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PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity

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Phosphatidylinositol 3-kinase (PI 3-kinase) has a regulatory 85 kDa adaptor subunit whose SH2 domains bind phosphotyrosine in specific recognition motifs, and a catalytic 110 kDa subunit. Mutagenesis of the p110 subunit, within a sequence motif common to both protein and lipid kinases, demonstrates a novel intrinsic protein kinase activity which phosphorylates the p85 subunit on serine at a stoichiometry of ~1 mol of phosphate per mol of p85. This protein-serine kinase activity is detectable only upon high affinity binding of the p110 subunit with its unique substrate, the p85 subunit. Tryptic phosphopeptide mapping revealed that the same major peptide was phosphorylated in p85α both *in vivo* in cultured cells and in the purified recombinant enzyme. N-terminal sequence and mass analyses were used to identify Ser608 as the major phosphorylation site on p85α. Phosphorylation of the p85 subunit at this serine causes an 80% decrease in PI 3-kinase activity, which can subsequently be reversed upon treatment with protein phosphatase 2A. These results have implications for the role of inter-subunit serine phosphorylation in the regulation of the PI 3-kinase *in vivo*.

Key words: autoregulation/dual specificity/PI 3-kinase/protein-serine kinase

Introduction

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 (PTKs) (reviewed in Schlessinger and Ullrich, 1992). Phosphatidylinositol 3-kinase (PI 3-kinase) has emerged as an important signal transducing molecule which is activated by diverse PTK receptors in a variety of cell types (Panayotou and Waterfield, 1993). There are currently few clues to the events triggered by activation of this enzyme since this occurs in diverse signalling pathways, but it is likely that phosphatidylinositol 3,4,5-trisphosphate (Stephens *et al.*, 1993), and possibly other 3-phosphoinositides, will act as some form of thus far unknown second messenger. Recent studies on the interaction of PI 3-kinase with the activated platelet-derived growth factor (PDGF) β-receptor (Fantl *et al.*, 1992; Valius and Kazlauskas, 1993) or with the polyoma middle T antigen/pp60$^{c\text{-}src}$ transforming complex (Ling *et al.*, 1992), support this conclusion, and thus suggest that these lipids will be important in the regulation of events leading to mitogenesis and cell transformation.

PI 3-kinase purified from rat brain or from bovine brain or thymus, exists as a heterodimer composed of 85 and 110 kDa subunits (Carpenter *et al.*, 1990; Morgan *et al.*, 1991; Shibasaki *et al.*, 1991; Fry *et al.*, 1992). Through cDNA cloning using protein sequence data, the primary structures of the p85 subunit (Escobedo *et al.*, 1991; Otsu *et al.*, 1991; Skolnik *et al.*, 1991) and the p110 subunit (Hiles *et al.*, 1992) have been elucidated. At least two distinct 85 kDa proteins, termed p85α and p85β, have been identified, but neither of these isoforms possesses PI 3-kinase activity (Escobedo *et al.*, 1991; Otsu *et al.*, 1991). Analysis of the primary sequence of p85 reveals a multidomain structure containing a number of non-catalytic modules previously identified by sequence homology analysis. Of particular note are the N-terminal Src homology 3 (SH3) region, two SH2 domains and a domain with significant sequence similarity to the product of the breakpoint cluster region (BCR) gene, which may possess a specific GAP activity as has been reported for proteins with this homology domain [Otsu *et al.*, 1991; reviewed in Fry (1992) and Pawson and Gish (1992)]. The presence of these distinct functional domains suggests that the p85 proteins have multiple interactive and regulatory roles.

Only a single form of p110 has been cloned to date and purified from bovine brain (Fry *et al.*, 1992; Hiles *et al.*, 1992), but protein purification studies using other tissues (Carpenter *et al.*, 1990; Shibasaki *et al.*, 1991) and PCR analysis (L.Macdougall and S.Volinia, unpublished observations) suggest that additional isoforms may exist. Expression studies have clearly demonstrated that the bovine p110 subunit can alone encode a PI 3-kinase activity (Hiles *et al.*, 1992). Amino acid sequence analysis of p110 does not reveal the presence of any characterized domains, although comparison of the p110 cDNA sequence with a yeast PI 3-kinase, Vps34p, shown to be involved in vesicle sorting, has allowed a putative lipid kinase consensus region to be defined at the C-terminal region (Herman and Emr,

1990; Herman *et al.*, 1992; Hiles *et al.*, 1992; Schu *et al.*, 1993).

