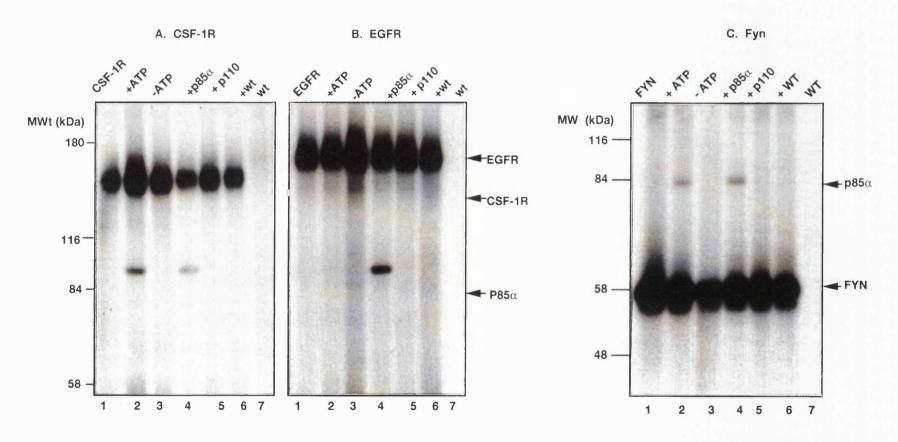


Figure 6.2 In vitro association of PI 3-kinase with protein tyrosine kinases.

An in vitro protein tyrosine kinase (PTK) assay was performed on anti-PTK immunocomplexes prepared from Sf9 cells infected with an appropriately infected baculovirus and treated as follows: anti-PTK immunoprecipitate, untreated (lane 1); anti-PTK immunoprecipitate pretreated with ATP and incubated with a p85 α /p110 containing Sf9 cell lysate (lane 2); anti-PTK immunoprecipitate, treated in the absence of ATP and incubated with a p85 α /p110 containing cell lysate (lane 3); anti-PTK immunoprecipitate pretreated with ATP and incubated with a p110-containing Sf9 cell lysate (lane 5); anti-PTK immunoprecipitate pretreated with ATP and incubated with a p110-containing Sf9 cell lysate (lane 5); anti-PTK immunoprecipitate pretreated with ATP and incubated with a cell lysate infected with wild-type (WT) virus (lane 6); anti-PTK immunoprecipitate of wild-type infected SF9 cells (lane7).

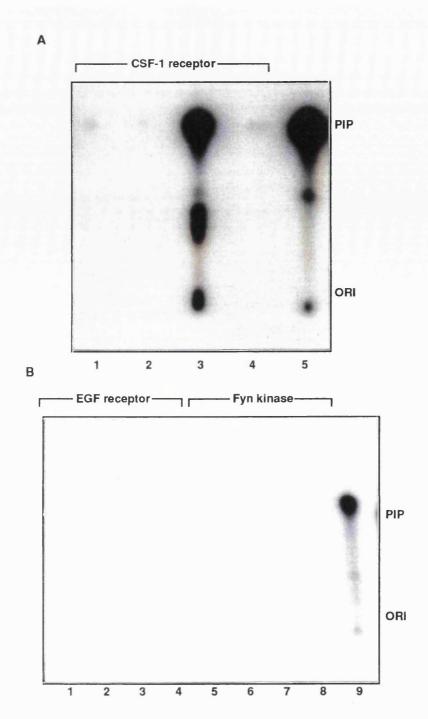


Figure 6.3. PI 3-kinase, partially purified from bovine brain, associates only with the activated CSF-1 receptor.

A. PI 3-kinase assays were performed on anti-CSF-1 receptor immunocomplexes prepared from Sf9 cells infected with a recombinant baculovirus expressing the CSF-1 receptor and treated as follows: ; untreated (lane 2); pretreated with ATP and incubated with a partially purified bovine brain PI 3-kinase (lane 3); treated in the absence of ATP and incubated with a partially purified bovine brain PI 3-kinase (lane 4); the amount of partially purified bovine brain PI 3-kinase used in the assay (lane 5); Lane 1 is a control showing anti-CSF-1 receptor immunoprecipitate of insect cells infected with wild type virus.

B. PI 3-kinase assays were performed on anti-EGF receptor (lanes 1-4) and anti-Fyn (lanes 5-8) immunocomplexes prepared from Sf9 cells infected with a recombinant baculovirus expressing the appropriate PTK and treated as follows: ; untreated (lanes 1 and 5); pretreated with ATP and incubated with a partially purified bovine brain PI 3-kinase (lanes 2 and 6); treated in the absence of ATP and incubated with a partially purified bovine brain PI 3-kinase (lanes 3 and 7); the amount of partially purified bovine brain PI 3-kinase used in the assay (lane 9); Control anti-EGF receptor (lane 4) and anti-Fyn (lane 8) immunoprecipitates of insect cells infected with wild type virus are shown.

6.2.3 The p85 β /p110 complex exhibits the same binding properties as the p85 α /p110 complex

Whether the p85β/p110 (βPI 3-kinase) complex would exhibit similar binding specificities to that shown by the recombinant αPI 3-kinase was examined next using essentially the same assay and conditions as that described above. The CSF-1 and the EGF receptors and pp59c-Fyn were immunoprecipitated from lysates of Sf9 cells that had been infected with the appropriate virus. Each kinase was then prephosphorylated in vitro in the presence of non-radiolabelled ATP and incubated with lysates of insect cells that had been coinfected with p85β and p110 viruses. Complexed proteins were then subjected to in vitro kinase assay and detected by autoradiography of SDS-PAGE gels or assayed for associated PI 3-kinase activity. The p85β/p110 complex was found to bind the phosphorylated CSF-1 and EGF receptors (Figure 6.3B, lane 2), but in contrast did not bind the non-receptor kinase pp59c-Fyn (Figure 6.3B, lane 2), thus displaying similar properties to those manifested by the recombinant p85α/p110 complex.

6.2.4 In vivo association of the recombinant PI 3-kinase with the CSF-1 and EGF receptors and with the non-receptor kinase pp59^{c-Fyn}.

The analyses described thus far have focused on the in vitro association of PI 3-kinase with PTKs. The interaction of protein-tyrosine kinases with PI 3-kinase in vivo was also examined. Insect cells were triply infected with PTK, p85α and p110 viruses (Figure 6.5, panels A, B and C, lanes 1-3). The results in Figure 6.5 show cells that were harvested 3 days post-infection and lysates immunoprecipitated with specific anti-PTK antibodies (Panels A, B and C, lane 1) or with antibodies to either the p85α (Panels A, B and C, lane 2) or the p110 (Panels A, B and C, lane 3) subunits of the PI 3-kinase complex. Immune complexes were washed several times with lysis buffer and then assayed for associated PI 3-kinase activity, or subjected to in vitro kinase assays and phosphoproteins detected by autoradiography of SDS-PAGE gels. The results in Figure 6.5 show that PI 3-kinase activity associated with the CSF-1 receptor, to a lesser degree with the EGF receptor and not at all with pp59c-Fyn in vivo (panels A, B and C, lane 1) as was observed in vitro (see Section 6.2.1 above).

6.2.5 Recombinant p85 proteins can block the binding of PI 3-kinase to CSF-1 receptor.

The next stage was to examine whether both $p85\alpha$ and $p85\beta$ proteins can bind to the same site on the CSF-1 receptor and in doing so mediate the interaction of PI 3-kinase with this receptor. Both of the p85 proteins were used in complementary studies to block the binding of $p85\alpha/p110$ PI 3-kinase activity to the CSF-1 receptor. CSF-1 receptor was immunoprecipitated and phosphorylated in vitro. The immunoprecipitates were

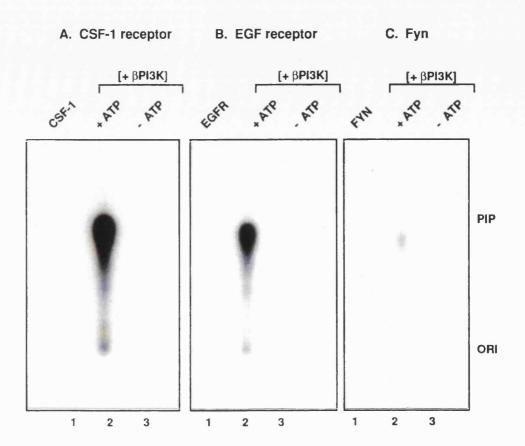


Figure 6. 4. The recombinant p85 β /p110 complex exhibits the same PTK binding specificities as the p85 α /p110 complex.

PI 3-kinase assays were performed on anti-PTK immunocomplexes (Panel A: anti CSF-1R; Panel B: anti EGFR; Panel C; anti-Fyn kinase) prepared from Sf9 cells infected with an appropriately infected baculovirus and treated as follows: anti-PTK immunoprecipitate, untreated (lane 1); anti-PTK immunoprecipitate pretreated with ATP and incubated with a p85 β /p110 containing Sf9 cell lysate (lane 2); anti-PTK immunoprecipitate, treated in the absence of ATP and incubated with a p85 β /p110 containing cell lysate (lane 3).

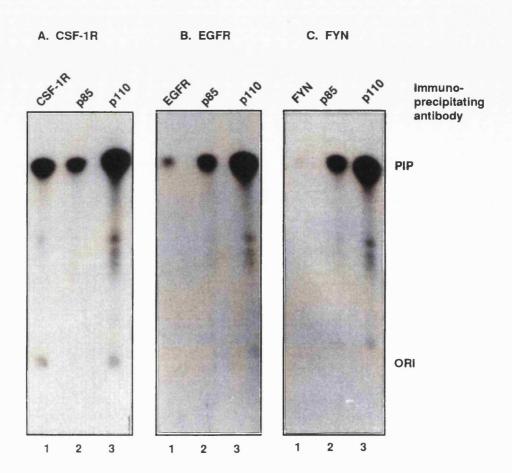


Figure 6.5. Reconstitution of active receptor-PI 3-kinase complex in insect cells

Insect cells were infected with a PTK, p85 α and p110 expressing baculovirus. Lysates of these cells were immunoprecipitated with PTK specific antibodies (lane 1), anti-p85 α antibodies (lane 2), and anti-p110 antibodies (lane 3). These immunoprecipitates were then assayed for associated PI 3-kinase activity.

incubated with either purified p85 α , p85 β , the p85 α /p110 complex alone, or with p85 α /p110 complex together with increasing concentrations of either p85 α or p85 β . After extensive washing immunocomplexes were assayed for associated PI 3-kinase activity. As can be seen in Figure 6.6, as the concentration of the recombinant p85 proteins added to the receptor was increased the amount of PI 3-kinase activity bound to the receptor decreased. At high concentrations, both p85 α and p85 β completely inhibit binding of PI 3-kinase activity to this receptor. These data suggest that both p85 proteins bind to the same site(s) on the CSF-1 receptor and in doing so mediate the interaction of PI 3-kinase with the CSF-1 receptor.

6.2.6 The SH2 domains of p85 α mediate the binding of the PI 3-kinase complex to the CSF-1 receptor.

To determine whether either of the SH2 domains of p85α were able to block the binding of PI 3-kinase to phosphorylated receptors, the CSF-1 receptor was immunoprecipitated from lysates of infected insect cells and prephosphorylated in vitro as described. The phosphorylated receptor was then incubated with GST-fusion proteins of the N- or the C-terminal SH2 domain of p85α, or with lysates of insect cells that had been infected with p85 and p110 viruses containing an excess of either the N- or the C-terminal SH2. Following binding, the immunocomplexes were washed several times with lysis buffer and then assayed for associated PI 3-kinase activity and/or subjected to in vitro kinase assays where the phosphoproteins were analysed by autoradiography of SDS-PAGE gels. The results in Figure 6.7 show that both SH2 domains associate with and are phosphorylated by the CSF-1 receptor in vitro (panel A, lanes 2-5). Further, both the N-and the C-terminal SH2 domains of p85α block the binding of PI 3-kinase activity to the CSF-1 receptor (Panel B, lanes 4 and 5).

6.2.7 Mechanisms of reconstitution of a receptor/PI 3-kinase complex.

The CSF-1 receptor/p85/p110 complex was reconstituted using a number of different procedures to determine whether p110 was able to bind p85 if the protein was already pre-bound to receptor, and also whether the phosphorylation state of the p85 protein affected the binding of the p110 protein. The three methods utilised were;

1. CSF-1 receptor was immunoprecipitated from infected insect cells, prephosphorylated in vitro in the presence of cold ATP and incubated with a lysate of insect cells infected with p85 α virus. Following several washes with lysis buffer, to remove unbound proteins, the in vitro formed receptor/p85 complex was incubated with a lysate from insect cells which had been infected with p110 virus.

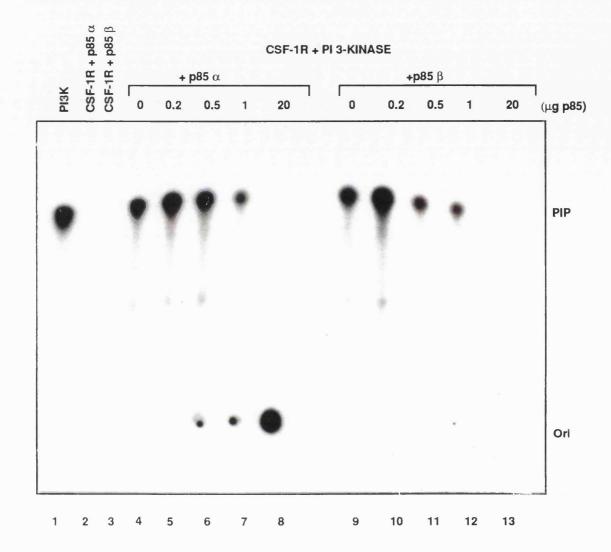


Figure 6.6 Blocking of in vitro association of p85 α /p110 with CSF-1 receptor by baculovirus p85 α and p85 β proteins.

Immunoprecipitated baculovirus expressed CSF-1 receptor was prephosphorylated by incubation with nonlabelled ATP and then exposed to either 1 μg of baculovirus purified p85 α or p85 β proteins (lanes 1 and 2 respectively), p85 α /p110 PI 3-kinase diluted in lysis buffer (lane 3), p85 α /p110 PI 3-kinase and increasing amounts of p85 α protein (0, 0.2 μg , 0.5 μg , 1 μg , 20 μg : (lanes 3 to 7 respectively), p85 α /p110 PI 3-kinase and increasing amounts of p85 β protein (0, 0.2 μg , 0.5 μg , 1 μg , 20 μg : (lanes 8 to 12 respectively). The samples were then assayed for receptor associated PI 3-kinase activity. The phosphorylated lipids were separated by TLC and visualised by autoradiography. Lane 1 is the amount of PI 3-kinase used in the assay. Ori and PIP markers indicate the positions of the TLC origin and PI 3-phosphate respectively.

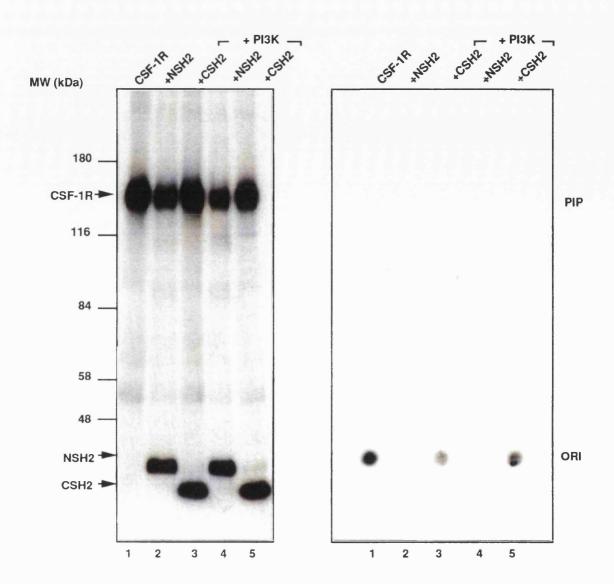


Figure 6. 7 Both the N- and the C-terminal SH2 block the binding of PI 3-kinase to the CSF-1R.

(A). The CSF-1 receptor was immunoprecipitated from lysates of infected cells and treated with ATP. The phosphorylated receptor was then incubated with lysis buffer (lane 1), lysis buffer containing GST-fusion proteins of the N- terminal SH2 domain (NSH2) (lane 2), GST-CSH2 (lane 3), lysate of Sf9 cells infected with p85 and p110 baculoviruses also containing GST-NSH2 (lane 4), lysate of SF9 cells infected with p85 and p110 baculoviruses also containing GST-CSH2 (lane 5). After binding immune complexes were washed and subjected to protein kinase assay. Phosphorylated proteins were detected by autoradiography of SDS-PAGE gels.

(B). Immune complexes treated as described in (A) were assayed for associated PI 3-kinase activity.

- 2. An in vivo formed CSF-1 receptor/p85 complex was immunoprecipitated using antireceptor antibodies from insect cells that had been coinfected with p85 α and CSF-1 receptor viruses. This immune complex was then incubated with lysates of insect cells that had been infected with p110 virus.
- 3. The CSF-1 receptor was immunoprecipitated from infected insect cells and incubated with a lysate of insect cells that had been coinfected with p85 α and p110 viruses.

All immune complexes, formed in the three methods described above, were washed with lysis buffer, and then assayed for associated PI 3-kinase activity or resolved by SDS-PAGE and proteins detected by Coomassie Blue staining. Comparable amounts of p85 were found to be associated with the CSF-1 receptor using all three methods (Figure 6.8A, lanes 2, 3 and 4). The results in Figure 6.8B show that p110 associated (on the basis of the observed PI 3-kinase activity) with the receptor bound p85 (lanes 2 and 3) as efficiently as the p85/p110 complex associated with phosphorylated receptor in vitro (lanes 4). Prior tyrosine phosphorylation of the p85 protein in vivo, by coinfection of the p85 protein with the CSF-1 receptor, also did not appear to affect the binding of p110 (see Chapters 3 and 5).

6.2.8 Detailed examination of the interaction of PI 3-kinase with the EGF receptor.

The molecular basis for the interaction between the PI 3-kinase and the EGF receptor is unclear although EGF can clearly stimulate PI 3-kinase activity in vivo (Carter and Downes, 1992; Soltoff et al., 1992). In these analyses qualitative comparisons reveal that the EGF receptor can bind to the recombinant PI 3-kinase, albeit with a lower affinity than the CSF-1 receptor. The EGF receptor has five major autophosphorylation sites that have been characterised in the carboxyl terminal tail of the receptor (Downward et al., 1984; Hsuan et al., 1989). However, these tyrosine autophosphorylation sites do not lie within the YXXM recognition motif shown to be required for high affinity binding of the PI 3-kinase (Cantley et al., 1991; Fantl et al., 1992; Kashishian et al., 1992). A Y920XXM motif is present in the kinase domain of the EGF receptor, but this has not been demonstrated to be an autophosphorylation site. In order to determine whether PI 3-kinase was binding to Y920 of the YXXM motif in the kinase domain, elastase treatment was used to remove the C-terminal tail of the EGF receptor.

6.2.8.1 The p85 α protein and PI 3-kinase do not bind the EGF receptor by its YXXM motif.

Elastase has been shown to remove the 15-20 kDa of the EGF receptor tail without degrading the receptor to smaller fragments (Gates et al., 1985). The EGF receptor was

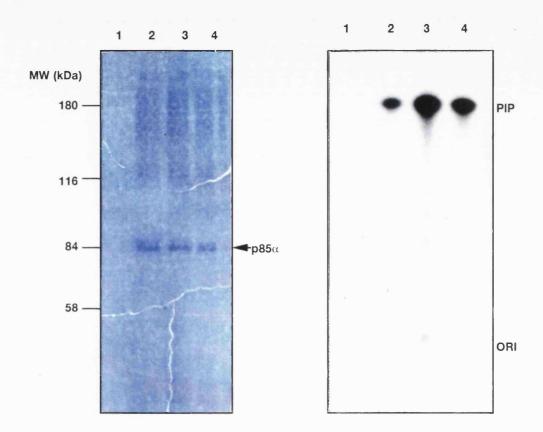


Figure 6. 8. Mechanisms of reconstitution of a receptor/PI 3-kinase complex

Anti-CSF-1 receptor immunoprecipitates prepared from Sf9 cells infected and treated as described: Untreated CSF-1 receptor alone (lane 1), immunoprecipitated CSF-1 receptor incubated with p85-containing Sf9 cell lysate, washed and then subsequently incubated with p110-containing Sf9 cell lysate (lane 2); Immunoprecipitated CSF-1 receptor incubated with lysate of Sf9cells that had been coinfected with p85 and p110 baculoviruses (lane 3); Anti-CSF-1 receptor immunoprecipitate of Sf9 cells that had been coinfected with CSF-1 receptor and p85 viruses. This receptor-p85 protein complex was then incubated with a p110-containing Sf9 cell lysate (lane 4). Immune complexes were then split in two. Half the sample was resolved on SDS-PAGE and proteins detected by Coomassie blue staining (A). The remainder of the sample was then assayed for associated PI 3-kinase activity (B).

immunoprecipitated from lysates of infected Sf9 cells and collected on Protein A-Sepharose beads. The immune complexes were washed several times in buffer and then incubated in the presence of elastase for increasing periods of time. The reaction was terminated by the addition of protease inhibitors and subjected to several washes in lysis buffer and then twice in kinase buffer. The EGF receptor was then phosphorylated in the presence of cold ATP. Excess ATP was removed by several washes in lysis buffer and the receptor was incubated with lysates of Sf9 cells infected with either p85 virus alone or lysates of insect cells that had been coinfected with p85 α and p110 viruses. The immune complexes were then subjected to in vitro kinase assays and the proteins detected by either Coomassie blue staining and autoradiography of SDS-PAGE gels, or by assay for associated PI 3-kinase activity. The results in Figure 6.9A show a Coomassie blue stain of the immunoprecipitated EGF receptor before elastase treatment (lane 1). Increasing time of elastase treatment resulted in a change in apparent molecular size of the EGF receptor from 180 kDa down to 150 kDa as the carboxyl terminal tail was detached (lanes 2-4). This process was accompanied by a parallel decrease in the amount of p85 protein which could bind to the receptor. Autoradiographic analysis of this gel revealed that although the truncated receptor was still heavily phosphorylated it was unable to bind $p85\alpha$ in vitro, indicating that $p85\alpha$ was binding specifically via a site in the tail (Figure 6.9B). The results in Figure 6.10 clearly demonstrated that the increased level of elastase treatment of the EGF receptor was also paralleled by a corresponding decrease in associated PI 3-kinase activity. Indeed, after 5 minutes of elastase treatment less than 10% of the PI 3-kinase activity was seen to be bound to the truncated EGF receptor relative to the wild type receptor.

6.2.8.2 Do the SH2 domains of p85 confer a specificity of PI 3-kinase binding the EGF receptor?

A partially purified bovine brain PI 3-kinase preparation was shown not to bind to the EGF receptor however, the recombinant PI 3-kinase clearly does bind this receptor (Otsu et al., 1991). I have already shown that both the isolated N- and the C- terminal SH2 domains of p85 associate with and are phosphorylated by the EGF receptor with similar affinities (Section 3.2.3.7/8). To investigate further the inability of the partially purified bovine brain preparation to bind the EGF receptor, p85 proteins containing either two N-(NNp85α) or two C- (CCp85α) terminal SH2 domains were constructed by P. Vicendo in our lab (Figure 6.11A). These constructs were then used to study if the lack of a particular SH2 domain, within the context of the PI 3-kinase complex, affected binding to the EGF receptor.

The NNp85 α and the CCp85 α proteins were translated from mRNA in reticulolysates. The translated recombinant p85 α proteins were then immunoprecipitated from

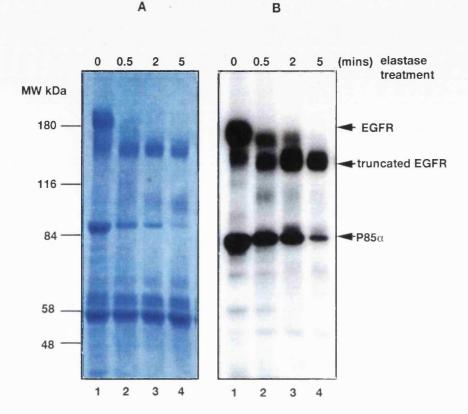
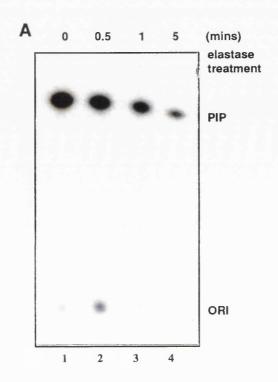


Figure 6. 9: The COOH-terminal tail of the EGF receptor binds p85. The EGF receptor was immunoprecipitated from lysates of infected Sf9 cells and treated with elastase for the indicated time periods (lanes 1-4). The immunoprecipitated receptor was then treated with ATP and then incubated with lysates of insect cells that had been infected with p85 expressing baculovirus. These protein complexes were subjected to protein kinase assay and SDS-PAGE. Proteins were detected by Coomassie Blue staining (A) and autoradiography of SDS-PAGE (B).



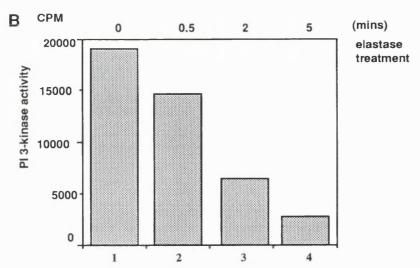


Figure 6.10: The COOH-terminal tail of the EGF receptor binds PI 3-kinase.

(A). The EGF receptor was immunoprecipitated from lysates of infected Sf9 cells and treated with elastase for the indicated time periods (lanes 1-4). The immunoprecipitated receptor was then treated with ATP and the immune complexes were incubated with lysates of insect cells that had been infected with p85 and p110 baculoviruses. These protein complexes were then assayed for associated PI 3-kinase activity.

(B). Graphical representation of the amount of PI 3-kinase activity (CPM) associated with the EGF receptor treated as described in (A).

reticulolysates using monoclonal antibodies that recognise the SH3 domain of p85α. Following several washes in lysis buffer the immunoprecipitated protein was incubated with either a lysate of Sf9 cells that had been infected with p110 virus or with lysis buffer alone. The immune complexes were washed again in lysis buffer and then incubated with a lysate of Sf9 cells that had been infected with the EGF receptor virus. To ensure maximal activation of the EGFR, the lysate was preincubated in the presence of ATP, Mg²⁺ and Mn²⁺. This reaction was quenched by the addition of EDTA to chelate all free Mg²⁺ and Mn²⁺ ions and then sodium orthovanadate added to inhibit tyrosine phosphases and PMSF to inactivate any remaining proteases. After binding, the immune complexes were washed several times in lysis buffer and then subjected to in vitro kinase assays which were analysed by autoradiography of SDS-PAGE gels, and assayed for associated PI 3-kinase activity. The results in Figure 6.11C show the wild type p85a protein can associate with p110 (lane 2) resulting in a significant elevation of PI 3-kinase activity over background (lane 1). The wild type p85α alone (Figure 6.11B, lane 1) and in complex with p110 (Figure 6.11B, lane 2) were both found to associate with the activated EGF receptor. Comparison with NNp85α and CCp85α reveals similar results. Both the NNp85 α and CCp85 α are able to bind p110 (Figure 6.11C, lanes 4 and 6) indicating that the SH2 domain of p85 does not influence the binding of p110. In addition, both NNp85α and CCp85α either alone or in complex with p110 are able to bind the phosphorylated EGF receptor (Figure 6.11B lanes 3-6).

6.3 Discussion

6.3A.1 Interaction of PI 3-kinase with the CSF-1 receptor and other members of the type III subfamily of protein tyrosine kinases

The recombinant p85α/p110 complex is here shown to bind the CSF-1 receptor, both in vivo and in vitro. This binding has also been observed in mammalian cells where PI 3-kinase was found to bind the activated CSF-1 receptor following 5 minutes ligand stimulation of a murine macrophage cell line and Balb 3T3 cells (Vartcovski et al., 1989). The association was concomitant with a 70% and 300% increase in the levels of cellular PI(3,4)P2 and PI(3,4,5)P3 respectively (Varticovski et al., 1989). The affinity of the interaction was similar to that observed with the highly characterised PDGF receptor (Shurtleff et al., 1990 and Chapter 7). Similarly, the v-Fms oncoprotein was also found to be physically associated with and caused activation of PI 3-kinase (Kaplan et al., 1987; Varticovski et al., 1989).

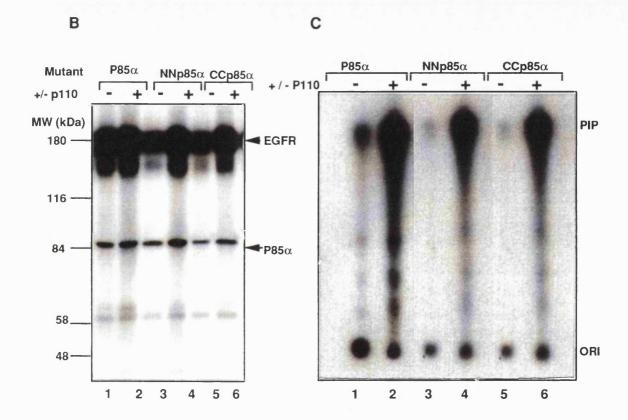


Figure 6.11: The EGF receptor can bind PI 3-kinase by its N- and the C- Terminal SH2 domains.

(A). Schematic showing the three p85 constructs.

(B). The wild type p85 (lanes 1 and 2), p85 containing two N-terminal SH2 domains (NNp85α) (lanes 3 and 4) and p85 containing two C-terminal SH2 domains (CCp85α) (lanes 5 and 6) were translated from mRNA in reticulocytes. The translated proteins were then immunoprecipitated from reticulolysates using anti-SH3 monoclonal antibodies. These immunoprecipitates were then either incubated with lysates of Sf9 cells that had been infected with 110 baculovirus (lanes 2, 4 and 6), or with lysis buffer (lanes 1, 3 and 5). After washing, these immune complexes were incubated with lysates of Sf9 cells that had been infected with EGF receptor expressing baculovirus pretreated with ATP, Mg2+ and Mn2+. Immune complexes were then subjected to protein kinase assay. Phosphorylated proteins were detected by autoradiography of SDS-PAGE gels.

(C). Immune complexes treated as described in (B) were assayed for associated PI 3-kinase activity.

The interaction between the recombinant PI 3-kinase expressed in baculovirus and the CSF-1 receptor was here found to require phosphorylation of the receptor. Indeed, a tyrosine kinase defective CSF-1 receptor has been shown to lack associated PI 3-kinase activity in immune complexes recovered from CSF-1 stimulated cells (Shurtleff et al., 1990). Further, this kinase negative receptor was found to associate with PI 3-kinase activity when phosphorylated in trans by a wild-type active receptor demonstrating that tyrosine phosphorylation of the receptor alone is sufficient to normalise binding to the inactive CSF-1 receptor (Shurtleff et al., 1990). Consistent with these analyses, constitutively active CSF-1 receptor containing a mutation at codon 301 (See chapter 1, section 1.1.1) associated with PI 3-kinase in the absence of CSF-1 stimulation and its activitiy was further enhanced with respect to wild type CSF-1 receptor in ligand stimulated cells (Shurtleff et al., 1990). Using deletion mutants, the site of interaction has been localised to the kinase insert of the CSF-1 receptor (Shurtleff et al., 1990; Reedjik et al., 1990; Choudhury et al., 1991). Antibodies that recognised this region were able to block the interaction with PI 3-kinase (Downing et al., 1991). In addition, a fusion protein of the isolated kinase-insert region phosphorylated on tyrosine was demonstrated to independently bind PI 3-kinase activity in vitro (Reedjik et al., 1992). The analyses revealing the requirement of a tyrosine phosphorylated CSF-1 receptor culminated with the identification of the in vivo phosphorylation site Y721, in the kinase insert region, as the specific site for PI 3-kinase binding (Reedjik et al., 1992).

Other PTK receptors within this subclass have also been observed to interact with PI 3-kinase. The kit receptor has been demonstrated to associate with PI 3-kinase both in stimulated cells and using isolated components (Lev et al., 1992; Rottapel et al., 1991; Reith et al., 1991; Herbst et al., 1992; Shearman et al., 1993). The interaction is again shown to be mediated by the kinase-insert region; as determined both by the use of deletion mutants (Lev et al., 1992) and by using a bacterial fusion protein containing this region (Rottapel et al., 1991). Likewise the kinase-insert of the PDGF α and β receptors was demonstrated to be responsible for its interaction with PI 3-kinase (Yu et al., 1991; Heiderahan et al., 1991; Coughlin et al., 1989; Severinson et al., 1990). Tyrosine residues 740 and 751 in the human PDGF β and analogous residues, Y731 and Y742, in the PDGF α have been mapped as specific binding sites for the PI 3-kinase (Yu et al., 1991; Kashishian et al., 1992). The interactions of PI 3-kinase and the PDGF β receptor are well documented and discussed more thoroughly in Chapter 8.

6.3A.2 Definition of a consensus PI 3-kinase binding sequence on protein tyrosine kinases

Examination of the PI 3-kinase binding sequences on receptor protein tyrosine kinases revealed they can all be described by the recognition motif pYXXM (Cantley et al., 1991). The tyrosine residue is known to be absolutely required for mediating the interaction with SH2 domains. The importance of the conserved methionine residue has been demonstrated experimentally where a PDGF receptor containing mutations at residues equivalent to M743 and M754 failed to associate with PI 3-kinase when expressed in COS-7 cells (Fantl et al., 1992; Shoelson et al., 1992). Further evidence in support of the YXXM motif as a recognition motif for PI 3-kinase binding came from the screening of a random peptide library with the SH2 domains of p85. Using this method, the YMXM peptide sequence was predicted as that which denoted the highest affinity of PI 3-kinase binding to activated receptors (Sonyang et al., 1993).

6.3A.3 The EGF receptor binds PI 3-kinase with lower affinity than the CSF-1 receptor.

The interactions of the PI 3-kinase with the EGF receptor are only poorly defined. The EGF receptor has been demonstated to associate with PI 3-kinase activity following ligand stimulation (Bjorge et al., 1990; Miller and Ascoli, 1990; Raffioni and Bradshaw, 1992). Further, an increase in the levels of PI(3,4)P2 and PI(3,4,5)P3 have been observed in cells following ligand stimulation (Carter and Downes et al., 1992; Soltoff et al., 1992; Pignataro and Ascoli, 1990). As mentioned above PI 3-kinase has been shown to bind with high affinity to the recognition sequence predicted as YXXM (Cantley et al., 1991). The EGF receptor contains a motif of this type in the kinase domain however, this has not been shown to be a major autophosphorylation site for the receptor (Downward et al., 1984; Hsuan et al., 1990). The results presented in this study clearly show that neither the p85 α protein nor the recombinant PI 3-kinase complex bind the YXXM sequence on the EGF receptor, but instead bind to the C-terminal tail. Indeed, Skolnik et al. (1991) cloned the human p85 α from an expression library using a highly phosphorylated C-terminal tail fragment of the EGF receptor.

The observed affinity of PI 3-kinase for the phosphorylated EGF receptor was found to be lower than that for the CSF-1 receptor both in vivo and in vitro. These data correlate with the known differences between the two receptors observed for stimulation of or association with PI 3-kinase activity in various cell types. The PDGF receptor associates with PI 3-kinase when present at low physiological levels (10-100³/cell) in a large variety of PDGF responding cell types (Kaplan et al., 1987; Auger et al., 1989; Coughlin et al., 1989). In contrast, stimulation by EGF has been observed in a few cell lines which share the common features of overexpressed EGF receptors (> 10⁶/cell) (Bjorge et al., 1990).

In addition, Hu et al (1992), have demonstrated that a greater amount of PI 3-kinase activity was immunoprecipitated from cells exposed to PDGF than those exposed to EGF, although the cells contained eight- to ten-fold more EGF receptors than PDGF receptors. This lower affinity association of the EGF receptor with PI 3-kinase is also reflected in the ability of EGF to elevate the levels of [32P] PI(3,4,5)P3 in Swiss 3T3 cells in comparison to PDGF (Jackson et al., 1992). Since it is known that the PI 3-kinase is not binding the YXXM on the EGF receptor, it can be presumed that association with any other tyrosine containing sequence will result in a lower affinity interaction (Songyang et al, 1993). It is also of interest that ErbB3 was shown to bind PI 3-kinase with affinities similar to that observed for the PDGF receptor (Fedi et al., 1994). Indeed, ErbB3 has six potential PI 3-kinase binds, including two of which have been predicted as autophosphorylation sites (Sonyang et al., 1993). Further, the EGF and Erb3 receptors may form heterodimers (Schlessinger and Ullrich, 1992). This might provide a basis for the potent activation of PI 3-kinase by EGF in some cells. Thus it may be surmised that the EGF receptor does bind PI 3-kinase in most cell lines examined and that the interaction is of lower affinity than that of the CSF-1 or PDGF receptors with PI 3-kinase.

These data do not, however, explain the complete lack of binding exhibited by the partially purified bovine brain preparation of PI 3-kinase to the EGF receptor. Preliminary evidence using a monoclonal antibody which binds to and specifically blocks the N-terminal SH2 domain of p85 α suggests that the association of the p85 α protein with the EGF receptor occurs via the N-terminal SH2 domain with very little, if any, role played by the C-terminal SH2 domain (End et al., 1993). However, as the CCp85α/p110 complex was seen to bind the EGF receptor as well as the NNp85α/p110 complex and both isolated N- and C- terminal SH2 domains of p85α bound the EGF receptor, this suggests that it is not the N-terminal SH2 domain which defines the specificity of interaction, but rather steric hindrance preventing the C-terminal tail of the EGF receptor having access to the 'distal' positioned SH2 (be it NSH2 or CSH2). Thus if the Nterminal SH2 domain of the partially purified bovine brain preparation is blocked by association with another protein or modified by phosphorylation (Kavenaugh et al., 1992), it is unavailable for binding to the C-terminal tail of the EGF receptor. In this context it is worth noting that Y580 has been mapped as an in vivo phosphorylation site in the N-terminal SH2 of p85 (Hayashi et al., 1993) and the tyrosine phosphorylated Nterminal SH2 domain is unable to bind the PDGF receptor (Kavenaugh et al., 1992). Thus, a possible explanation is as follows; the kinase insert region of both the CSF-1 and the PDGF receptors can access both the central and distal positioned SH2 domains of p85α (See Figure 6.12), and thus bind the bovine brain partially purified PI 3-kinase preparation. However, if the EGF receptor is sterically unable to access the the distal SH2 domain by nature of the conformation of the EGF receptor C-terminal tail and/or the

three dimentional structure of the p85/p110 complex, and can only bind the central SH2 domain (be it NSH2 or CSH2), any modification to the central SH2 domain will inhibit binding of the receptor to PI 3-kinase. This model is described in Figure 6.12.

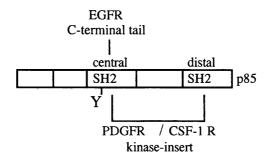


Figure 6.12. Schematic model for the mechanism of PI 3-kinase binding the EGF and the CSF-1/PDGF receptors.

6.3.A.4 The HGF receptor and PI 3-kinase.

The HGF receptor, which has been shown to bind the p85 α subunit with high affinity is also observed to bind the p85/p110 complex but with a much reduced affinity both in vivo and in vitro (Graziani et al., 1991; Bardelli et al., 1992). However, amino acid sequence analysis reveals the HGF receptor contains the Y₁₃₁₃MXM recognition motif and synthetic phosphopeptides containing this sequence and surrounding residues are found to bind PI 3-kinase in vitro (Ponzetto et al., 1993). Further, the affinity of this interaction is shown to be in the nM range, equivalent to that observed using Y740 and Y751 containing phosphopeptides derived from the PDGF receptor. (Panayotou et al., 1992; Felder et al., 1992). However, upon closer inspection it is found that Y1313 on the HGF receptor is not phosphorylated in vivo and mutation of this residue does not effect the binding of PI 3-kinase (Ponzetto et al., 1993). These data highlight the hazards of using sequence data alone to determine PI 3-kinase binding sites. Subsequently, using a combination of competition experiments carried out with synthetic phosphopeptides and association experiments using Tyr-Phe mutants, Y1349 and Y1356, contained within a YVXV motif, were identified as in vivo binding sites for the PI 3-kinase (Ponzetto et al., 1993). In addition, the affinity of interaction of these phosphopeptides with PI 3-kinase was shown to be two orders of magnitude lower than that observed using phosphopeptides containing sequences surrounding Y740 and Y751 which is the PI 3kinase binding site on the PDGF receptor (Ponzetto et al., 1993; Fantl et al., 1992). Thus, these data suggest that in some cases PI 3-kinase will bind to sequences that do not conform to the YMXM canonical consensus sequence, however they do so with lower affinity. It should be noted that Y1173 on the EGF receptor conforms to the YXXV motif on the HGF receptor, which may in part, be responsible for mediating the observed lower affinity interaction of receptor with PI 3-kinase.

6.3A.5 Study of the interaction of PI 3-kinase with the NGF and FGF receptors

PI 3-kinase has also been shown to coprecipitate, using antiphosphotyrosine antibodies, with the activated NGF receptor from stimulated PC12 cells (Carter and Downes, 1992; Raffioni and Bradshaw, 1992; Soltoff et al., 1992). The interaction is demonstrated to be of 8-fold lower affinity than that with the activated EGF receptor in the same cells (Carter and Downes, 1992; Obermeier et al., 1993). Further, the amount of PI 3-kinase activity immunoprecipitated with the activated receptor paralleled increases in the levels of cellular PI(3,4,5)P3 (Carter and Downes, 1992; Soltoff et al., 1992). In a similar analysis bFGF was found to elicit activation of PI 3-kinase activity and association with the FGF receptor (Raffioni and Bradshaw, 1992; Jackson et al., 1992), albeit with lower affinity than that observed with the NGF receptor (Raffioni and Bradshaw, 1992). Using phosphotyrosine-containing synthetic peptides and with NGF receptors containing specific point mutations, Y751 was identified a site binding site for p85 (Obermeier et al., 1993). However, there appears to be evidence that suggests that the NGF receptor may activate an 100-110 kDa phosphoprotein which acts as an adapter to mediate the interaction between the NGF receptor and PI 3-kinase (Ohmichi et al., 1991). As the p85 protein has been shown to exhibit a distinct binding specificity to the p85/p110 complex, association studies using Y751 mutated NGF receptor with PI 3-kinase rather than p85 alone will allow the problem to be resolved.

6.3A.6 An adapter protein mediates the interaction of the Insulin receptor with PI 3-kinase.

Most of the receptor PTKs described so far appear to interact directly with the PI 3kinase, however, this is not the case with the insulin and IGF receptors. Initial studies demonstrated that PI 3-kinase activity could be immunoprecipitated with antiphosphotyrosine antibodies following insulin stimulation of CHO cells (Ruderman et al., 1990; Endemann 1990). This was shown to be concomitant with an increase in the levels of PI(3,4)P₂ and PI(3,4,5)P₃ (Ruderman et al., 1990; Kelly and Ruderman, 1993). However, only 3-10% of PI 3-kinase activity was recovered in anti-insulin receptor immunoprecipitates from the same cell lysates (Ruderman et al., 1990; Endemann 1990; Backer et al., 1992a; Backer et al., 1993; Zhang et al., 1993). In addition, the insulin receptor contains a single Y₁₃₂₂XXM motif in the C-terminal region; deletion of which does not affect ligand induced activation of PI 3-kinase (Backer et al., 1992a; Yonezawa et al., 1992a). Further, treatment of insulin-stimulated cells with bifunctional cross linkers has been found to cause a significant increase in the amounts of PI 3-kinase activity found associated with the insulin receptor (Yonezawa et al., 1991). These results suggest that in contrast to the other receptor protein tyrosine kinases as discussed above, a linker protein may mediate the association of PI 3-kinase with the receptor. Consistent with this suggestion PI 3-kinase has been found to associate strongly with a protein called

IRS-1 in insulin stimulated cells (Sun et al., 1991; Backer et al., 1992a). Tyrosyl phosphorylation of IRS-1 has been shown to be required for it to bind PI 3-kinase in vitro (Backer et al., 1992; Myers et al., 1992; Giorgetti et al., 1993). Further, the activation of PI 3-kinase was reduced in cells expressing mutant receptors that phosphorylate IRS-1 poorly (Yonezawa et al., 1992a; Kapeller et al., 1991; Backer et al., 1992a; 1993). Indeed, the levels of insulin stimulated PI 3-kinase activity has been shown to parallel tyrosine phosphorylation of IRS-1 (Backer et al., 1993; Kelly and Ruderman, 1993). In addition, overexpression of IRS-1 in cells has been shown to result in an increased PI 3-kinase activation (Backer et al., 1992a; Backer et al., 1993).

The IRS-1 protein contains 9 putative PI 3-kinase binding sites, six of these tyrosyl residues are located in YMXM motifs and three are in YXXM motifs. Synthetic peptides based on the sequence of these of these YXXM motifs all act as good substrates for the insulin receptor (Shoelson et al., 1992) and bind PI 3-kinase with similar affinities. In addition, these synthetic peptides inhibit the formation of the PI 3-kinase-IRS-1 complex in vitro (Yonezawa et al., 1992; Myers et al., 1992; Backer et al., 1992). Moreover, peptides containing either Y740, Y751 and flanking sequences of the PI 3-kinase binding site on the PDGF receptor or residues surrounding Y315 from the mT antigen are able to block the interaction of PI 3-kinase with IRS-1 in vitro (Backer et al., 1992). This highlights the similarity in srtucture of regions from different signalling proteins that bind the SH2 domains of PI 3-kinase, and also suggests that only a small region on both the PI 3-kinase and IRS-1 protein are actually involved in this specific interaction between the two proteins. Analysis of the phosphorylated IRS-1 has made it possible to identify Y608 and Y939 as in vivo phosphorylation sites, both contained in the YMXM motif, which preferentially bind SH2 domains of p85 with high affinity (Sun et al, 1993). The IGF receptor has also been observed to activate PI 3-kinase (Kapeller et al., 1991; Giorgetti et al., 1993), and again this interaction is mediated by the IRS-1 protein (Giorgetti et al., 1993). The necessity for the presence of an adapter protein in insulin stimulated metabolic responses is unclear, although it should be noted that insulin stimulation of cells results in the translocation of ~50% of the total pool of cytosolic PI 3-kinase to the plasma membrane and is presumambly a reflection on the number of phosphorylation sites available for binding of PI 3-kinase on the IRS-1 protein as opposed to those on the insulin receptor itself. (Backer et al. 1993; Folli et al., 1992). This is in sharp contrast to translocation to the membrane of ~5% of the total pool of cytosolic PI 3-kinase in PDGF and other ligand stimulated cells (Discussed in further detail in Chapter 8). It remains to be determined whether PI 3-kinase plays a greater role in insulin stimulated rather than PDGF stimulated metabolic responses but the employment of a distinct adapter protein by a phosphorylated protein tyrosine kinase as a docking site for signalling molecules adds another dimension to the regulation of signalling responses by insulin.

6.3A.8 Non-receptor protein tyrosine kinases utilise an alternative method for interacting with PI 3-kinase.

The activated non-receptor PTK pp59c-Fyn is here shown to bind the p85 proteins (Chapter 3), but is unable to bind both the recombinant PI 3-kinase and the bovine purified PI 3-kinase. This suggests that the p110 protein may be modulating and influencing binding specificity, perhaps through steric effects, indicating that physiologically, there exist constraints other than the availability of an appropriate tyrosine phosphorylated residue in a suitable primary sequence context. In support of this concept, the p85 α protein has been shown to bind directly and with high affinity to the insulin receptor in vitro (Yonezawa et al., 1992; Hayashi et al., 1993). However, a much lower affinity interaction is observed for PI 3-kinase and the insulin receptor in vivo (Endemann et al., 1990; Ruderman et al., 1990) and in vitro (Horn et al., 1994). Similarly, the p85 α protein and PI 3-kinase displayed differential affinities for binding a panel of tyrosine phosphorylated peptides (Fry et al., 1992).

Recently published results suggest that non-receptor protein tyrosine kinases may be binding the PI 3-kinase through their SH3 domains. Wages et al. (1992) have demonstrated that mutations in the SH3 domain of pp60^{V-Src} reduce its association with PI 3-kinase. More direct evidence has shown that the SH3 domains of pp59^{C-Fyn} and pp60^{V-Src} bind PI 3-kinase activity from T cells and non-transformed cell lines (Plieman et al., 1993; Liu et al, 1993; Prasad et al., 1993). Further, the interaction between the SH3 domain of the non-receptor PTK and PI 3-kinase has been shown to be concomitant with activation of the PI 3-kinase, analogous to the manner receptor PTKs activate the SH2 domain of p85 (Plieman et al., 1994; Carpenter et al., 1993a). It will be interesting to examine if a non-receptor protein tyrosine kinase SH3 domain is able to induce a conformational change in the p85 protein which may thus induce activation of the PI 3-kinase.

The binding site on the p85α protein for non-receptor protein tyrosine kinase SH3 domains has been identified as the proline rich region flanking the Bcr domain (Liu et al., 1993; Kapeller et al., 1994; Pleiman et al., 1994). Studies using the baculovirus expressed p85α protein suggest that its SH3 domain is bound in an intermolecular manner to its proline-rich region; hence the p85α-SH3 domain binding site is unavailable for interaction with exogenous SH3 domain-containing proteins (Gout et al., 1993). This would explain why once the p85α protein is in complex with the p110, pp59c-Fyn is only sterically able to bind PI 3-kinase by its SH3 domain, and thus in our case the interaction with the recombinant material is unable to take place. In a cellular situation, a third protein may be required to unlock the PI 3-kinase. It was interesting that the association of PI 3-kinase from lysates of pp60v-Src transformed cells was mediated by the SH2

domain of pp56^{c-lck} binding a phosphotyrosine residue on PI 3-kinase (Vogel and Fujita., 1993). This suggests that there exist differentially modified forms of PI 3-kinase. A tyrosine phosphorylated form of PI 3-kinase may preferentially bind with high affinity to the SH2 domain of a non-receptor kinase, whereas a non-phosphorylated form may bind to the SH3 domain of Src-family kinase. Utilisation of these distinct domains for interaction of the PI 3-kinase, presumably results in the activation of distinct effectors.

The Src family of non-receptor kinases have also been observed to interact with PI 3-kinase in an indirect manner, similar to the adapter mechanism utilised by the insulin receptor. In cells transformed by polyoma virus, the polyoma middle T antigen (mT) forms a complex with the activated protein-tyrosine kinase pp60c-Src. This results in tyrosine phosphorylation of mT and leads to complex formation with PI 3-kinase (Talmage et al., 1989). The mT/pp60c-Src complex has been demonstrated to bind both p85α (Otsu et al., 1991) and purified PI 3-kinase in vitro (Auger et al., 1992). Such complex formation has been shown to require an active Src kinase as although an inactive pp60c-Src will associate with mT, an interaction with PI 3-kinase was not observed (Auger et al., 1992). The major binding site for PI 3-kinase on mT has been identified as Y315 and flanking residues which lie within the sequence motif YXXM (Talmage et al., 1990). In addition it should be noted that phosphopeptides based on residues around this region are observed to block the interactions between mT/pp60c-Src and the SH2 domains of p85 in competition experiments (Yoakim et al., 1992).

6.3B.1 The p85 β /p110 PI 3-kinase complex

The recombinant p85 β /p110 complex exhibits identical binding specificities as the recombinant p85 α /p110 complex. Indeed, both the p85 α and the p85 β proteins are shown to compete for binding to the CSF-1 receptor. Whether this is a true reflection of what is happening in cells remains to be examined in further detail. It should be noted that activation of T-cells via the T-cell receptor (TCR)/CD3 complex is found to cause differential phosphorylation of the p85 α and the p85 β proteins (Reif et al., 1993), revealing the potential for divergent regulation and function of these two PI 3-kinase isoforms.

6.3B.2 The SH2 domains of p85 are required to mediate the interaction of the catalytic subunit of PI 3-kinase with activated receptors.

The work described here shows that although the p110 subunit did possess PI 3-kinase activity when expressed alone in insect cells it required either the p85 α or the p85 β protein to mediate its binding to receptors. These observations reinforce the requirement for two subunits to regulate PI 3-kinase. The ability of the recombinant baculovirus

expressed p85 proteins to block the binding of PI 3-kinase to the CSF-1 receptor demonstrated that both the p85 α and the p85 β proteins bound a similar region of the receptor (Reedijk et al., 1992). A similar observation was made by Escobedo et al, (1991a), who demonstrated that the murine p85 α homologue could compete with PI 3-kinase activity in NIH 3T3 lysates for binding to the PDGF β receptor phosphorylated in vitro. Reconstitution analyses of the receptor-p85 α /p110 complex revealed that p110 is able to bind a receptor-p85 α complex and tyrosine phosphorylation of the p85 α protein does not affect the binding of p110. This provides preliminary evidence that the p110 protein does have the ability to undergo the biological functions inherent of the cellular PI 3-kinase, if present in the cell in a free, uncomplexed form.

In Chapter 3, I showed that the SH2 domains of p85 bound receptor protein tyrosine kinases as independent protein modules. In this Chapter these studies are extended to show that both the N- and the C-terminal SH2 domains of p85 were able to effectively block the binding of PI 3-kinase activity to the CSF-1 receptor and both SH2 domains efficiently mediated the binding of PI 3-kinase activity with the EGF receptor. Likewise, the C-terminal SH2 domain of p85 α has been shown to block the binding of full-length p85α and PI 3-kinase to the phosphorylated PDGF receptor (Klippel et al., 1992). These data provide further evidence in support of the concept that the SH2 domains of p85a mediate the binding of PI 3-kinase to receptors, and that the p85 protein acts as an adapter subunit required to bring p110 and hence PI 3-kinase association with activated PTKs. In the studies carried out here both SH2 domains are observed to function efficiently using these particular assay methods. However, other methods of analysis employed to measure reported receptor interactions with the recombinant N- and C- terminal SH2 domains of p85\alpha have shown that while both SH2 domains are capable of binding to sequences containing the YXXM motif, in particular Y740 and Y751 of the PDGF receptor, the two domains show distinct specificities for such related sequences. Thus the N-terminal SH2 domain of p85 binds with high affinity to Y751 but not Y740 while the C-terminal SH2 binds strongly to both sites (Panayotou et al., 1993a). Klippel et al. (1992) have also presented data suggesting that a high affinity interaction between the PDGF receptor and the p85α subunit is maintained by the C-terminal SH2 domain. Likewise, the C-terminal SH2 domain of p85α was consistently shown to complex more efficiently with the phosphorylated kinase-insert region of the CSF-1 receptor (Reedjik et al., 1992), a phosphopeptide containing both PI 3-kinase binding sites, Y1349 and Y1356, on the HGF receptor, and mT (Yoakim et al., 1992). Consistent with the notion that the two SH2 domains of p85 behave as independent modules it is of note that the N-terminal SH2 domains shows only 37% sequence identity with the C-terminal SH2. Furthermore, both SH2 domains have been found to interact with distinct sets of proteins when used as affinity probes to bind ³⁵S-labelled proteins (Yoakim et al., 1992). In addition it has been shown that the C-terminal SH2 domain of p85 specifically blocked PI 3-kinase activation in oocytes, while the N-terminal SH2 domain had no effect (Muslin et al., 1993). This suggests that both SH2 domains may have inherent the property to function independently with distinct effectors in different cell lines.

There is also considerable debate that as well as functioning as distinct modules, the two SH2 domains of p85 act in concert to produce a synergistic effect. Examples for this model have been observed where a fusion protein containing both the N-and the Cterminal SH2 domains of P85a was found to bind the phosphorylated CSF-1 receptor kinase-insert with greater affinity than did either SH2 domain alone (Reedjik et al., 1992). Consistent with this observation, PI 3-kinase was found to bind a phosphopeptide containing the two PI 3-kinase binding sites on the PDGF receptor Y740 and Y751, with much higher affinity than singly phosphorylated peptides (Kazlauskas and Cooper, 1992). Moreover, Carpenter et al, (1993) have shown that the addition of a peptide containing pY740 and pY751 causes significant activation of the PI 3-kinase, however a peptide phosphorylated at only a single site, either Y740 or Y751, does not produce a similar effect. Similar results were observed using peptides containing Y315 and Y322 from the polyoma virus middle T antigen (Carpenter et al, 1993), and Y1349 and Y1356 of the HGF receptor (Ponzetto et al., 1993). Taken together these results suggest that while both SH2 domains of p85 are initially required for high affinity receptor interaction and activation of PI 3-kinase in stimulated cells, there remains the possibility that one of the SH2 domains may then be released from this association leaving it free to bind and thus activate distinct effectors.

6.3C Conclusion.

Structural analysis of the PI 3-kinase has been used to define a heterodimeric enzyme which consists of a multidomain 85 kDa adapter subunit and a 110 kDa catalytic subunit. Although the p110 subunit is sufficient for catalysis, the p85 subunit is a key component required for binding of the enzyme to receptors. The p110 protein is seen to influence the binding specificity of the p85 protein, a process affected perhaps by steric effects or mediated by a physically induced change in the conformation of the p85 protein. Indeed, as the inter-SH2 domain of p85 is observed to bind the catalytic subunit, it is not difficult to imagine how the binding of p110 may influence the binding of the p85 SH2 domains to activated receptors and also result in the activation of PI 3-kinase. The CSF-1 and EGF receptors and the non-receptor kinase Fyn exhibit differential binding affinities and distinct mechanisms of activation for the PI 3-kinase complex. A hierarchial activation of PI 3-kinase activity, by a number of receptors ie. PDGF>IGF-1>EGF>FGF receptor, was also noted in Swiss 3T3 cells (Jackson et al. 1992). This variability in PI 3-kinase activation may be important to influence downstream effectors and thus stimulate

different biological responses (Discussed in Chapter 9). The structure of PI 3-kinase has been probed in detail however, very little is known of the mechanism for its regulation. The following Chapters will address this issue.

Chapter 7: PI 3-kinase is a dual specificity enzyme: Autoregulation by an intrinsic protein-serine kinase activity.

7.1 Introduction

While structural analysis of the p85 and p110 subunits has proceeded at a rapid pace, the basis for regulation of the PI 3-kinase enzymatic activity remains poorly understood. Several possible modes of regulation in vivo have been suggested (reviewed in Panayotou et al., 1992a). Since the p85 subunit binds receptors through its SH2 domains, regulation of the enzymatic activity intrinsic to the associated catalytic subunit could result from physical translocation of the enzyme to the membrane permitting access to substrate. Alternatively, interactions between the subunits which result in the activation of the PI 3kinase activity intrinsic to the p110 subunit may take place. For example changes induced in the p85 protein which occur upon its binding to protein tyrosine kinases which are then transmitted to the associated catalytic domain may also result in enzymatic activation. Phosphorylation of the p85 or the p110 subunits on tyrosine, serine or threonine residues might also regulate kinase activity. PI 3-kinase was originally identified as a phosphotyrosyl protein (Kaplan et al., 1987; Courtneidge and Heber., 1987) immunoprecipitated from quiescent cells. Subsequently it was shown to be phosphorylated on both serine and threonine residues (Kaplan et al., 1987; Cohen et al., 1990; Reif et al., 1993). At the start of the work described in this Thesis, bovine brain PI 3-kinase had been shown to copurify with an associated protein-serine kinase activity (R. Dhand, F. Ruiz-Larrea, M. Fry; unpublished data). A report from Carpenter et al. (1993a) was published that showed that a similar activity, which he termed PIK kinase, copurified with and might regulate rat liver PI 3-kinase.

7.1.1 Aim

To further investigate the nature of the associated protein-serine kinase and its possible role in the regulation of the PI 3-kinase, the wild type and mutant recombinant PI 3-kinase subunits have been used alone or in a complex to show that the p110 subunit of PI 3-kinase has both an intrinsic phosphoinositide kinase activity and a protein-serine kinase activity with unique specificity for p85. A high stoichiometry of phosphorylation of p85 by this activity has been established and shown to take place on a single serine residue on the p85 protein. Phosphorylation of this specific serine residue is then shown to regulate the PI 3-kinase activity. The role of such a dual specificity kinase in signal transduction processes is discussed.

7.2 Results

7.2.1 Recombinant PI 3-kinase expressed in Sf9 cells has an associated protein kinase activity

A protein-serine/threonine kinase activity has been reported in immunoprecipitates of PI 3kinase (Carpenter et al., 1993a; Reif et al., 1993). However, it has remained unclear from these studies whether this activity represented a tightly bound cellular enzyme, or whether the activity was intrinsic to a component of the PI 3-kinase complex. The process of cDNA cloning and subsequent expression of the two subunits of the enzyme has allowed a more detailed investigation of the associated protin-serine kinase. Insect cells (Sf9) were infected with baculoviruses which mediated expression of either p85 α or p85 β alone, or were coinfected with a virus which expressed p110. The p85 α /p110 and the p85 β /p110 complexes were then either immunoprecipitated with antibodies directed against the p85 or p110 subunit, or the complexes were bound to a Y751 phosphopeptide affinity column (Otsu et al., 1991; Fry et al., 1992). The bound proteins were then used in protein kinase assays. The results shown in Figure 7.1A demonstrated that in the absence of p110, neither p85 α nor p85 β could be phosphorylated in vitro (lanes 5 and 6). However, when the insect cells were coinfected with viruses expressing p110, both the p85 α and the p85 β proteins were found to become phosphorylated as analysed following immunoprecipitation using antibodies directed to either subunit of the complex (Figure 7.1A, lanes 7-8 and 10-11), or after binding to the phosphopeptide column (Figure 7.1A, lanes 9 and 12). The p110 protein was however not phosphorylated significantly under these conditions and no other phosphorylated proteins were detected in precipitations with either antibodies or with the phosphopeptide affinity beads from control infected cells (Figure 7.1A, lanes 1-4). Analysis of the metal ion requirements of this kinase activity showed it to be completely dependent on the presence of Mn^{2+} (Figure 7.1Bb, lane 2).

The substrate specificity of the protein kinase activity associated with the PI 3-kinase complex from Sf9 cells infected with recombinant baculoviruses was then investigated further. Phosphoamino acid analysis of the PI 3-kinase complex affinity purified using Y751 phosphopeptide and then phosphorylated in vitro was performed exactly as described above. The results shown in Figure 7.2A revealed that the p85 α subunit contained exclusively phosphoserine. The level of phosphate incorporated in the p110 subunit in vitro under identical conditions was too low to allow phosphoamino acid analysis. To examine the phosphorylation state of the PI 3-kinase complex in vivo, insect cells were coinfected with p85 α and p110 expressing baculoviruses and metabolically labelled with $^{32}P-PO_4$. Lysates of these cells were bound to the Y751 phosphopeptide beads which were then washed and SDS buffer eluted proteins visualised by Coomassie Blue staining

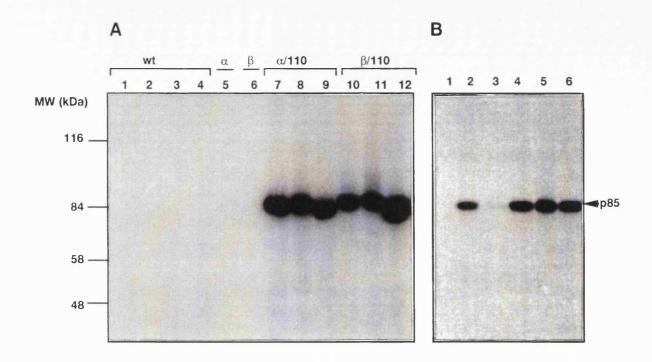


Figure 7.1. Both p85 α and p85 β are substrates for a Mn2+ dependent protein kinase activity.

(A) Sf9 cells were infected with wild type (wt) virus (lanes 1-4); p85 α (lane 5); p85b (lane 6); or were coinfected with p85 α /p110 viruses (lanes 7, 8, and 9) or with p85 β /p110 viruses (lanes 10, 11 and 12). Lysates of these cells were precipitated using polyclonal, affinity purified antibodies raised against p85 α (lanes 1 and 7); monoclonal antibodies raised against p85 α (lanes 2 and 10); polyclonal, affinity purified antibodies raised against p110 (lanes 3, 8 and 11) or bound to an immobilised Y751 phosphopeptide (lanes 4, 5, 6, 9, and 12). Samples were subjected to in vitro protein kinase assays and analysed by SDS-PAGE and autoradiography.

(B) Lysates of insect cells coinfected with p85 α /p110 viruses were immunoprecipitated using anti-p85 polyclonal antibodies. Samples were then subjected to protein kinase assays in the presence of 0 mM Mn2+/0 mM Mg2+ (lane 1), 2 mM Mn2+/12 mM Mg2+ (lane 2), 0 mM Mn2+/10 mM Mg2+ (lane 3), 5 mM Mn2+/5 mM Mg2+ (lane 4), 10 mM Mn2+/0 mM Mg2+ (lane 5), 12 mM Mn2+/2 mM Mg2+ (lane 6), and analysed by SDS-PAGE and autoradiography.

after SDS-PAGE gel analysis. Autoradiography of these gels revealed a much higher level of phosphate incorporated into p85 α than into p110 with respect to the amount of protein present as determined by Coomassie Blue staining (Figure 7.2B, lanes 1 and 2). Phosphoamino acid analysis revealed that the p85 α contained both phosphoserine and phosphothreonine when labelling was carried out in vivo, while the p110 subunit contained only phosphoserine (Figure 7.2Ci and 7.2Cii).

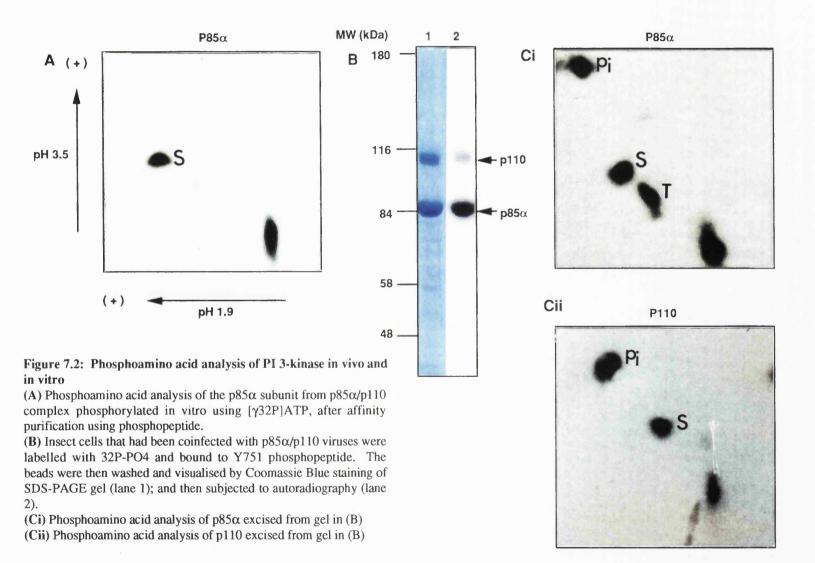
Anti-p110 immunoprecipitates of $p85\alpha/p110$ were used to study the kinetics of phosphorylation. The Km (ATP) for the phosphorylation of $p85\alpha$ was measured as approximately 4 μ M. The stoichiometry of phosphorylation of $p85\alpha$ was then measured in the presence of excess ATP (50 μ M). Approximately 0.9 mol of phosphate was incorporated per mol of $p85\alpha$ protein.

7.2.2 The protein-serine kinase activity is intrinsic to the p110 subunit.

Several methods were used to determine whether the protein-serine kinase activity described above was intrinsic to the p110 catalytic subunit of the PI 3-kinase or due to the presence of an associated insect cell kinase activity.

7.2.2.1 Mutagenesis of p110 abolishes both kinase activities

Neither the p85 α nor p85 β protein amino acid sequences exhibit any recognisable motifs related to those found in protein kinases (Hanks et al., 1988), or to those of ATP- or to GTP-binding domains in other proteins (Saraste et al., 1990). Thus, the p110 subunit, which does have known kinase motifs and can transfer phosphate from ATP to PI, is the most likely candidate to possess an intrinsic protein-serine kinase activity. The p110 subunit contains amino acids which are conserved in the active sites of known protein kinases and the yeast PI 3-kinase, Vps34p (Hiles et al., 1992). The DRHNSN sequence is known to be essential for binding the nucleotide phosphate moieties and for phosphotransferase activity in classical protein kinases (Taylor et al., 1992). To study the functional significance of this sequence which is clearly shared between the phosphoinositide kinase and known protein kinases, site-directed oligonucleotide mutagenesis was employed to construct a point mutation which converted arginine 916 to proline (R916P) within the DRHNSN motif of bovine p110. Expression of the p110-R916P in insect cells was clearly shown to produce a PI 3-kinase with an enzymatically inactive p110 (Chapter 4, Section 4.2.2). The expression and binding capabilities of the mutant p110-R916P were commensurate with those of wild type p110, which suggested that the mutation had disrupted the catalytic site of the protein without completely disrupting the structure of the p110 protein.



This mutant p110-R916P was then employed to study the effect of this mutation on the associated protein-serine kinase activity. Insect cells were coinfected with either p85 α and wild type p110 or with p85 α and mutant p110-R916P viruses. Lysates of these cells were immunoprecipitated with antibodies directed against the p110 subunit and the immunoprecipitated proteins were then phosphorylated in vitro. The p85 α subunit was seen to be phosphorylated in vitro when in complex with wild type p110 (Figure 7.3A, lane 1). However, in sharp contrast, the p85 subunit associated with the mutant p110-R916P was not phosphorylated in this assay (Figure 7.3A, lane 2). Neither the wild type p110 nor the mutant p110-R916P alone were observed to autophosphorylate (Figure 7.3A lanes 3 and 4).

Since p110 is able to bind p85 α in vitro, as well as in vivo, the possibility that p110 could exhibit a trans-kinase activity by binding to, and phosphorylating p85 α in vitro was examined. p85\alpha from insect cells was bound to phosphopeptide beads which were washed stringently. The beads were then either incubated in lysis buffer, or with lysates of insect cells that had been infected with either wild type p110 or with mutant p110-R916P. The resultant complexes were washed, phosphorylated in vitro, then analysed by SDS-PAGE and autoradiography. The p85α protein expressed alone was not phosphorylated (Figure 7.3B lane 3), while the p85 α that has been bound in vitro to wild type p110 was heavily phosphorylated (Figure 7.3B lane 1). In contrast p85α complexed with mutant p110-R916P was not phosphorylated (Figure 7.3B lane 2). To ensure that the phosphorylation observed on the p85α was not exclusively associated with p85 expressed in the insect cell system, bacterially expressed p85 α as a glutathione S-transferase (GST) fusion protein was employed as an alternative source of this protein. The experiment was then carried out as described above using this material. Following binding, in vitro kinase assays revealed that the GSTp85α alone had no associated protein kinase activity (Figure 7.3C, lane 3), but upon association with wild type p110, this p85 α fusion protein, became heavily phosphorylated (Figure 7.3C, lane 1). Again no phosphorylation of GSTp85a bound to the mutant p110-R916P was detected (Figure 7.3C, lane 2).

7.2.2.2 No other proteins are detected in association with p85 and p110

To complement the mutagenesis data and ensure there were no other proteins associated with the PI 3-kinase complex, insect cells were infected with either wild type virus, p85α or the p85α/p110 viruses and were biosynthetically labelled with ³⁵S methionine. Lysates of these cells were then incubated with Y751 phosphopeptide beads. After several washes radiolabelled proteins released by SDS buffer were analysed by SDS-PAGE and detected by autoradiography. The results shown in Figure 7.4 do not reveal any proteins bound to the affinity phosphopeptide from insect cells infected with wild type baculovirus.

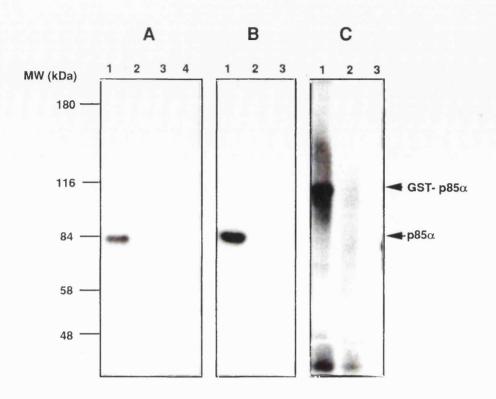


Figure 7.3. p110 possesses intrinsic protein-serine kinase activity.

(A) Insect cells were infected with viruses as follows: p85 α and p110 (lane 1); p85 α /mutant p110-R916P (lane 2); p110 alone (lane 3); mutant p110-R916P alone. Anti-p110 immunoprecipitates of these samples were then subjected to in vitro phosphorylation and analysis by autoradiography.

(B) p85α was affinity purified using the Y751 phosphopeptide column from Sf9 cells and then incubated in vitro with lysates of Sf9 cells infected with p110 (lane 1); mutant p110-R916P (lane 2); untreated (lane 3). Washed samples were then subjected to in vitro protein kinase assays and then analysed by SDS-PAGE and autoradiography.

(C) GST-p85 α was bound to affinity resin and then treated as described in (B).

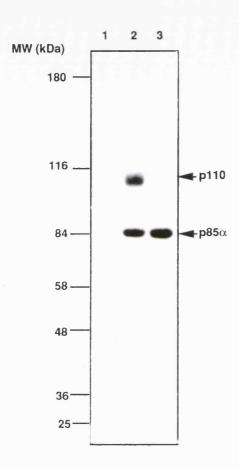


Figure 7.4. 35S-methionine labelling of the PI 3-kinase complex.

Insect cells infected with wild type virus (lane 1); p85 α and p110 viruses (lane 2); or p85 α alone (lane 3) were labelled with [35S]-methionine for 16 h. Lysates of these cells were then bound to a Y751 phosphopeptide column. After several washes radiolabelled proteins were analysed by SDS-PAGE and autoradiography.

Furthermore, from cells that had been infected with either $p85\alpha$ alone, or $p85\alpha$ together with p110, no other associated proteins were detected even after a three day autoradiographic analysis. It is thus clear that an extrinsic kinase associated with $p85\alpha$ or p110 would have to be present at a very low stoichiometry.

7.2.2.3 Both kinase activities have a similar thiol requirement

PI kinases isolated from a number of eukaryotic sources are known to be sensitive to treatment with sulphydryl-modifying reagents (Hou et al., 1988; Scholz et al., 1991), as indeed are certain other kinases (Muirhead et al., 1986; Maru and Witte, 1992). To address whether PI 3-kinase and/or the associated protein-serine kinase might have a similar thiol requirement, the p85α/p110 complex or p110 alone was immunoprecipitated from insect cells using antibodies directed against the p110 subunit. Samples were then either left untreated (Figure 7.5A, lane 1 and 7; Figure 7.5B lane 1) or were treated with 5,5'-Dithiobis[2-Nitrobenzoic acid] (Nbs₂) and analysed on SDS gels (Figure 7.5A, lanes 3-6 and 9-12; Figure 7.5B lanes 3-6). Approximately 95% of the PI 3-kinase activity was lost when the samples were incubated with 0.3 mM Nbs₂ (Figure 7.5A, lanes 3 and 9) and a similar loss of protein-serine kinase activity was also observed (Figure 7.5B, lane 3). Both catalytic activities could be restored by incubating the modified enzyme with increasing concentrations of DTT (Figure 7.5A, lanes 4-6 and 10-12; Figure 7.5B, lanes 4-6). Incubation of either the PI 3-kinase complex or p110 with DTT alone at a final concentration of 300 mM produced a slight activation of both the PI 3-kinase activity (Figure 7.5A, lanes 2 and 8) and the protein-serine kinase activity (Figure 7.5B, lane 2). Both inactivation by Nbs2, and reactivation by DTT, showed essentially identical dose response-curves for both lipid and protein kinase activities (Figure 7.5C). It is likely that the loss of activity seen here is due to the formation of a disulphide bond between an essential but as yet unidentified cysteine residue(s) and thionitrobenzoate (Nbs⁻) anion(s) in the catalytic domain .

7.2.3 Complex formation is an absolute requirement for the phosphorylation of p85 by p110

Several proteins including enolase, a mixture of histones, and casein were tested to determine whether they could act as substrates for the p110 protein-serine kinase activity. However, to date no substrates, other than the p85 proteins, have been identified (data not shown).

To ascertain whether binding of the p85 α protein to p110 was a prerequisite for it to act as a substrate for the protein-serine kinase activity, various GST-subdomain fusion proteins of p85 α (Figure 7.6A) were utilised as potential substrates for the protein-serine kinase

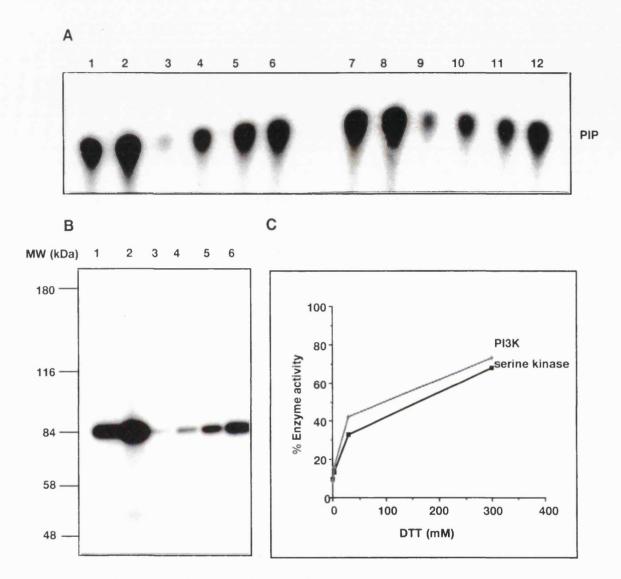


Figure 7.5. Thiol requirement for catalysis.

- (A) Insect cells were infected with p85¢/p110 viruses (lanes 1-6) or infected with p110 alone (lanes 7-12). In vitro PI 3-kinase assays were performed on anti-p110 immunoprecipitates of these cells that had been treated as follows: untreated (lanes 1 and 7); 300 mM DTT (lanes 2 and 8); treated with 0.3 mM Nbs2 (lanes 3 and 9); immunoprecipitates pretreated with 0.3 mM Nbs2 incubated with 3 mM DTT (lanes 4 and 10), 30 mM DTT (lanes 5 and 11), 300 mM DTT (lanes 6 and 12).
- (B) Lysates of Sf9 cells that had been coinfected with $p85\alpha/p110$ baculoviruses were immunoprecipitated with anti-p110 antibodies and treated as follows; untreated (lane 1); 300 mM DTT (lane 2); 0.3 mM Nbs2 (lane 3); pretreated with 0.3 mM Nbs2 and then incubated with 3mM DTT, 30 mM DTT, 300 mM DTT (lanes 4, 5, 6, respectively). Samples were then subjected to in vitro kinase assays and analysis by SDS-PAGE. Phosphorylated proteins were visualised by autoradiography.
- (C) Dose-curves for inactivation by Nbs2, and reactivation with increasing concentrations of DTT, for both kinase activities.

activity. The p110 protein was immunoprecipitated from insect cells, and then equal amounts of each GST fusion protein, or the GST protein alone, were mixed with p110 and the proteins allowed to phosphorylate in vitro. Phosphorylated proteins were analysed by SDS-PAGE and autoradiography. The inter-SH2 region of p85 α has been shown to be responsible for binding to the p110 protein (Chapter 5). In particular this interaction requires residues 478-574, since their deletion in a mutant p85 α N-C Δ 478-514 renders the protein totally unable to bind p110 or PI 3-kinase activity when compared to a similar construct without the deletion p85 α N-C. Results in Figure 7.6C show that the only proteins that were phosphorylated by p110 were those to which it has been shown to bind, that is the full-length p85 α and p85 α N-C domain which includes the N- and the C-terminal SH2 domains and the region in between. The inter-SH2 domain alone is known to bind PI 3-kinase activity but was not phosphorylated in this assay. These results could be explained if the in vitro phosphorylation sites for the kinase reside within one of the two adjacent SH2 domains or in the regions flanking the inter-SH2 domain used in this study (see below). Together these data suggest that the protein-serine kinase activity of p110 can only be detected upon high affinity binding of p110 with its specific substrate p85, and the presence of the various unbound subdomains of p85 alone are not sufficient for activation of the kinase and phosphorylation to take place.

7.2.4 Identification of the site of phosphorylation on p85 α

Phosphoamino acid analysis of the p85 α subunit which was affinity purified from Sf9 cells showed that it contained phosphoserine (Figure 7.2). However, the identification of this site(s) in mammalian cells in vivo remained to be determined. The p85 subunit, phosphorylated both in vivo and in vitro, from a variety of sources was used in experiments designed to address this issue. Phosphopeptide mapping was performed on PI 3-kinase purified, using a Y751 phosphopeptide column, from insect Sf9 and bovine SGBAF-1 cells which had been labelled with phosphate in vivo. The phosphorylation sites labelled in vitro were also examined by two methods. Firstly, using PI 3-kinase bound to a phosphopeptide column from Sf9 cells that had been coinfected with p85α and p110 viruses. As a second approach, bacterially expressed GSTp85 α and GSTp85 α N-C was bound to Glutathione-Sepharose beads and associated in vitro with p110 immunopurified from lysates of p110 infected Sf9 cells. The p85 proteins from both sources were phosphorylated in the presence of $[\gamma^{32}P]ATP$. These samples were resolved on SDS-PAGE gels, the p85α proteins identified by autoradiography and excised from the gel. Following trypsin digestion, the p85α protein digests were subjected to analysis by reverse-phase H.P.L.C and radioactivity in the eluted fractions was detected by analysing Cerenkov radiation. As can be seen from the results shown in Figure 7.7, the same major phosphopeptide was detected in maps of p85 α from all the different preparations, whether the proteins had been labelled in vitro or in vivo. As the bacterial GSTp85 α N-C,

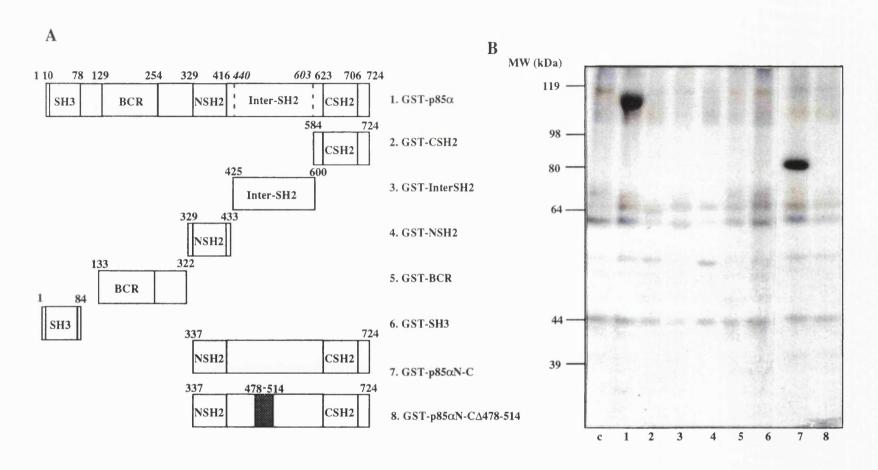


Figure 7.6. p85 α is only a substrate for p110 when associated in a complex.

- (A) Full-length GST-p85 α (1) and deletion mutants (2-8).
- (B) p110 immunoprecipitated from insect cells was incubated with 1 μ g of each of the fusion proteins 1-8 described in (A). GST alone incubated with immunoprecipitated p110 was used as a control (lane c). These proteins were then subjected to in vitro kinase assays in the presence of [γ 32P]ATP. Phosphoproteins were analysed by SDS-PAGE and visualised by autoradiography.

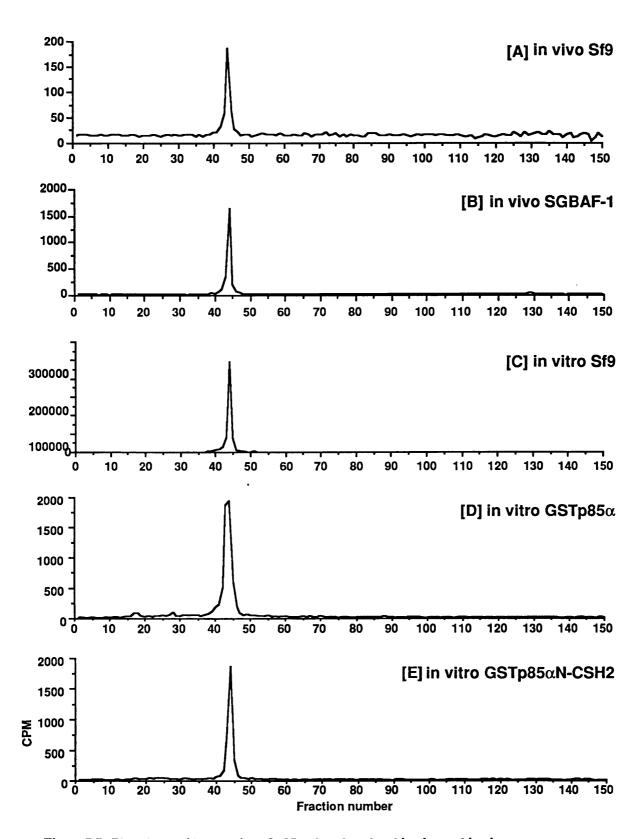


Figure 7.7. Phosphopeptide mapping of p85 α phosphorylated in vivo and in vitro. (A) Phosphopeptide-purified PI 3-kinase from Sf9 cells coinfected with p85 α /p110 viruses and labelled with phosphate in vivo. (B) PI 3-kinase treated as above from SGBAF-1 cells labelled with phosphate in vivo. (C) Phosphopeptide-purified PI 3-kinase from Sf9 cells coinfected with p85 α /p110 viruses phosphorylated in vitro in the presence of [γ 32P]ATP. GST-p85 α (D) or GSTp85 α N-CSH2 (E) bound to Glutathione-Sepharose beads, and then incubated with p110 containing Sf9 cell lysates. Complexed proteins were phosphorylated in vitro in the presence of [γ 32P]ATP. All samples were resolved on SDS-PAGE, the p85 protein identified by autoradiography and excised from the gel. Following trypsin digestion, the p85 proteins were subjected to analysis by reverse-phase H.P.L.C. as described in experimental procedures, and eluted fractions were counted for Cerenkov radiation.

phosphorylated in vitro by p110 and digested with trypsin contains the same phosphopeptide, this data suggests that the phosphoserine residue(s) resides in the C-terminal half of the p85 protein. The phosphopeptide was purified from a tryptic digest of a large scale preparation of p85 from Sf9 cells coinfected with p85α and p110 viruses, phosphorylated in vitro, and was then subjected to mass and N-terminal sequence analysis by N. Totty and O. Truong. Automated amino terminal Edman degradation identified the amino terminus of the phosphopeptide and suggested that it had the sequence LNEWLGNENTEDQYSLVEDDEDLPHHDEK. Mass analysis revealed a mass of 3583 Da (data not shown) which was consistant with a single phosphorylation site on this peptide since the mass of the peptide containing no phosphorylated residues should be 3484 Da. Taken together with the phosphoamino acid analysis, which indicated that the molecule was exclusively phosphorylated on serine in vitro, these results established that Ser-608 is the major site of phosphorylation. Phosphothreonine was also detected in the phosphoamino acid analysis of Sf9 cells labelled in vivo (Figure 7.2), however, it was not possible to recover a peptide containing a phosphothreonine residue(s).

7.2.5 Regulation of PI 3-kinase

The possible effect of this protein-serine/threonine kinase on the PI 3-kinase activity was next investigated. Lysates of insect cells that had been coinfected with viruses expressing the p85α and the p110 proteins were immunoprecipitated with antibodies directed against the p110 subunit. These immunocomplexes were phosphorylated in the presence of $[\gamma^{32}P]ATP$ for increasing periods of time (Figure 7.8A, lanes 1-7). After each incubation the reaction was stopped by washing extensively with lysis buffer containing 10 mM EDTA to chelate and remove excess MnCl₂. These immunocomplexes were then divided into two and half was used for analysis of proteins by SDS-PAGE and autoradiography while the remaining sample was subjected to PI 3-kinase assay. Results in Figure 7.8B clearly demonstrate that the increased level of phosphorylation seen on the p85α subunit is paralleled by a corresponding decrease in PI 3-kinase activity. Indeed, after 20 minutes incubation of the enzyme in the presence of MnCl₂ and $[\gamma^{32}P]ATP$, approximately 80% of the PI 3-kinase activity is lost. This effect can be reversed upon treatment of the inactivated enzyme with phosphatases. Results in Figure 7.8C show that treatment of the serine phosphorylated enzyme (lane 2), with either phosphoprotein phosphatase 2A (lane 3), or with alkaline phosphatase (lane 4) removed $[\gamma^{32}P]ATP$ from the p85α subunit. Parallel PI 3-kinase assays of these phosphatase-treated samples revealed restoration of activity (Figure 7.8D, lanes 1-4).

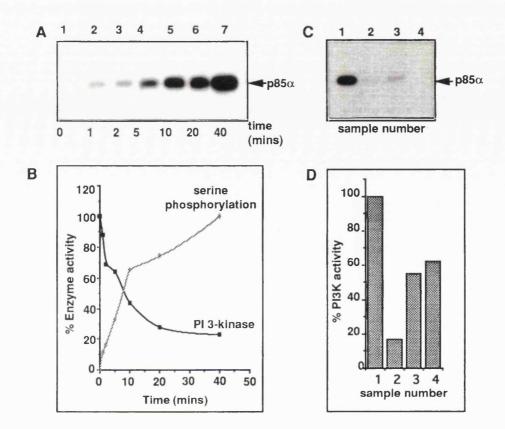


Figure 7.8. Serine Phosphorylation of the p85 α inhibits PI 3-kinase activity.

(10A) Anti-p110 immunoprecipitates of insect cells that had been coinfected with p85 α /p110 viruses were subjected to in vitro phosphorylation in the presence of [γ 32P]ATP for 0, 1, 2, 5, 10, 20 and 40 min, (lanes 1-7 respectively). After each time period, reactions were stopped by extensive washing with lysis buffer, containing 10 mM EDTA to chelate MnCl2. Samples were then split into two, half subjected to analysis on SDS-PAGE and autoradiography (10A) and the remainder subjected to in vitro PI 3-kinase assays (10B).

(10C) Effect of phosphatase treatment. PI 3-kinase was immunoprecipitated from insect cells that had been coinfected with p85 α /p110 viruses and maximally phosphorylated in vitro as described above. The sample was then left untreated (lane 2) or was subjected to treatment with protein phosphatase 2A (lane 3), or alkaline phosphatase (lane 4). Lane 1 is unphosphorylated sample. At this stage the sample was split in two, half subjected to analysis on SDS-PAGE and autoradiography and the remainder assayed for PI 3-kinase activity as shown in 10D.

(10D) lane 1; unphosphorylated PI 3-kinase, lane 2; $[\gamma 32P]ATP$ phosphorylated PI 3-kinase, lane 3; protein phosphatase 2A treatment of $[\gamma 32P]ATP$ phosphorylated PI 3-kinase, lane 4; alkaline phosphatase treatment of $[\gamma 32P]ATP$ phosphorylated PI 3-kinase.

7.3 Discussion

Several laboratories have observed a protein-serine kinase activity associated with PI 3-kinase isolated from a variety of sources (Kaplan et al., 1987; Carpenter et al., 1993a; Reif et al., 1993; R. Dhand, F. Ruiz-Larrea, M. Fry unpublished data). The expression of recombinant subunits of the PI 3-kinase complex described here has permitted a comprehensive investigation of the origin of this activity.

7.3.1 Structural and functional analysis of the catalytic domain of p110

An earlier analysis of the primary amino acid sequence of p110 had revealed that this protein exhibits a limited but significant sequence similarity to regions of the catalytic domains of protein kinases while possessing unique motifs which may define a lipid kinase concensus sequence (Hiles et al., 1992). The catalytic domains of protein kinases are modular structures in which regions of high sequence conservation are interspersed with regions where a great deal of variation is tolerated (Hanks et al., 1988). The DRHNSN sequence is part of a concensus motif that resides in subdomain VI which forms the central core of the catalytic domain (Hanks et al., 1988). These residues are found in diverse classical protein kinases that use ATP as a phosphate donor. To further examine the nature of the kinase activity intrinsic to p110, the mutated DRHNSN sequence (R916P) was used to demonstrate that although the resultant p110 protein still binds to the p85 subunit, the mutation resulted in the concomitant loss of both PI 3-kinase and protein-serine kinase activity, strongly suggesting that this protein can act as both an phosphoinositide kinase and a protein-serine kinase. The p110 protein studied here does not autophosphorylate significantly in vitro, unlike the p110 associated with the rat PIK kinase, which is phosphorylated (Carpenter et al. 1993a). This difference may be because the preparation of Carpenter et al. (1993a) contains two distinct types of p110 which are phosphorylated to varying degrees. The upper band is the more heavily phosphorylated p110 and this has recently been cloned and identified as p110 β (Hu et al., 1993). Indeed, in contrast to the bovine p110 (equivalent to the lower band in the p110 preparation by Carpenter et al., 1993a), the recombinant p110 β was shown to become phosphorylated in an in vitro kinase assay. This may represent a divergent pathway for differential regulation of the two isoforms of p110. The possibility that PI 3-kinase can phosphorylate the hydroxyl groups on both an amino acid side chain and phosphoinositides is somewhat surprising, but the identification of protein kinases that can phosphorylate tyrosine, serine and threonine residues also brought many previously held assumptions into question (Lindberg et al., 1992).

Both enzyme activities were found to be sensitive to sulphydryl modifying agents; the bovine PI 3-kinase and protein-serine kinase activities were inactivated upon treatment with Nbs₂ and could be reactivated with DTT. This observation suggested that cysteine residues are located close to or in the ATP-binding domain of the PI 3-kinase and that their sulphydryl groups play a role in interacting with ATP. Interestingly, a mutant p110 with Cys905 converted to Arg905 (C905R) in the catalytic domain of p110, (a residue analogous to Cys721 in Vps34p), is also inactive when expressed in Cos cells (Ian Hiles, unpublished data). A thiol requirement for catalysis has been observed in some but not all kinases (Hou et al., 1988; Scholz et al., 1991). Three-D structural data from crystallography of pyruvate kinase show that this enzyme contains two conserved cysteine residues in close proximity to the active site (Muirhead et al., 1986).

It is also interesting that the BCR protein is not only a Mn²⁺-dependent protein-serine kinase, but also uses an unusual nucleotide-binding domain containing paired cysteine residues (Maru and Witte, 1991). Phosphorylase kinase is a dual specificity serine/threonine and tyrosine kinase with a thiol requirement for catalysis. It is of particular interest that the specificity of phosphorylation is determined by divalent cations, where Mg²⁺ causes seryl phosphorylation of substrate and Mn²⁺ activates tyrosine phosphorylation (Yuan et al., 1993). Also of note is the dual specificity phosphatase encoded by vaccinia virus which employs a common catalytic mechanism using an essential cysteine residue for hydrolysis of both tyrosine and serine phosphoproteins (Guan et al., 1991).

PI 3-kinase has been described to be specifically inhibited by Wortmannin (Acaro et al., 1993). This inhibition was also observed for the protein-serine kinase activity of p110, substantiating further that p110 contains a dual specificity kinase domain (R. Woscholski, P. Parker, R. Dhand unpublished data). Since the phosphoinositol lipid and the protein-serine kinase activities of p110 appear to have a common thiol requirement for catalysis and both activities are inhibited by Wortmannin, it appears plausible to suggest that the protein-serine kinase activity is intrinsic to the PI 3-kinase complex and a single catalytic site on this protein may display a dual substrate specificity.

7.3.2 Substrate analysis

The p85 proteins are the only known substrates for the protein-serine kinase activity of p110. This unique specificity may be the result of a conformational change induced by subunit interactions which activate the serine kinase activity, or that the p110 subunit lacks a classical protein substrate binding pocket and requires its substrate to be brought into close proximity to the active site as a consequence of a high affinity interaction. This does however establish that p110 can carry out a transphosphorylation reaction and opens the

possibility that other cellular proteins might also be suitable targets for this kinase activity. The substrate specificity for the protein-serine kinase described here is more restricted than that reported by Carpenter et al (1993a), who demonstrated that casein was also a substrate for purified rat liver PIK kinase. The origin of these differences will await more detailed studies of the recombinant rat PI 3-kinase. Phosphopeptide mapping of tryptic digests showed that a common, major peptide was phosphorylated in immunoprecipitates of p85a from insect, bacterial and bovine cells, both in vivo and in vitro. This data strongly suggests that the results observed during in vitro analysis, are relevant to the in vivo systems. Detailed protein chemical analyses have identified Ser-608 in the inter-SH2 region as the major phosphorylation site in vitro. A serine residue is present at an analogous position in p85β, moreover, surrounding residues are well-conserved in both subunits, which suggests that this site is important as a regulatory motif for this family of proteins. Tyr-607 on p85 α was recently identified as a major phosphorylation site by the insulin receptor in vivo (Hayashi et al., 1993) in cells overexpressing p85. The possible significance of this phosphorylation event for regulation of the enzyme, when operating as a component of a membrane-associated signal transducing complex, remains to be determined.

7.3.3 Serine phosphorylation as a mode of PI 3-kinase autoregulation

The novel observation shown here, that PI 3-kinase also has an intrinsic protein-serine kinase activity adds another dimension to the potential for regulation of its enzyme activity. It has been demonstrated that the p85α subunit of the PI 3-kinase immunoprecipitated from quiescent cells is phosphorylated exlusively on serine and threonine residues. Only following stimulation of the cells with a growth factor is the p85 subunit seen to become phosphorylated on tyrosine residues (Roche et al., 1994) with a corresponding increase in total cellular PI 3-kinase activity (Chapter 8). Further, in polyoma virus middle Ttransformed cells, protein phosphatase 2A has been found associated with the mT complex (Pallas et al., 1990). One could surmise that PI 3-kinase associates with the mT/pp60^{c-src} complex and is activated by dephosphorylation of the exposed Ser-608 by the protein phosphatase 2A. Results presented here define a Mn²⁺-dependent protein-serine kinase activity, that phosphorylates the p85 subunit and causes down-regulation of PI 3-kinase in vitro. Consistent with these results, insulin stimulation of okadaic acid treated 3T3-L1 adipopeytes, which inhibits the action of serine phosphatases, produced a dose-dependent inhibition of the activation of PI 3-kinase (Jullian et al., 1993). Further as expected, the total PI 3-kinase activity in cells was shown to remain the same regardless of insulin stimulation. In addition, the ability of the p110 to autoregulate its PI 3-kinase activity might account for the reduced activity of the enzyme with respect to the monomeric p110 form reported by Shibasaki et al. (1991; 1992).

7.3.4 Conclusion

This study defines PI 3-kinase as dual specificity enzyme with an intrinsic PI 3-kinase and protein serine kinase activity. Further, phosphorylation of the p85 protein on ser-608 has been shown to autoregulate the enzyme. At present it is difficult to determine the exact role of this dual specificity kinase in vivo. However, the observed use of the Ser-608 site for p85α proteins phosphorylated either in vivo in different cell types or in vitro, and the potent regulatory effect of the phosphorylation of the p85 protein on the PI 3-kinase complex, suggests that it may have physiological relevance in vivo. The following Chapter will examine the possible physiological relevance of this, and other results obtained throughout the course of this thesis, in intact cells.

Chapter 8: The interaction of PI 3-kinase with the PDGF receptor in NIH 3T3 cells

8.1 Introduction

Extensive studies of the structure and function of the PDGFB receptor have made it the the prototype for a related family of protein-tyrosine kinase receptors which share structural similarities (Subclass III) that includes the CSF-1 (c-fms) and p145c-kit (c-kit) receptors. These receptors are characterised by their extracellular ligand-binding domain which contains five immunoglobulin-like repeats and a hydrophilic amino acid sequence of variable length, which can be considered as an insert in the basic kinase domain structure (Ullrich and Schlessinger, 1992). The PDGF receptor triggers a cellular response by a mechanism which involves ligand-induced receptor dimerisation followed by trans or autotransphosphorylation of the receptor on multiple tyrosines (Schlessinger, 1988). The phosphotyrosine residues of the activated receptor serve as a "coded template" to direct the binding of receptor to specific intracellular substrates. Following activation, receptor complexes move to coated pits and are rapidly internalised. Subsequently, these receptors enter distinct cellular sorting pathways leading to their degradation or renewal of ligand and recycling to the cell surface. The structural determinants that mediate targeting of these receptors are contained in their cytoplasmic domains (Reviewed in Schlessinger and Ullrich, 1990).

The PDGFβ receptor was the first receptor for which PI 3-kinase activity was shown to be bound when it was demonstrated that antiphosphotyrosine immunoprecipitates from PDGF-stimulated cells contained up to 50 fold more PI 3-kinase activity than those prepared from control cells (Kaplan et al., 1987; Whitman et al., 1987). An involvement of the PDGF receptor was subsequently proven using antibodies specific for the receptor (Coughlin et al., 1989). Further, PDGF stimulation of cells was shown to result in a concomitant temporal increase in the levels of PI(3,4)P2 and PI(3,4,5)P3 in intact cells (Auger et al., 1989). Receptor association with PI 3-kinase was shown to be mediated by a tyrosine phosphorylated kinase-insert (Coughlin et al., 1989; Severinsson et al., 1990; Escobedo et al., 1991a; Kazlauskas and Cooper, 1989; Kazlauskas and Cooper, 1990). More detailed analysis led to the identification of Y751, a known autophosphorylation site in the kinase-insert region of the human PDGFβ receptor, as being partially responsible for binding PI 3-kinase in vivo (Kazlauskas and Cooper, 1989) and in vitro (Kazlauskas and Cooper, 1990; Escobedo et al., 1991a). A murine based peptide phosphorylated on residues equvalent to Y740 on the human PDGF receptor also blocked

association and this residue was subsequently also found to be phosphorylated in vivo (Kashishian et al., 1992). Mutagenesis studies and competition analyses using phosphopeptides revealed that Y740 and Y751 were both involved in binding PI 3-kinase activity both in vivo and in vitro (Kashishian et al., 1992; Fantl et al., 1992). Other structural features of the kinase-insert were demonstrated not to be important for binding PI 3-kinase as determined by deleting regions of ~10 amino acids either side of Y740 and Y751 (Kashishian et al., 1992). These results were therefore consistent with PI 3-kinase binding with high affinity to peptides of only five residues in length.

8.1.1 Aim

Through the course of this study the interaction of isolated and reconstituted components of the PI 3-kinase complex with protein-tyrosine kinases has been examined both in vitro and in vivo in insect cells. It is rapidly emerging that a family of PI 3-kinases exist (Discussed in more detail in Chapters 3 and 4) and this suggests that some analyses using antiphosphotyrosine and anti-receptor antibodies to examine the interaction of protein tyrosine kinases with "the PI 3-kinase" may have produced ambiguous results. The generation of reagents that specifically recognise the individual domains of the PI 3-kinase complex has facilitated examination of the functioning of this particular enzyme in mammalian cells using a distinct approach. Antibodies raised against the regulatory and catalytic subunits of the PI 3-kinase complex, and those that recognise the PDGF receptor have been used to examine the kinetics of receptor-PI 3-kinase association. I have found that ligand stimulation of NIH 3T3 cells results in a rapid, but transient association of the enzyme with the PDGF receptor. This event was further shown to elicit activation of the associated pool of PI 3-kinase activity. These results are discussed with respect to the analyses performed using the recombinant bovine enzyme.

8.2 Results

8.2.1 PI 3-kinase activity transiently associates with the PDGF receptor following ligand stimulation

To examine the kinetics of receptor-PI 3-kinase complex formation, NIH 3T3 murine fibroblasts which endogenously express the PDGFβ receptor at high levels were grown to 80-90% confluence and then starved for 36 h in 0.5% FCS to bring them to quiscence. The cells were stimulated with saturating concentrations of PDGF-BB for varying periods of time. For example, unstimulated 0, 10, 30, 60, 120, 240, min or overnight. The cells were then washed with PBS and lysed immediately on ice. These lysates were immunoprecipitated with an excess of anti-PDGF receptor antibodies as determined by titration experiments (data not shown). The immunocomplexes were collected on Protein

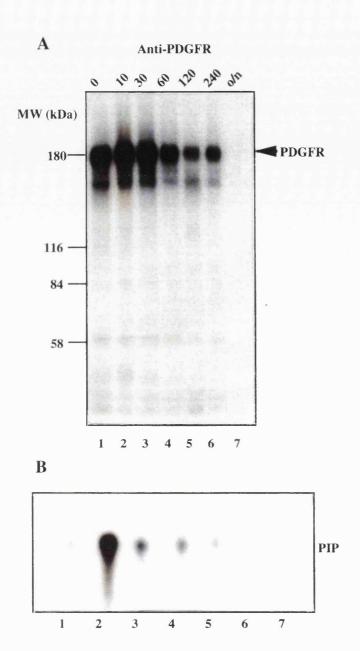


Figure 8.1: PI 3-kinase activity transciently associates with the PDGF receptor following ligand stimulation.

Near confluent and quiesced NIH 3T3 cells were stimulated with PDGF-BB at 37°C for the indicated times, ie. 0 (lane 1), 10 min (lane 2), 30 min (lane 3), 60 min (lane 4), 120 min (lane 5), 240 min (lane 6), overnight (o/n) (lane 7). Lysates were prepared from these cells and precipitated using polyclonal antibodies raised against the PDGF receptor. Samples were split in two and half subjected to in vitro kinase assays and analysed by SDS-PAGE and autoradiography (panel A). The remainder was then assayed for associated PI 3-kinase activity (panel B).

A-Sepharose beads and washed several times in lysis buffer, after which each sample was split. Half was assayed for associated PI 3-kinase activity while the remainder was subjected to in vitro protein-kinase assays and analysed by autoradiography of SDS-PAGE gels. The results in Figure 8.1A show activation of the PDGF receptor upon ligand stimulation as revealed by the enhanced in vitro autophosphorylation observed (compare lanes 1 and 2). The level of receptor was seen to decrease as judged by its capacity to autophosphorylate in the presence of Mn²⁺ and $[\gamma^{32}P]ATP$ (compare lanes 2-6), presumably due to receptor downregulation with increasing time after ligand stimulation. Examination of the same immunoprecipitates for associated PI 3-kinase activity, revealed no association with the PDGF receptor in unstimulated cells (Figure 8.1B, lane 1). However, ligand activation resulted in a burst of PI 3-kinase activity which transiently associated with the receptor and was then rapidly lost from the immunoprecipitates. Maximal PI 3-kinase activity was recovered from the receptor precipitates at the earliest time point examined upon ligand activation. By 30 min most of the PI 3-kinase activity had returned to its basal level in resting cells, although a high level of receptor phosphorylation still remained (Figure 8.1 panels A and B, lanes 3 and 4).

8.2.2 There is no significant change in the levels of total cellular PI 3-kinase activity following ligand stimulation

The interaction of PI 3-kinase with the PDGF receptor has always been examined using either receptor-specific antibodies or anti-phosphotyrosine antibodies. The availability of antibodies that specifically recognised each subunit of the PI 3-kinase complex independently allowed a more specific examination of the receptor-PI 3-kinase complex in cells. Thus, to probe further the interaction between the PDGF receptor and PI 3-kinase, the same lysates of NIH 3T3 fibroblasts that had been used to immunoprecipitate receptor were immunoprecipitated with an excess of either anti-p85 α or anti-p110 antibodies. These immunoprecipitates were then assayed for associated PI 3-kinase activity or subjected to in vitro protein kinase assays where phosphoproteins were identified by autoradiography of SDS-PAGE gels.

The results in Figure 8.2Ai show that a p85 α protein could be immunoprecipitated and phosphorylated both in unstimulated cells (lane 1) and from cells exposed to ligand for any length of time (lanes 2-7). The absence of a phosphoprotein of molecular size 110 kDa should be noted. The PDGF receptor is seen to be present in the enzyme complex, at the earliest point examined following exposure of the cells to ligand. By 30 min breakdown of the complex has already commensed. This is exactly the reverse of the events described in the anti-PDGF receptor immunoprecipitations above. Analysis of the PI 3-kinase activity associated with the p85 α antibody (Figure 8.2Bi) revealed that

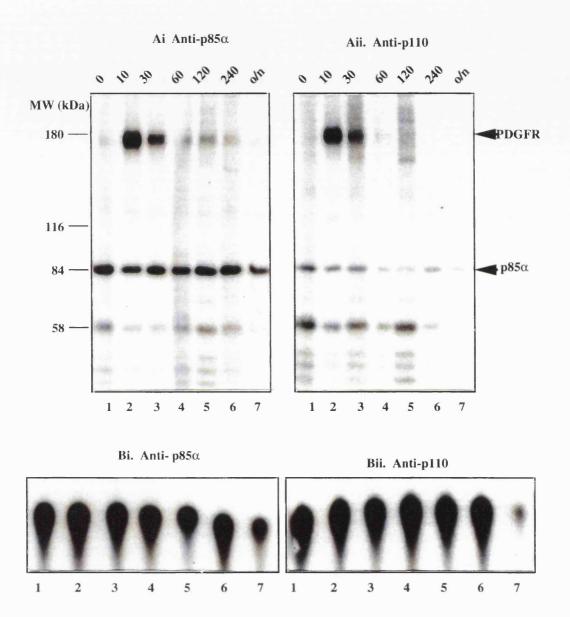


Figure 8.2. There is no significant decrease in the level of PI 3-kinase activity with continuous ligand stimulation.

Near confluent and quiesced NIH 3T3 cells were stimulated with PDGF-BB at $37^{\circ}C$ for the indicated times, ie. 0 (lane 1), 10 min (lane 2), 30 min (lane 3), 60 min (lane 4), 120 min (lane 5), 240 min (lane 6), overnight (o/n) (lane 7). Lysates were prepared from these cells and precipitated using polyclonal antibodies raised against the p85 α protein (panel Ai and Bi) and the p110 subunit (panel Aii and Bii). Samples were split in two and half subjected to in vitro kinase assays and analysed by SDS-PAGE and autoradiography (panel Ai and Aii). The remainder was then assayed for associated PI 3-kinase activity (panel Bi and Bii).

increasing periods of PDGF stimulation resulted in no significant decrease in the level of PI 3-kinase activity. Together with the results in Figure 8.1 this suggests that over a 24 h period PI 3-kinase down-regulation is an independent event to receptor turnover. The results observed with the anti-p110 antibody are essentially identical to those witnessed using the p85 α -specific antibody (Figure 8.2, panels Aii and Bii), and imply that p85 α and p110 are most likely to be in a complexed state for the majority time in the cell. In particular, as the results observed in anti-p110 immunoprecipitations (Figure 8.2Bii), mirrored those in the equivalent anti-p85 immunoprecipitations (Figure 8.2Ai), this suggested that the p85/p110 complex dissociates from the PDGF receptor as a single event, rather than through selective loss of the catalytic subunit of PI 3-kinase.

8.2.3 The amounts of methionine labelled PI 3-kinase subunits remain constant following ligand stimulation

To verify the amounts of p85 α and p110 protein immunoprecipitated by the respective antibodies at the various times of ligand stimulation, NIH 3T3 cells were biosynthetically labelled with 35 S methionine overnight prior to further biochemical analyses. The cells were then stimulated for the same periods of time as already described. Lysates prepared from these cells were imunoprecipitated using antibodies that recognise the p85 α protein (Figure 8.3B) and the PDGF receptor (Figure 8.3C) and were collected on Protein A-Sepharose beads. After several washes radiolabelled proteins released by SDS-PAGE buffer were analysed by SDS-PAGE and detected by autoradiography. As a second antibody was used in these immunoprecipitations, an anti-mouse antibody alone was used as a control (Figure 8.3A).

The results in Figure 8.3B show a constant ratio of the p85 α protein and the p110 protein present in the cell. The total amounts of p85 α and the p110 protein, as indicated by the arrow, remained unchanged over the course of ligand stimulation. Immunoprecipitations performed using the anti-110 antibodies revealed identical but weaker results, due to the lower affinity of these antibodies. These results also showed that the p110 protein was always present in the p85 α immunoprecipitations but was not always seen to be phosphorylated (compare Figure 8.2Ai with Figure 8.3B). The PDGF receptor is observed in cells for 30 min post-ligand stimulation, after this time the levels of receptor are observed to decrease presumably because the receptor is degraded (Figure 8.3C).

8.3.4 PI 3-kinase activity rapidly associates with the PDGF receptor upon ligand stimulation

The results shown in Figures 8.1 and 8.2 give only an outline of receptor binding PI 3-kinase so the kinetics of the association were examined in detail. Identical experiments as those already described were performed but with earlier time points of PDGF

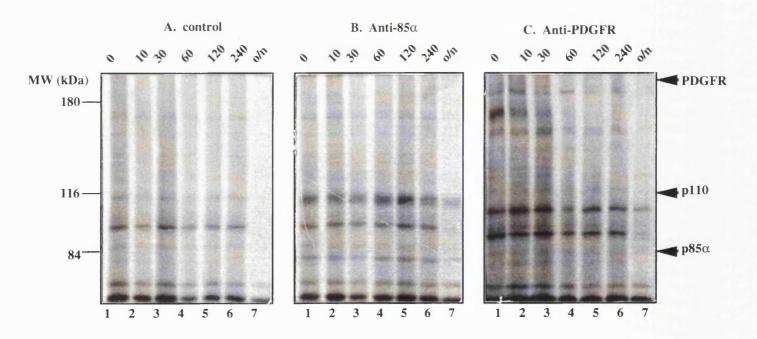


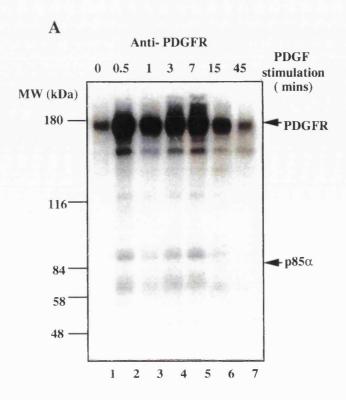
Figure 8.3: The amounts of 35S-methionine labelled PI 3-kinase remains constant with continous ligand stimulation.

Near confluent and quiesced NIH 3T3 cells were were labelled with [35S]-methionine for 16 h and then stimulated with PDGF-BB at 37°C for the indicated times, ie. 0 (lane 1), 10 min (lane 2), 30 min (lane 3), 60 min (lane 4), 120 min (lane 5), 240 min (lane 6), overnight (o/n) (lane 7). Lysates were prepared from these cells and precipitated using polyclonal antibodies raised against the p85α protein (Panel B) and the PDGF receptor (Panel C). Anti-mouse antibodies bound to Protein-A Sepharose beads was used as a control (Panel A). After several washes radiolabelled proteins were analysed by SDS-PAGE and autoradiography.

stimulation to study the kinetics of binding. Thus, 80-90% confluent, quiescent NIH 3T3 cells were stimulated with saturating concentrations of PDGF for the indicated time periods ie., unstimulated 0, 0.5, 1, 3, 7, 15 and 45 mins and were then the cells were lysed. The lysates were immunoprecipitated with an excess of anti-PDGF receptor antibodies and these were assayed for associated PI 3-kinase activity or subjected to in vitro protein kinase assays. The products of the PI 3-kinase assay were analysed by autoradiography of TLC plates and phosphoproteins detected by autoradiography of SDS-PAGE gels. The results shown in Figure 8.4A reveal activation of the PDGF receptor at 30 s following exposure to ligand. Indeed, the receptor was seen to remain maximally phosphorylated for up to 7 min after PDGF stimulation of cells. The p85α protein was seen as a faint phosphoprotein 30 s following stimulation. Analysis of receptor associated PI 3-kinase activity (Figure 8.4B) revealed a sharp increase in the activity associating with the activated PDGF receptor, reaching a maximum at 1 min and then rapidly declining, even though the receptor remains highly phosphorylated (Figure 8.4A, lanes 2-5).

8.2.5 PI 3-kinase is activated upon binding ligand

Lysates of NIH 3T3 fibroblasts that had been used to immunoprecipitate the PDGF receptor were also immunoprecipitated with an excess of anti-p85α or anti-p110 antibodies. These immunoprecipitates were either phosphorylated in vitro, resolved on SDS-PAGE and analysed by autoradiography or assayed for associated PI 3-kinase activity. The results in Figure 8.5Ai show $p85\alpha$ to be phosphorylated both in the presence and in the absence of receptor. The PDGF receptor is found to become associated with PI 3-kinase following 30 s ligand stimulation of cells and remains associated for up to 7 min, after which the level of receptor present in the enzyme complex begins to decrease. It is of particular interest that a phosphoprotein of 110 kDa is also visible for the time period 0.5-7 min of ligand stimulation, and the p85 α protein concurrently appears as a doublet for this time period. Analysis of the PI 3-kinase activity associated with the p85\alpha antibody (Figure 8.5B) revealed a 2-fold increase in total PI 3-kinase activity upon PDGF stimulation, indeed, maximal PI 3-kinase activity was recovered from the anti-p85α precipitates 3 min following receptor activation. Following 15 min exposure of cells to ligand, the PI 3-kinase activity had begun to fall to the basal level observed associated with the receptor before stimulation of the cells. This peak increase in activity correlates with the appearance of the 110 kDa phosphoprotein and the p85 phosphorylated doublet (Figure 8.5Ai and 8.5B, lanes 2-5). The results observed with the antibody raised to the p110 protein are essentially identical to those using the antibody that recognises the p85α subunit Figure 8.5Aii and 8.5B), reinforcing the observation that the p85α and the p110 subunits of PI 3-kinase are commonly associated in a tight complex in the cell.



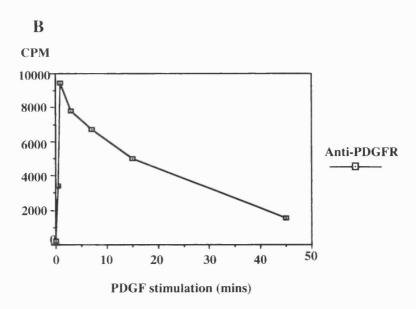
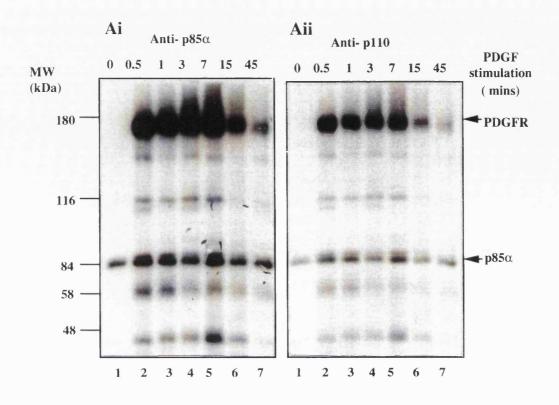


Figure 8.4. PI 3-kinase activity rapidly associates with the PDGF receptor following ligand stimulation.

Near confluent and quiesced NIH 3T3 cells were stimulated with PDGF-BB at 37°C for the indicated times, ie. 0 (lane 1), 0.5 min (lane 2), 1 min (lane 3), 3 min (lane 4), 7 min (lane 5), 15 min (lane 6), 45 min (lane 7). Lysates were prepared from these cells and precipitated using polyclonal antibodies raised against the PDGF receptor. Samples were split in two and half subjected to in vitro kinase assays and analysed by SDS-PAGE and autoradiography (A). The remainder was then assayed for associated PI 3-kinase activty (B).



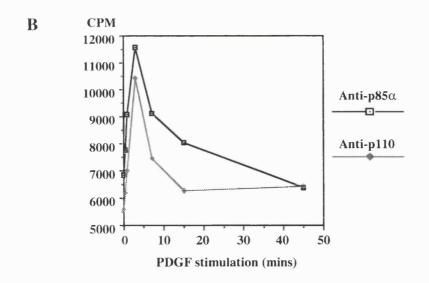


Figure 8.5. PI 3-kinase is activated upon binding ligand.

Near confluent and quiesced NIH 3T3 cells were stimulated with PDGF-BB at $37^{\circ}C$ for the indicated times, ie. 0 (lane 1), 0.5 min (lane 2), 1 min (lane 3), 3 min (lane 4), 7 min (lane 5), 15 min (lane 6), 45 min (lane 7). Lysates were prepared from these cells and precipitated using polyclonal antibodies raised against the p85 α protein (**panel Ai**) and the p110 subunit (**panel Aii**). Samples were split in two and half subjected to in vitro kinase assays and analysed by SDS-PAGE and autoradiography (**panel Ai and Aii**). The remainder was then assayed for associated PI 3-kinase activity (**panel B**).

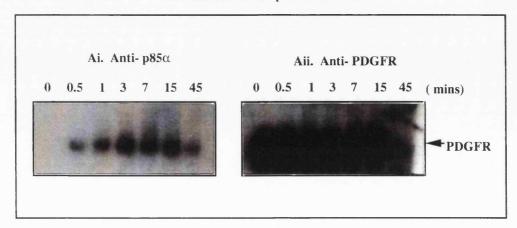
8.2.6 Quantitation of p85 α and PDGF receptor present in the cell following growth factor stimulation

In order to examine the fraction of the total pool of PI 3-kinase that was actually in association with the receptor, the following approach was utilised. NIH 3T3 cells were stimulated with saturating concentrations of PDGF for the indicated time periods between 0.5 and 45 min and were then lysed. These lysates were immunoprecipitated with an excess of antibodies that recognised either the PDGF receptor or the p85 α subunit of PI 3-kinase. These immunoprecipitates were resolved on SDS-PAGE gels, and then subjected to Western blot analysis using antibodies directed to either the p85α protein or the PDGF receptor. The proteins were labelled with ¹²⁵I-Protein A and detected by autoradiography. The results in Figure 8.6 (panel Ai) show an anti-PDGF receptor immunoblot which reveals the amount of PDGF receptor which is associated with the p85α protein in an anti-85α immunoprecipitation. In panel Aii the amount of total PDGF receptor that can be immunoprecipitated from this lysate is shown. Qualitative analysis reveals that approximately 5% of the total PDGF receptor present is participating in complex formation with PI 3-kinase. In parallel, examination of the anti-p85α western blot of the anti-p85α (Figure 8.6B, panel Bi) and the anti-PDGF receptor (Figure 8.6B, panel Bii) immunoprecipitations reveals that of the total amount of p85 α protein present, approximately 5% is found in association with the receptor. Thus, at a given time only 5% of the total pool of PI 3-kinase and PDGF receptors are cooperating in complex formation. This implies that the 2 fold increase in PI 3-kinase activity, recovered from the anti-85α and anti-p110 immunoprecipitations in PDGF stimulated cells, may actually be much greater but is masked by the large pool of inactive PI 3-kinase that is not in association with the receptor.

8.2.7 The p85 subunit of the PI 3-kinase is phosphorylated on serine residues in unstimulated cells

The predominant presence of a phosphorylated p85α protein in both unstimulated and stimulated cells, led me to examine the phosphorylation state of the components of the complex. NIH 3T3 fibroblasts were grown to near confluence and then serum starved. The cells were stimulated with saturating concentrations of PDGF for 0.5, 1 and 3 min. The cells were then washed with PBS and lysed immediately on ice. These lysates were immunoprecipitated with anti-p85α antibodies and collected on Protein A-Sepharose beads. Following several washes in lysis buffer the immune complexes were phosphorylated in vitro and resolved on SDS-PAGE. The gels were subjected to KOH treatment, which selectively removes phosphoserine residues, which are more labile to alkaline hydrolysis, and were then analysed by autoradiography. The results shown in Figure 8.7A reveal that the p85α protein becomes phosphosphorylated in

A. Anti-PDGF receptor western blot



B. Anti-p85α western blot

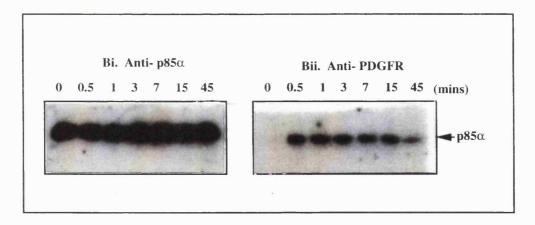


Figure 8.6: Quantitation of p85 α and PDGF receptor present in the cell following growth factor stimulation.

Near confluent and quiesced NIH 3T3 cells were stimulated with PDGF-BB at 37° C for the indicated times, ie. 0 (lane 1), 0.5 min (lane 2), 1 min (lane 3), 3 min (lane 4), 7 min (lane 5), 15 min (lane 6), 45 min (lane 7). Lysates were prepared from these cells and precipitated using polyclonal antibodies raised against the p85 α protein (Panel Ai and Bi) and the PDGF receptor (Panel Aii and Bii). These immunoprecipitations were subjected to Western blot analysis using antibodies directed to the PDGF receptor (A) and the p85 α protein (B).

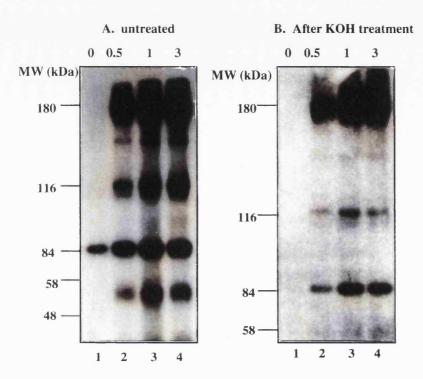


Figure 8.7. The p85 α subunit of PI 3-kinase is phosphorylated on serine residues in unstimulated cells. Near confluent and quiesced NIH 3T3 cells were stimulated with PDGF-BB at 37 $^{\circ}$ C for the indicated times, ie. 0 (lane 1), 0.5 min (lane 2), 1 min (lane 3), 3 min (lane 4). Lysates were prepared from these cells and precipitated using polyclonal antibodies raised against the p85 α protein. These samples were subjected to in vitro kinase assays and then analysis by SDS-PAGE and autoradiography (A). Samples prepared as described in (A) and the SDS-PAGE gel is treated with KOH at 55 $^{\circ}$ C for 30 min (B).

immunoprecipitates from both stimulated and unstimulated cells (lanes 1 and 2). Following alkaline treatment the p85α protein immunoprecipitated from unstimulated cells was found to be phosphorylated on serine residues (Figure 8.7B, lanes 1 and 2). In PDGF stimulated cells it was however observed to become phosphorylated on tyrosine when in association with the receptor (compare Figure 8.7A and 8.7B, lanes 2-4). It should also be noted that the 110 kDa protein was also phosphorylated on tyrosine residues following growth factor activation of cells (Figure 8.7B, lanes 2-4). Thus, these results suggest that tyrosine phosphorylation may play a role in the activation of PI 3-kinase in stimulated cells.

8.3 Discussion

The binding of PDGF to its receptor rapidly activates its intrinsic protein tyrosine kinase activity and elicits a cascade of biochemical responses that can in some cells culminate in cell division. The association of PI 3-kinase with the activated PDGF receptor has been well documented since it was first demonstrated that a PI 3-kinase activity was immunoprecipitated from PDGF treated cells with an antiphosphotyrosine antibody (Kaplan et al., 1987; Whitman et al., 1987). The interaction of PI 3-kinase has since been examined with several other protein tyrosine kinase receptors. These include the receptors for CSF-1 (Varticovski et al., 1989), EGF (Bjorge et al., 1990), insulin (Endenmann et al., 1990; Rudermannn et al., 1990), IGF-1 (Kapeller et al., 1991), NGF (Soltoff et al., 1992) c-kit (Lev et al., 1992), HGF (Graziani et al., 1991) and ErbB2 (Peles et al., 1992). Due to the lack of reagents that recognise the two subunits of PI 3kinase these analyses were generally perfored using antibodies that recognised either the receptor or phosphotyrosine. As it is now becoming clear that there exist a family of PI 3-kinases, the generation of antibodies to both the regulatory p85 α and the catalytic p110 subunit has here permitted a detailed analysis of the interaction of PI 3-kinase with the PDGF receptor in cells using a more specific approach to that previously utilised.

8.3.1 Biosynthesis of PI 3-kinase

The results presented in this study show that the interaction of PI 3-kinase activity with the activated PDGF receptor was observed both rapidly and transiently upon ligand stimulation of cells. The observed decrease in the ability of the PDGF receptor to become autophosphorylated in the continual presence of PDGF-BB is presumably a reflection of the receptor being internalised as it is unable to accumulate on the cell surface and is subsequently degraded. Similar results have also been observed in other studies where the binding of PDGF-BB to its receptor has been shown to first induce internalisation of the ligand-receptor, followed secondly by its degradation (as measured

by release of low Mr ¹²⁵I radioactivity from labelled cells and pulse chase experiments using radiolabelled methionine). The half-life for the PDGF receptor has been measured as two hours (Keating et al, 1988; Severrinson et al., 1990). However, the level of PI 3-kinase activity was not seen to decrease significantly over these periods of stimulation. A recent study by Kapeller et al, (1993) showed that the phosphorylated PDGF receptor and activated PI 3-kinase could be found in clatharin coated vesicles within two min of exposure to PDGF, indicating that both receptor and PI 3-kinase entered the endocytic pathway together and were then internalised. Moreover, a PDGF receptor mutant lacking PI 3-kinase binding sites failed to internalise, suggesting that PI 3-kinase regulates the trafficking of receptor tyrosine kinases (Joly et al., 1994). In addition, insulin stimulation has been shown to result in the activation of PI 3-kinase in low density microsomal fractions which include the endocytic vesicles (Kelly et al., 1992). As the major pool of PI 3-kinase present in the cell fails to associate with the receptor, a small receptor associated pool of PI 3-kinase may be internalised together with the receptor, and the remaining bulk of PI 3-kinase must presumably utilise another mechanism.

8.3.2 Kinetics of receptor-PI 3-kinase interaction

Further analysis into the kinetics of receptor-PI 3-kinase association revealed that complex formation, upon growth factor stimulation of cells, was very rapid. PI 3-kinase activity was observed to bind to the receptor at 0.5 min following exposure to PDGF and then to decline sharply after 1 minute, although the receptor remained phosphorylated at this point. Consistent with this observation anti-receptor immunoprecipitations of PDGF stimulated Swiss 3T3 cells (Kazlauskas and Cooper., 1990) and immunoprecipitations with anti-phosphotyrosine antibodies of PDGF stimulated Balb/3T3 cells (Kaplan et al., 1987), have shown that PI 3-kinase activity binds the activated receptor within 1 min, maximal binding being observed at 2-3 min with a half-life of 15 min post-ligand stimulation. Essentially the same kinetics of interaction of the PI 3-kinase have been noted in studies with ErbB2 (Peles et al., 1992) and the CSF-1 receptor (Varticovski et al., 1989). The kinetics of NGF and EGF stimulation of PI 3-kinase activity have also been investigated in PC12 cells by antiphosphotyrosine immunoprecipitations. It was found that whereas EGF displayed kinetics of activation similar to those observed with PDGF, NGF elicited a more delayed response. That is, PI 3-kinase activation was maximal following 5 min of ligand stimulation and could still be detected 2 h post stimulation (Carter and Downes., 1992). This is of interest since NGF initiates neuronal differentiation in PC12 cells, while EGF causes cellular proliferation.

8.3.3 Stoichiometry of receptor-PI 3-kinase interaction

A two fold activation of total PI 3-kinase activity was observed here upon stimulation with PDGF. This is consistent with the 5-fold activation of PI 3-kinase activity previously observed upon CSF-1 stimulation of NIH 3T3 cells (Choudhury et al., 1991). However, only a small proportion of the total PI 3-kinase activity present in the cell was actually demonstrated here to be associated with the receptor at any given time. Thus, the observed small increase in the total pool of PI 3-kinase presumably masks a greater enhancement of PI 3-kinase activity associated with receptors. Similar results have been shown for PI 3-kinase activity associated with the activated PDGF receptor in Balb/3T3 cells (Escobedo et al., 1989), CSF-1 receptor and ErbB2 in NIH 3T3 cells (Downing et al., 1991; Peles et al., 1992). In addition, only a small (3-10%) fraction of PI 3-kinase associates directly with the activated insulin receptor in vivo (Sun et al., 1991); Backer et al., 1992; Zhang et al., 1993) and in vitro (Yonezawa et al., 1992). However, between 25% and 70% of the total pool of PI 3-kinase activity present in the cell associates with the IRS-1 protein upon insulin stimulation of cells (Backer et al. 1993a;1993; Folli et al., 1992). This may be a reflection on the number of phosphorylation sites available for binding of PI 3-kinase on the IRS-1 protein as opposed to those on the insulin receptor itself.

8.3.4 Mechanisms of PI 3-kinase activation

8.3.4.1 Tyrosine phosphorylation

The physical binding of substrates to receptor protein tyrosine kinases can markedly lower the Km for phosphorylation making the SH2-containing proteins preferred substrates of receptor protein tyrosine kinase activity; a process which may contribute to their activation. Although one study has reported tyrosine phosphorylation of the p85α protein was not detected unless both p85α and the PDGF receptor were overexpressed in the cell (Hu et al., 1992), tyrosine phosphorylation of PI 3-kinase directly associated with receptor was observed in this study and has been documented by several other independent groups. Thus NGF, EGF, and bFGF were shown to stimulate the tyrosine phosphorylation of p85 in PC12 cells and NIH 3T3 cells (Soltoff et al, 1992; Raffioni and Bradshaw., 1992). Similarly, tyrosine phosphorylation of p85 has been observed in Balb/C 3T3 cells, NIH 3T3 and smooth muscle cells following PDGF stimulation (Kaplan et al., 1987; Kavenaugh et al., 1992; Soltoff et al, 1992; Auger et al., 1989). Furthermore, a correlation between ligand stimulated tyrosine phosphorylation of p85 and an increase in the specific activity of PI 3-kinase has also been observed in a number of cell types (Raffioni and Bradshaw, 1992; Hayashi et al., 1992). Consistent with the concept that tyrosine phosphorylation of PI 3-kinase by an activated protein tyrosine kinase can also lead to an increase in PI 3-kinase activity, is the report that a decrease in PI 3-kinase activity follows treatment of the purified bovine brain enzyme with a phosphotyrosyl protein phosphatase (Ruiz-Larrea et al., 1993). Three sites of tyrosine phosphorylation have been mapped to residues 368, 580 and 608 of p85 (Hayashi et al., 1993). Mutagenesis of these sites will be required to investigate their possible role in PI 3-kinase regulation.

It is intriguing that the activation of the cellular PI 3-kinase is accompanied by the appearance of a tyrosine phosphorylated p110 protein in the protein kinase assays reported here. It is clear that the p110 protein is not phosphorylated in resting cells as shown here or in studies of the recombinant bovine p110 (See Chapters 4 and 7). Roache et al., (1994) have also specifically shown tyrosine phosphorylation of p110 to be coincident with the ligand driven activation of PI 3-kinase in PDGF-stimulated fibroblasts both in vivo and in vitro. In addition, phosphorylated p110 has been observed in intact Balb/C3T3 cells in response to PDGF (Kavenaugh et al., 1992), insulin stimulated Fao cells (Okamoto et al., 1993) and NGF stimulated PC12 cells (RAffioni and Bradshaw, 1992). It is also of interest that the appearance of phosphorylated p110 coincides with the appearance of a p85α phosphoprotein doublet, where one member of the doublet is distinctly phosphorylated on tyrosine. Whether tyrosine phosphorylation is responsible for partially activating PI 3-kinase is still a matter of debate, although as described here, tyrosine phosphorylation does appear to be, at the very least, coincident with activation of PI 3-kinase.

It has been demonstrated that the p85α subunit of the PI 3-kinase complex immunoprecipitated from quiescent cells is phosphorylated mainly on serine and threonine residues, and only following growth factor stimulation are the p85 and p110 subunits phosphorylated on tyrosine residues with a corresponding increase in PI 3-kinase activity. The observation that the PI 3-kinase is a dual specificity enzyme (Dhand et al., 1994a; Chapter 7) with an intrinsic protein-serine kinase activity explains the predominant presence of serine phosphorylated p85 in cells (Kaplan et al., 1987; Kavenaugh et al., 1992; Reif et al., 1993; Carpenter et al., 1993; Dhand et al., 1994a). It is tempting to speculate that serine and tyrosine phosphorylation on the PI 3-kinase may provide a means of down- and up-regulating the enzyme in cells. Tyrosine phosphorylation may either act as an 'off switch' for serine phosphorylation or tyrosine phosphorylation may overcome the down-regulatory effects of serine phosphorylation by inducing some conformational change in the PI 3-kinase.

8.3.42 Induced Conformational changes

However, tyrosine phosphorylation of the PI 3-kinase cannot alone be responsible for its activation, since the PI 3-kinase complex has not been observed to be phosphorylated on tyrosine residues when in a tertiary complex containing the insulin receptor and the IRS-1

protein (Goirgetti et al., 1993; Okamoto et al., 1993; Yonezawa et al., 1992, 1992a; Backer et al., 1992; Folli et al., 1992). Although it is interesting to note that PI 3-kinase activation was found to correlate more closely with the extent of tyrosyl phosphorylation of the IRS-1 complexed to PI 3-kinase than to the amount of p85 bound to IRS-1 (Kelly and Ruderman, 1993). Indeed, tyrosyl phosphorylated IRS-1 has been shown to directly stimulate the activity of the PI 3-kinase in vitro (Meyer et al., 1992; Giorgetti et al., 1993; Backer et al., 1992). Further, the addition of tyrosine phosphorylated peptides that correspond to potential PI 3-kinase binding sites on the insulin receptor and insulin receptor substrate IRS-1, have been found to directly activate PI 3-kinase in vitro (Backer et al., 1992; Meyer et al., 1992; Gioretti et al., 1992). Similar results have been observed using phosphorylated peptides derived from the known binding sites for PI 3-kinase on polyoma middle T antigen and the PDGF receptor (Carpenter et al., 1993) and the HGF receptor (Ponzetto et al., 1993), with doubly phosphorylated peptides containing two PI 3-kinase binding sites, being far more efficient at activating PI 3-kinase than singly phosphorylated peptides. These results suggest that changes induced in the p85 proteins upon binding of phosphorylated protein tyrosine kinases may be transmitted to the associated catalytic domain and result in its activation. Evidence for conformational changes taking place has been demonstrated using circular dichroism and flouresence measurements of p85 and in particular its N-terminal SH2 domain in studies of the binding of a phosphotyrosine-containing peptide corresponding to Y751 in the kinase insert region of the PDGF receptor to p85 (Panayotou et al., 1992; Shoelson et al., 1993). Similar conclusions were reached by Waksman et al, (1992), where changes in the crystal structure of the v-src SH2 domain were observed between complexed and complexed forms.

8.3.4.3. Translocation

The most obvious mechanism for activation of the PI 3-kinase must be the physical translocation of the enzyme from its cytosolic location to the membrane where there is an enrichment of its lipid substrates PI(4)P and PI(4,5)P₂. The importance of subcellular localisation of the PI 3-kinase for its correct functioning has been demonstrated through the use of non-transforming mutants of the polyoma middle T antigen. In these experiments the creation of mutations in the NPXY box, responsible for localising the proteins to the plasma membrane, allowed association of polyoma middle T antigen with the PI 3-kinase, but since the resulting complex was not correctly localised it failed to elevate the cellular levels of 3-phosphoinositides (Ling et al., 1992). Similar results were observed in studies where the myristylation domain of v-Abl was mutated (Varticovski et al., 1991). Evidence for the ligand induced translocation of PI 3-kinase comes from Zhang et al. (1992) who have shown that in thrombin stimulated human platelets PI 3-kinase rapidly becomes associated with membrane cytoskeletal components and is

activated. Similarly, PDGF stimulation of fibroblasts has been shown to cause activation of membrane-bound PI 3-kinase (Susa et al., 1992). Subcellular fractionation studies of insulin stimulated cells showed PI 3-kinase activity was translocated to the membrane fraction within 1 min of exposure to ligand (Giorgetti et al., 1992; Kelly et al., 1992; Kelly and Ruderman, 1993).

8.3.5 Conclusion

The recent generation of a plethora of reagents to the PI 3-kinase complex will facilitate further studies investigating the regulation of this enzyme in vivo and provide more conclusive evidence refining the biological functioning of PI 3-kinase in the cell. The following Chapter examines the regulation of PI 3-kinase in a number of different cell systems by diverse receptor types and speculates on the possible role of this enzyme in signal transducing processes.

Chapter 9: Discussion

9.1 Agonist activation of PI 3-kinase

Results presented in this thesis have probed the function of the two distinct subunits of PI 3-kinase. The association of the p85 and p110 subunits has been shown in Chapter 5 to be a non covalent interaction mediated by the helical inter-SH2 domain of p85 and the Nterminal region of p110. The p110 subunit, described in Chapter 4, contains the catalytic domain of the enzyme and has been shown to possess kinase activity towards both proteins and phosphoinositides (Chapter 7). This subunit is active alone, however, it is unable to mediate interactions with activated protein tyrosine kinases (PTK) receptors. The binding of PI 3-kinase to receptors is a physiological requirement for the enzyme since its lipid substrates and the effectors it generates are located in the plasma membrane. The PI 3-kinase-PTK interaction is mediated by the SH3 and SH2 domains of the multimodular p85 subunit (Chapters 3 and 6). In addition to this 'bridging' function, the p85 protein plays a regulatory role. The data presented in Chapter 7 demonstrate that serine phosphorylation of p85 by p110 down-regulates the PI 3-kinase activity of the p85-p110 complex. Taken together the observations made using recombinant expression systems, in vitro analyses and the data from intact cell studies described in Chapter 8 suggest the following model for the regulation of PI 3-kinase in growth factor stimulated cells.

9.1.1 Regulation by receptor PTKs

Intrinsic serine phosphorylation of the p85 subunit maintains the catalytic activity of PI 3-kinase at a basal level in resting cells. Ligand stimulation could then cause activation of PI 3-kinase by a combination of factors. Firstly, the translocation of the enzyme from its cytosolic location to the plasma membrane and binding to the activated receptor PTK through engagement of its SH2 domains. Subsequent proximity to its lipid substrates, PI(4)P and PI(4,5)P, the action of a serine phosphatase and tyrosine phosphorylation of the PI 3-kinase complex may then promote further activation of the enzyme. Tyrosine phosphorylation of the p85 α protein may then also serve as a switch for dissociation from the activated receptor. Although speculative, this model provides a possible basis for the regulation of PI 3-kinase in growth factor stimulated cells.

9.1.2 Regulation by non-receptor tyrosine kinases

In this thesis, the role of PI 3-kinase in growth factor stimulated pathways involving receptor PTKs has been examined in detail, however, there is also much experimental evidence for the regulation of PI 3-kinase by non-receptor PTKs activated by cytokine, T-

cell antigen and G-protein coupled receptors. The most significant difference in structure of the cytokine and T-cell Antigen receptors compared to the growth factor PTK receptors can be seen in the cytoplasmic domains, which seem to contain no sequences associated with enzymatic activity. Intracellular signal transduction from these receptors probably works via activation of a non-receptor PTK, such as Src family kinases.

T-cell antigen and cytokines have been shown to cause a rapid activation of PI 3-kinase which results in an increase in the levels of PI(3,4,5)P3 (Remillard et al., 1991; Merida et al., 1991; Ward et al., 1992; Thompson et al., 1992). As was observed for receptor PTKs in Chapter 6, there appear to be three basic mechanisms for the activation of PI 3-kinase by these agonists.

- 1. Firstly, PI 3-kinase may associate directly with the activated receptor. For example, the SH2 domain of PI 3-kinase has been shown to bind directly to the tyrosine phosphorylated C-terminal tail of the Erthropoietin (Epo) receptor following stimulation of cells (Mayeux et al., 1993; Miura et al., 1994). Indeed in some cases, as was shown with the B cell antigen receptor mIgM in complex with CD19, PI 3-kinase bound specifically to the canonical PI 3-kinase binding motif, YXXM on CD19 which had been phosphorylated by the Src family kinase pp56^{c-lck} (Yamanishi et al., 1992; Tuveson et al., 1993).
- 2. In other instances, the interaction between the receptor and PI 3-kinase is mediated by a Src family kinase. The pp59c-Fyn and pp56c-lck mediate the interaction of PI 3-kinase with the IL-2, IL-3 and GM-CSF receptors (Augustine et al., 1991; Taichman et al., 1993; Corey et al., 1993). Furthermore, the interaction between PI 3-kinase and the CD4-pp56c-lck complex in T-cells has been shown to be mediated by the SH3 domain of pp56c-lck binding to the proline-rich region of PI 3-kinase (Thompson et al., 1992; Prasad et al., 1993a).
- 3. In the final case, an adapter protein, analogous to IRS-1, has been shown to mediate the interaction between PI 3-kinase and the receptor. IL-4 can activates PI 3-kinase (Wang et al., 1992), and PI 3-kinase activity has been seen to associate with a 170 kDa phosphoprotein termed 4PS (IL-4 induced phosphotyrosine substrate). 4PS is highly homologous to the insulin receptor substrate, IRS-1. Indeed, antibodies against IRS-1 have been shown to cross react with 4PS and a recent study has shown that in myeloid cells, which have IL-4 receptors but lack 4PS, IRS-1 is able to substitute for this 4PS deficiency and confer IL-4 dependent proliferative properties to this cell line (Wang et al., 1993). It remains to be determined if 4PS is a docking protein for other substrates of the IL 4 receptor.

9.1.3 Regulation by G-protein coupled receptors

G-protein coupled receptors are a class of seven transmembrane domain receptors that do not contain an intrinsic PTK activity or associate with non-receptor PTKs but have been found to transduce their signal by coupling to cytoplasmic, heterotrimeric G-proteins, consisting of α , β and γ subunits (Reviewed in Lefkowitz, 1993).

The possibility that G-protein mediated transduction pathways are implicated in the regulation of PI 3-kinase was first suggested by observations showing that receptors coupled to G-proteins, such as those activated in platelets stimulted by thrombin and neutrophils activated by f-Met-Leu-Phe (FMLP), can stimulate accumulation of PI(3,4,5)P₃ (Kucera and Rittenhouse, 1990; Stephens et al., 1991). This idea was strengthened by studies suggesting that pertussis toxin, which selectively inactivates some G-protein α-subunits, can block FMLP stimulated PI(3,4,5)P₃ accumulation (Traynor-Kaplan et al., 1989; Corey et al., 1993) and that mastoparan and GTP[γS], which can mimic the molecular interaction between the receptor and the G-protein, can stimulate PI(3,4,5)P₃ production in neutophils and permeabilised platelets respectively (Kucera and Rittenhouse, 1990; Norgamer et al., 1992). More recent studies suggest that FMLP might regulate PI 3-kinase activities in neutrophils via two discrete G-protein dependent pathways (Stephens et al., 1993; Vlahos and Matter, 1992).

9.1.3.1 Indirect activation of PI 3-kinase by G-protein coupled receptors.

One pathway employs an indirect route which requires the activation of Src-related PTKs. Consistent with this is the sensitivity of the response to the tyrosine kinase inhibitor staurosporine and its slower activation kinetics relative to those observed for other G-protein effectors (Stephens et al., 1993; 1993a). This non-receptor PTK associated activity makes a relatively small contribution (~5-10%) to the initial synthesis of PI(3,4,5)P₃ in FMLP-activated neutrophils (Stephens et al., 1993; Vlahos and Matter, 1992). However, in platelets which overexpress Src family PTKS (Permutter et al., 1988), this pathway may become the major mechanism by which, for example, thrombin can regulate PI 3-kinase activity. Indeed, a thrombin dependent association between a PI 3-kinase activity and pp60^{c-Src}/pp59^{c-Fyn} has been detected in platelets (Gutkind et al., 1990). However, there is no compelling evidence for tyrosine phosphorylation of any of the components of PI 3-kinase by pp60^{c-Src}/pp59^{c-Fyn} (Mitchell et al., 1990; Guinebault et al., 1993).

9.1.3.2. Direct activation of PI 3-kinase by G-protein coupled receptors

The second mechanism results in a PI 3-kinase activity which is staurosporine resistant and produces ~90% of the PI(3,4,5)P3 that accumulates during the first 10-20 seconds in the presence of FMLP (Stephens et al., 1993; 1993a). This mechanism is distinct from

that described previously and may depend on a direct interaction between a PI 3-kinase activity and activated G-protein subunits. Indeed, a distinct PI 3-kinase activity has been described in neutrophils and U937 cells which is not immunologically recognised by reagents to the characterised PI 3-kinase (Otsu et al., 1992; Gout et al., 1992; Hiles et al., 1992), is chromatographically distinct and displays unique responses to kinase inhibitors (Stephens et al., 1993; 1994). Further, a purified form of this PI 3-kinase activity becomes activated by $\beta\gamma$ G-protein subunits in vitro (Stephens et al., 1994) This suggests that the G-protein regulated pathways might contain a PI 3-kinase isoform that is distinct from that regulated by PTKs analogous to the situation for PLCs.

9.1.3.3. Regulation by small GTP-binding proteins

In addition, it is interesting to note that the p85 subunit contains a Bcr domain that shows extensive homology with a GAP domain found in a family of proteins which interact with small molecular weight guanine nuucleotide binding proteins of the Ras super family, Rac and Rho (Reviewed in Fry, 1992a; Hall, 1992; Chapter 1, section 1.6.2). Although no GAP activity for p85 has been detected a recent report suggests that Rho appears to mediate the effects of GTP[γS] on a PI 3-kinase activity in platelets (Zhang et al., 1993a). Similarly, the stimulation of PI 3-kinase by lysophosphatidic acid (LPA), which activates G-protein coupled receptors, was also shown to be regulated by a Rho-dependent pathway (Kumagai et al., 1993). In addition to Ras related proteins acting in concert with G-protein coupled receptors to regulate PI 3-kinase, a more direct interaction is also suggested by the coprecipitation of Ras with PI 3-kinase (Sjolander et al., 1991). PI 3-kinase has also been shown to associate with RasGAP (Sjolander and Lapetina, 1992). Taken together these results suggest that GTP binding proteins may directly regulate PI 3-kinase.

The studies described in section 9.1 suggest that most receptors appear to have an inherent ability to activate PI 3-kinase. An important factor underlying variability in a distinct cell type response may thus be the magnitude and duration of the signal evoked within the target cell by a particular ligand (Discussed in detail in section 9.3). The diverse affinities and the different mechanisms of interaction of various receptors with PI 3-kinase (Discussed in Chapter 6 and above) will presumably influence downstream signalling pathways and the ability of a particular ligand to elicit a biological response.

9.2 PI 3-kinase: A key signal transducing element?

9.2.1 The consequence of PI 3-kinase activation

Although the precise mechanism of PI 3-kinase activation by the diverse series of receptors described above may vary, the consequence is the production of 3-

phosphorylated phosphoinositides. PI(3,4)P2 and PI(3,4,5)P3 accumulate rapidly from negligible levels upon ligand stimulation, while there is little change in the levels of PI3P (Auger et al., 1989; Varticovski et al., 1989; Ruderman et al., 1990; Ward et al., 1992; Thompson et al., 1992; Corey et al., 1993; Jackson et al., 1992; Kucera and Rittenhouse, 1990; Stephens et al., 1991). An exception to this observation is in the yeast S. cerevisiae, which accumulate high levels of PI(3)P (Auger et al., 1989; Schu et al., 1993). Data from a number of studies examining the time course of accumulation and subsequent metabolism of the individual 3-phosphoinositides following stimulation indicate that PI(3,4,5)P3 is most likely to be a critical signalling molecule (Traynor et al., 1988; Stephens et al., 1991; Hawkins et al., 1992; Jackson et al., 1992). In support of this hypothesis purified PI 3-kinase has a lower Km for PI(4,5)P2 than for either PI(4)P or PI (Carpenter et al., 1990). PI(3,4)P2 and PI(3,4,5)P3 have also been detected in transformed cells independent of exogenous ligand stimulation. Cells tranformed by polyomavirus mT have elevated levels of PI(3,4)P2 and PI(3,4,5)P3 whereas cells expressing non-transforming mutants have much lower levels of these lipids (Serunian et al., 1990; Uleg et al., 1990). In addition, cells transformed with the Abl oncogene accumulate PI(3,4)P2 and PI(3,4.5)P3 (Varticovski et al., 1991) whereas those transfected with non-transforming variants have low levels of these phospholipids.

9.2.1.1 The biosynthetic source and interconversion of 3-phosphorylated inositides

The metabolic pathway involved in the formation of PI(3,4)P₂ and PI(3,4,5)P₃ is currently unclear. The most plausible mechanism suggests that both these lipids are synthesised by 3-phosphorylation of cellular PI(4)P and PI(4,5)P respectively (Auger et al., 1989; Stephens et al., 1991; Hawkins et al., 1992). This hypothesis is supported by the finding that purified preparations of PI 3-kinase can phosphorylate PI(4)P and PI(4,5)P₂ to produce PI(3,4)P₂ and PI(3,4,5)P₃ in vitro (Carpenter et al., 1990). Other reports have suggested another model for the biosynthesis of these lipids where a PI(3)P 4-kinase (Yamanishi et al., 1992) is suggested to phosphorylate PI(3)P. The PI(3,4,5)P₃ which accumulates in cells may then be synthesised via a PI(3,4)P2-directed 5-kinase activity (Cunningham et al., 1990; Cunningham and Majerus, 1991) ie, PI 3-kinase PI(3)P <u>4-kinase</u> PI(3,4)P <u>5-kinase</u> PI(3,4,5)P. Another possibility is that $PI(3,4,5)P_3$ is the primary product of PI 3-kinase and that a ligand stimulatable lipid phosphatase may produce PI(3,4)P₂, by dephophorylating PI(3,4,5)P₃ Evidence for such a pathway is based on the time course of lipid production which shows that the initial accumulation of PI(3,4,5)P₃ in ligand stimulated cells is followed after a lag period by the accumulation of PI(3,4)P₂ (Stephens et al., 1991; Hawkins et al., 1992; Kucera and Rittenhouse, 1990; Traynor-Kaplan et al., 1988;1989). Further support for this mechanism comes from the observation that PI(3,4,5)P₃ is rapidly metabolised in lysates of either 3T3 cells (Hawkins et al., 1992) or neutrophils (Stephens et al., 1991) by a predominantly membrane associated PI(3,4,5)P3 5-phosphatase. Studies of the metabolism of PI(3,4)P2 in neutrophil lysates indicate that it is metabolised by an inositol lipid 4-phosphatase activity that generates PI(3)P. Similarly, PI(3)P can be degraded by an inositol lipid 3-phosphatase (Lips et al., 1989; Caldwell et al., 1991).

9.2.2 The biological significance of PIP3 generation

Despite substantial progress in understanding how various receptors can activate PI(3,4,5)P₃ synthesis, little is known of the biological significance of this response. It is established that, in contrast to the conventional PI pathway, various PLCs from bovine brain (Serunian et al., 1989; Lips et al., 1989) are unable to hydrolyse the lipid products of PI 3-kinase. However, the knowledge that so many diverse receptors can mobilise 3-phosphorylated inositol lipid metabolism via a single type of lipid kinase activity and the fact that ligand stimulated PI(3,4,5)P₃ is removed from cells at a very rapid rate (Traynor-Kaplan et al., 1988;1989; Jackson et al., 1992 Carter and Downes, 1992) support the notion that these lipids may themselves serve as second messengers.

As virtually all mitogens stimulate PI 3-kinase a great deal of emphasis has been placed on the idea that this pathway is necessary for the mechanism by which growth factors stimulate mitogenesis. There is a very clear correlation in some systems between the presence of PI 3-kinase and transformation of cells. For example, mutants of mT, pp60^c-Src or pp60^v-Src which are unable to activate PI 3-kinase are unable to sustain transformation in their host cell (Kaplan et al., 1987; Courtneidge and Heber, 1987; Serunian et al., 1990; Uleg et al., 1990; Varticovski et al., 1991; Druker et al., 1992; Ling et al., 1992). In support of this notion the oncogenic Neu receptor is found to be permanently coupled to PI 3-kinase (Peles et al., 1992).

However, whether PI 3-kinase is sufficient for generating a signal essential for mitogenesis is less clear. Deletion of the kinase-insert region of the PDGFβ receptor leads to the generation of a receptor with a severely reduced mitogenic potential in Balb/c 3T3 fibroblasts (Escobedo and Williams, 1988; Coughlin et al., 1989) and CHO cells (Severinsson et al., 1990). Individual mutations of either of the two sites of PI 3-kinase binding have been shown to have only a partial effect (Yu et al., 1992; Fantl et al. 1992), whereas point mutations of both of the PI 3-kinase binding sites on the PDGF receptor can completely block its mitogenic response in a normal murine mammary gland fibroblast (NMuMG) cell line (Fantl et al., 1992) and in human HepG2 hepatoma and dog epithelial TRMP cells (Valius and Kazlauskas, 1993).

Another growth factor receptor well characterised with respect to its interaction with PI 3-kinase is the CSF-1 receptor. Deletion of the entire kinase-insert of the CSF-1 receptor,

which contains the binding site for PI 3-kinase, has been shown to abrogate the ability of the CSF-1 receptor to alter cell morphology and increase growth rate in response to CSF-1 in Rat-2 fibroblasts (Van der Greer and Hunter, 1993). However, the same mutant receptor expressed in NIH 3T3 fibroblasts (Choudhury et al., 1990; Reedijk et al., 1990; Shurtleff et al., 1990) was still able to transmit a reduced mitogenic response. Further, mutation of Y697, the PI 3-kinase binding site, only has a limited effect on the ability of the CSF-1 receptor to stimulate growth in stimulated Rat-2 cells (Van der Greer and Hunter, 1993). This suggests that in Rat-2 fibroblasts at least, while PI 3-kinase may play a major role in mitogenesis, other signalling molecules that also bind the kinase insert region such as Grb2 (Van der Greer and Hunter, 1993; Section 1.2.5.1), may be able to compensate for a CSF-1 receptor unable to bind PI 3-kinase.

All these results are in conflict with data from a number of studies where deletion of the kinase-insert of the PDGF α receptor containing the binding sites for PI 3-kinase (Heidarhan et al., 1991), point mutations of Y740 and Y751 of the PDGF β receptor (Kazlauskas et al., 1992), mutation of the equivalent residue in PDGF α , Y731 and Y742 (Yu et al., 1991) and deletion of the kinase insert of the CSF-1 receptor (Taylor et al., 1989) had no obvious effect on mitogenic signalling by these receptors in a hematopetic 3D cells, TRMP and NIH 3T3 cells. Thus in this case, alternative signalling systems can be used to generate a mitogenic response and this is discussed in more detail in Section 9.3.

Taken together these data suggest that while activation of PI 3-kinase may be important for generating a proliferative signal from certain receptors in some cells types, its primary role is probably in controlling a more general aspect of intracellular signalling important in diverse signalling pathways. Indeed, the activation of PI 3-kinase has not only been observed in proliferating cells but also in NGF induced differentiation of PC12 cells (Carter and Downes, 1992), and terminally differentiated cells such as FMLP and thrombin stimulated neutrophils (Traynor et al., 1988; Stephens et al., 1991) and platelets respectively (Kucera and Rittenhouse, 1990; Nolan and Lapetina et al., 1991). Thus the PI 3-kinase signal is confined not only to mitogenesis but also cellular differentiation and other biological responses.

The observations described above stimulate consideration of where PI 3-kinase might function in signal transduction. Evidence has been presented that shows PKC ζ may be activated in vitro specifically by PI(3,4,5)P3 (Nakanishi et al., 1993a). PKC ζ is a ubiquitous isoform of PKC which is not thought to be activated by phorbal esters or diacylglycerol and whose physiological targets are presently unknown (Asaoka et al., 1992). However, this interaction has yet to be shown to take place in vivo. Also

intriguing is the finding that PKCζ can function in a Ras-dependent pathway in mitogenic signal transduction (Berra et al., 1993). Thus, if the 3-phosphoinositides are required to activate PKCζ, one can speculate that PI 3-kinase and Ras function in the same signalling pathway. Indeed, there are several lines of evidence which implicate this. Anti-Ras immunoprecipitates from Ras transformed rat liver epithelial cell lines coimmunoprecipitate PI 3-kinase activity (Sjolander et al., 1991). Moreover, PI 3-kinase activity in the anti-Ras immunoprecipitates was shown to be increased following insulin or IGF-1 treatment (Sjolander et al., 1991). Also a mutant PDGFβ receptor (Y740F/Y751F), defective in binding PI 3-kinase, was unable to stimulate activation of Ras, although mutants that were unable to bind either GAP or PLCy had no effect (Satoh et al., 1993; Fantl et al., 1992). Moreover, PI 3-kinase was shown to function upstream of Ras and Raf in CHO cells in the insulin stimulated signalling pathway leading to the transcriptional activation of the c-fos serum response element (Yamauchi et al., 1993). It is noteworthy that Downes and Carter (1991) have proposed a possible role for 3phosphoinositides in the kinase cascade leading to the activation of MAP kinase, the S6 ribosomal protein p90Rsk and subsequently mitogenesis. If this is the case it may explain why PI 3-kinase is activated in mitogenic pathways by PDGF and CSF-1 (Fantl et al., 1992; Van der Greer and Hunter, 1993), as well as in non-mitogenic pathways such as NGF stimulation of PC12 cells (Carter and Downes, 1992), insulin stimulation of adipocytes (Giorgetti et al., 1992; Kelly et al., 1993), or GMCSF and FMLP stimulation of neutrophils (Gomez-Cambronero et al., 1989). In addition, in mammalian cells the p70 S6 kinase pathway is known to be sensitive to the immunosupressant rapamycin (Kuo et al., 1992; Price et al., 1992). Thus the finding that a yeast PI 3-kinase, TOR2, may also be a target of rapamycin (Kunz et al., 1992), further fuels speculation that a lipid kinase product may be required for the activation of another signalling pathway implicated in mitogenesis.

9.2.3 Potential targets of PI 3-kinase action

9.2.3.1. Development

Consistent with a role for PI 3-kinase functioning upstream of MAPK activation, is the observation that injection of oocytes with the C-terminal SH2 domain of p85 blocked progesterone induced MAP kinase (Muslin et al., 1993). Further, this SH2 domain was found to inhibit Xenopus oocyte maturation, an effect which could be overturned by coinjection of c-mos which directly activates the MAP kinase pathway (Muslin et al., 1993). Similar results were seen using insulin as a stimulus (Chang et al., 1993). These results suggest not only a physiological function for PI 3-kinase but also that the enzyme functions upstream of c-mos and MAP kinase. It is also of interest that PKC ζ has been shown to be necessary for oocyte maturation (Berra et al., 1993).

9.2.3.2. Regulation of the cytoskeletal network.

Mitogenic proliferation is closely associated with extensive changes in the actin cytoskeleton including cell rounding and membrane ruffling. A number of studies have suggested a role for inositol lipids in the regulation of the cytoskeletal network (Stossel., 1989; Goldschmidt-Clermont, 1990). PI(4,5)P₂ has been proposed to bind profilin which inhibits actin polymerisation, and bind gelsolin which severs actin filaments (Lasing and Lindberg, 1985; Goldschmidt-Clermont etal., 1990). In addition, the rapid rise and fall in PI(3,4,5)P₃ levels in neutrophils very closely parallels actin polymerisation and subsequent depolymerisation in vivo (Erbele et al., 1990). Although these studies implicate PI(4,5)P₂ in actin polymerisation, a role for 3-phosphorylated inositides seems equally plausible.

There is further evidence which suggests a more direct function for PI 3-kinase in actin rearrangement. The Rho and Rac family of small molecular weight G-proteins have been shown to control arrangement of the actin cytoskeleton (Hall, 1992; Ridley and Hall, 1992; Ridley et al., 1993). Since PI 3-kinase possesses a Bcr domain, with homology to Rho and Rac GAPs, it might disrupt actin organisation by interacting with or stimulating the GTPase activity of Rac and Rho (Otsu et al., 1991; Fry, 1992). Indeed, an activated fraction of PI 3-kinase has been found to translocate to the membrane cytoskeleton upon thrombin stimulation of platelets (Zhang et al.,1992; Grondin et al., 1991). In addition, immunofluorescence analyses have localised p85 to the microtubule cytoskeleton in serum-starved cells (Kapeller et al., 1993) perhaps suggesting a role for PI 3-kinase in resting cells.

In transfection experiments a mutant form of the PDGFβ receptor that lacks the kinase-insert domain is incapable of inducing either actin reorganisation or mitogenesis in CHO cells (Sevrrinson et al., 1990). This mutant receptor does not bind PI 3-kinase activity. Moreover, a PDGFβ receptor containing mutations of both tyrosine residues 740 and 751 to which PI 3-kinase binds, is unable to rearrange actin filaments (observed as membrane ruffling) when stably expressed in porcine aortic endothelial (PAE) cells (Wennstromm et al., 1994; 1994a). More definitive evidence for the involvement of PI 3-kinase in membrane ruffling was observed in studies which overexpressed a mutant 85 kDa regulatory unit of PI 3-kinase, defective in binding the catalytic p110 subunit (Characterised in Chapter 6) in PAE cells or in CHO cells. PDGF and insulin stimulation, respectively, of these cells revealed both cell lines were incapable of exhibiting membrane ruffling (Wennstromm et al., 1994; 1994a; Kotani et al., 1994). Interestingly, unlike PDGF, EGF stimulation of these cells induces membrane ruffling by an alternative mechanism (Kotani et al., 1994). In addition, exposure of CHO and PAE

cells to the potent PI 3-kinase inhibitor wortmannin blocked membrane ruffling (Wennstromm et al., 1994; 1994a; Kotani et al., 1994).

As well as membrane ruffling, PI 3-kinase has also been shown to be involved in the chemotactic cell motility response (Wennstrom et al, 1994; Kundra et al., 1994). An essential feature of the chemotactic response is the formation of lamellipodia with membrane ruffles extending in the direction of movement. Studies have shown that PDGF receptor mutants lacking PI 3-kinase binding sites fail to mediate chemotaxis (Wennstrom et al, 1994; Kundra et al., 1994).

9.2.3.3. Protein trafficking

Further clues to the possible function of PI 3-kinase are provided by the observation that the catalytic subunit of the bovine PI 3-kinase is closely related to the yeast PI 3-kinase Vps34p (Herman and Emr, 1990; Hiles et al., 1992; Schu et al., 1993). Vps34p has been implicated in the targeting of proteins to the yeast vacuole, a vesicle analogous to the mammalian lysosome, as well as in vacuole morphogenesis (Reviewed in Herman et al., 1992; Chapter 4). This allows some speculation for a possible role of a PI 3-kinase in membrane trafficking and vesicle morphogenesis (Panayotou and Waterfield, 1992 trends).

A possible function for PI 3-kinase would thus be in the internalisation of the activated receptor complexes with which it associates. Indeed, tyrosine phosphorylated PDGFβ receptor and activated PI 3-kinase are found in isolated clathrin coated vesicles within two minutes after exposure of cells to ligand, hence indicating that both receptor and PI 3-kinase enter the early stages of the endocytic pathway together in 3T3-L1 fibroblasts (Kapeller et al., 1993). Moreover, a PDGF receptor mutant lacking PI 3-kinase binding sites failed to internalise, suggesting that PI 3-kinase regulates the trafficking of receptor tyrosine kinases (Joly et al., 1994). In addition, insulin stimulation of adipocytes was shown to result in the activation of PI 3-kinase in low-density microsomal fractions which include endocytic vesicles (Giorgetti et al., 1992). This is relevant as adipocytes are a major insulin target for glucose transport and utilisation, and insulin has also been shown to stimulate PI 3-kinase activity in these cells. In addition glucose transport requires vesicular trafficking. Using dominant-negative p85 mutants which are unable to interact with p110, it was shown that insulin stimulated activation of PI 3-kinase was required for glucose uptake by the translocation of GLUT 1 glucose transporters from the intracellular vesicle pool to the plasma membrane in CHO cells (Hara et al., 1994).

Yeast geneticists have identified more than 40 proteins which are involved in the biogenesis and maintanance of the yeast vacuole. Vps1p is such a protein that was identified during a screen for proteins deficient in protein sorting (Wilsbach and Payne, 1993). This protein has a mammalian homologue in the form of dynamin which was originally described as a microtubule binding protein with an intrinsic GTPase activity (Obar et al., 1990). Mutational studies have linked dynamin to endocytosis and as an intermediate step in coated vesicle formation (Chen et al., 1991a; Herskovitz et al., 1993; Van der Bliek et al., 1993). Further, the dynamin GTPase activity can be regulated by the direct binding of the p85 SH3 domain (Gout et al., 1993) and acidic phospholipids such as PI (Tuma et al., 1993) suggesting that this protein may be a target for the activated PI 3-kinase. Moreover, dynamin has a homologue in Drosophilia termed *shibire* (Vater et al., 1992) indicating the evolutionary conservation of a signalling pathway.

9.3 Specificity of growth factor signalling pathways

The ability of a cell to respond to external signals is determined firstly by the specificity of growth factor-receptor interaction and the restricted expression of genes encoding these proteins during development (Rappolee et al., 1988; Wilkinson et al., 1988; Matsui et al., 1990). Additional levels of regulation and diversity can be achieved by expression of polymorphic forms of ligand and receptor PTKs. For example, the PDGF receptor α and β isoforms bind distinct substrates (Heidaran et al., 1993). Furthermore, whereas both isoforms of the PDGF receptor can transduce a mitogenic response, only the β -receptor mediates chemotaxis and actin reorganisation in the form of circular membrane ruffles (Errikson et al., 1992). Similarly, the signalling and biological responses elicited by different FGF receptors, which respond to distinct but related factors, has been found to differ substantially (Wang et al., 1994).

The pleiotropic function of receptor-PTKs in development is also determined by the array of downstream signal transduction pathways that are activated by the receptor. The PDGF receptor is able to engage or activate a diverse complement of signalling molecules including PI 3-kinase, GAP, PLCy, Raf-1, pp60c-Src, Vav, SHPTP2-GRB2-Sos and Nck (Reviewed in Pawson and Schlessinger, 1993; Chapter 1). However, the EGF and CSF-1 receptors activate only subsets of these intracellular substrates. Such complementation may explain the synergistic effects observed when growth factors are applied together (Rozengurt, 1986) and the differential potencies of various mitogens in particular cells. Variation in the kinetics of activation of growth factor stimulated signalling pathways will further enhance diversity.

There is a growing body of literature indicating that growth factor receptors are able to initiate more than one signal transduction pathway. PLCy has been shown to play a central role in signal transduction, but using specific PDGF receptor mutants, was proposed not to be of primary importance in the presence of PI 3-kinase in any particular cell type (Valius and Kazlauskas, 1993; Satoh et al., 1993). This suggests that the PI 3kinase- and PLCy-mediated pathways, may converge and thus, as for the CSF-1 receptor, the absence of PLCy binding may be compensated for. The signal to initiate DNA synthesis may involve more than just activation of Ras (Valius and Kazlauskas, 1993). MAP kinases been shown to be activated by three distinct pathways that coexist within one cell type involving either Ras, PKC or calcium. Depending on the cell type (Rat-1, NIH, Swiss 3T3), growth factors may connect to one or more of these pathways (Burgering et al., 1993). PDGF has been shown to stimulate multiple signalling pathways by the activation of PLCy, GAP and PI 3-kinase. Inhibition of one of these pathways using specific PDGF receptor mutants may or may not affect Ras activation, depending on which cell type expresses the receptor (Satoh et al., 1993; Valius and Kazlauskas, 1993).

An interesting example of the most diverse effects of growth factor mediated signalling is observed in PC12 cells upon the addition of EGF and NGF. The biological effects promoted by EGF are primarily proliferative, while NGF is a differentiation factor. Although the biological effects of these two growth factors are thought to be clearly defined, the initial signalling events elicited by these two growth factors do not appear to differ. Both NGF and EGF induce a similar set of immediate early genes (Greenberg et al., 1985; Bartel et al., 1989) and the same set of cellular proteins are activated, including substrates such as PLCy and PI 3-kinase (Kim et al., 1991; Vetter et al., 1991; Downes and Carter, 1992). Further, both growth factors stimulate the activation of Ras (Bar-Sagi and Feramisco, 1985; Hagag et al., 1986; Thomas et al., 1992; Wood et al., 1992; Qui and Green, 1991). However, these signalling pathways culminate with NGF inducing neurite outgrowth and EGF inducing proliferation. However, recent data from several laboratories has suggested a link between the sustained action of signalling molecules and theinduction of neurites in PC12 cells. For example NGF induces prolonged activation of Ras, PI 3-kinase and MAP kinase, whereas EGF only transiently activates these molecules in PC12 cells (Qui and Green, 1991; Carter and Downes, 1992; Traverse, 1992).

The concept is rapidly emerging that the choice of cell used to study a mitogenic response is going to be crucial to defining the role of a specific growth factor, particularly regarding the requirement for a distinct signalling molecule. Most such studies have involved the use of receptors mutated at specific sites to prevent recruitment of a

signalling protein, and then a test of the effects of this recruitment on growth factor induced DNA synthesis. However, the picture emerging suggests that the nature of protein-protein interactions is more complex than originally anticipated and more than one protein may bind to a particular site on the activated receptor molecule. For example, both PI 3-kinase and the Nck bind Y751 on the PDGFB receptor (Fantl et al., 1992; Nishimura et al., 1993) and both PLCy and Syp have been shown to bind Y1009 on the PDGFβ receptor (Ronnenstad et al., 1992; Lechleider et al., 1993). Thus, mutation of either of these residues will result in defects produced by the loss of more than one signal protein. It should be kept in mind that these assays have employed cell lines which contain no endogenous receptors, hence, the signalling pathways downstream of the transfected receptor may have been downregulated and thus not reflect the true physiological situation. In addititon, the recipient cells are invariably semi-transformed immortal cell lines in which one or more of the signalling pathways under investigation could have already been altered or constitutively stimulated. Thus, the recruitment of a particular enzyme by receptors may be redundant if molecules downstream in the signalling cascade are already activated. A further complication arises from the fact that transfected receptors are often overexpressed and therefore lower affinity interactions, using alternative autophosphorylation sites may become more prominent.

Against this background the observed intricate network of protein-protein interactions that regulate the activation of signalling pathways by tyrosine kinases reveals a complex picture for the mechanism of cellular transformation. However, the recent discovery that critical genes for the regulation of the cell cycle have been functionally conserved from yeast to man suggests that basic features of cell growth control will be conserved throughout the eukaryotic kingdom. This may allow definition of signal transducing molecules that are central to growth and development in all species and may ultimately lead to the development of drugs that will provide a potential means for inhibiting activated signalling pathways.

Abbreviations

Acceped abbreviations in this thesis are listed in Biochem. J. (1992) **281**, 11–12. In addition the following abbreviations are used:

 β ARK β -Adrenergic receptor kinase

ALL Acute lymphocytic leukemia

BSA Bovine serum albumin
CHO Chinese hamster overy

CML Chronic myelogenous leukemia
CSF-1 Colony stimulating factor-1

DAG Diacylglycerol

DMEM Dulbecco's modified Eagle's medium

DTT Dithiothreitol

ECL Enhanced chemiluminescence

EGF Epidermal growth factor

Epo Erthropoietin

ERK Extracellular signal-regulated kinase

FCS Foetal calf serum

FGF Fibroblast growth factor

FMLP N-formylmethionyl-leucyl-phenylalanine

FSBA Flourosulphonylbenzoyladenosine

GAP GTPase activating protein

GM-CSF Granulocyte macrophage colony-stimulating factor

GNRF Guanine nucleotide releasing factor

GST Glutathione S-transferase HGF Hepatocyte growth factor

IAA Indoleacrylic acid

IFN Interferon

IgG Immunoglobulin

IGF-1 Insulin-like growth factor-1

Ins Inositol

IL- Interleukin-

IPTG Isopropyl-2-D-thiogalactopyranoside

IRS-1 Insulin recepter substrate

KLH Keyhole limpet haemocyanin

LPA Lysophosphatidic acid

MAP2 Microtubule-associated protein 2

MAP kinase Mitogen-activated protein kinase

mT middle T

NF1 von Recklinghausen neurofibromatosis (type I)

NGF Nerve growth factor

NP-40 NonidetP-40

PAE Porcine aortic endothelial

PAO Phenyl arsine oxide

PBSA Dulbecco's phosphate buffered saline solution A, pH 7.2

PDGF Platelet-derived growth factor

PEG Polyethylene glycol
PH Pleckstrin homology
PI Phosphatidylinositiol

PI 3-kinase Phosphatidylinositol 3-kinase

PKC Protein kinase C
PLC Phospholipase C

PMSF Phenylmethylsulphonyl fluoride

PTK Protein tyrosine kinases

PTPase Phosphatase

PVDF Polyvinylidene difluoride

RSV Rous sarcoma virus

Sf9 Spodoptera frugiperda-9 SH2/3 domain Src homology 2/3 domain

SV40 Simian virus 40

TCA Trichloroacetic acid

TCR T cell receptor

TGF- β Transforming growth factor- β TLC Thin layer chromatography

TPA 12-O-tetradecanoyl 13-phorbol acetate
Tween 20 Polyoxyethylenesorbitan monolaurate

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