While structural analysis of the p85 and p110 subunits has proceeded at a rapid pace, the basis for the regulation of PI 3-kinase activity remains poorly understood. There are several ways by which regulation might be achieved *in vivo* (reviewed in Panayotou and Waterfield, 1992). Since p85 binds receptors through its SH2 domains, regulation of enzymatic activities intrinsic to the associated catalytic subunit could result from physical translocation of the enzyme to the membrane with access to substrate or by interactions between the subunits which result in the activation of the PI 3-kinase activity intrinsic to the p110 subunit. Evidence for the physical translocation 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). The latter mechanism involves changes induced in the p85 protein upon binding to PTKs which are then transmitted to the associated catalytic domain and result in its activation. Such a change has been detected when the p85 subunit of PI 3-kinase and in particular its N-terminal SH2 domain bind to a phosphotyrosine-containing peptide, Y_{751}, which includes Tyr_{751} of the PDGF β-receptor, the novel PI 3-kinase binding site (Panayotou *et al.*, 1992). Moreover, tyrosine phosphorylated peptides that correspond to potential PI 3-kinase binding sites on the insulin receptor substrate, IRS-1, or the intact IRS-1 protein, have been shown to increase the activity of PI 3-kinase *in vitro* (Backer *et al.*, 1992; Carpenter *et al.*, 1993a; Giorgetti *et al.*, 1993). Alternatively, phosphorylation on the p85 or the p110 subunit, for example on tyrosine, serine or threonine residues could regulate kinase activity. PI 3-kinase was originally identified as a phosphotyrosyl protein (Courtneidge and Heber, 1987; Kaplan *et al.*, 1987) and Hayashi *et al.* (1992) have recently shown a correlation between insulin stimulated tyrosine phosphorylation of p85 and an increase in the specific activity of PI 3-kinase. The report that a decrease in PI 3-kinase activity follows treatment of the enzyme with a phosphotyrosyl protein phosphatase (Ruiz-Larrea *et al.*, 1993) is consistent with the concept that tyrosine phosphorylation of PI 3-kinase by an activated PTK can also cause an increased PI 3-kinase activity. Serine and threonine phosphorylation of the components of the PI 3-kinase complex may also play a role in regulation of the enzyme as several studies have shown that the p85α subunit of the PI 3-kinase immunoprecipitated from quiescent cells is phosphorylated on serine and threonine residues (Kaplan *et al.*, 1987; Cohen *et al.*, 1990; Reif *et al.*, 1993). The purified bovine brain preparation of PI 3-kinase has been shown to copurify with an associated protein serine kinase activity (R.Dhand, M.Fry and F.Ruiz-Larrea, unpublished data) and a recent report from Carpenter *et al.* (1993) showed that a similar activity, which they termed PIK kinase, may copurify with and regulate rat liver PI 3-kinase.

To investigate further the nature of the associated protein-serine kinase and its possible role in the regulation of the PI 3-kinase, we have used the wild type and mutant recombinant PI 3-kinase subunits 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. We have established a high stoichiometry of phosphorylation and also identified the single serine phosphorylation site on the p85 protein. In addition we show that this specific serine phosphorylation can regulate the PI 3-kinase activity. The role of such a dual specificity kinase in signal transduction processes is discussed.

Results

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 3-kinase (Kaplan *et al.*, 1987; Cohen *et al.*, 1990; Carpenter *et al.*, 1993b; Reif *et al.*, 1993). However, it has remained unclear from these studies whether this activity represented a tightly bound cellular enzyme, or 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 have allowed us to make a more detailed investigation of the associated serine kinase. Insect cells (Sf9) were infected with baculoviruses which mediated expression of either p85α or p85β alone, or were coinfected with a virus which expressed p110. The p85α/p110 and the p85β/p110 complexes were then either immunoprecipitated with antibodies directed against the p85 or p110 subunit, or bound to a Y_{751} phosphopeptide affinity column (Otsu *et al.*, 1991; Fry *et al.*, 1992). p85β has been shown to reconstitute with p110 to form an active complex as efficiently as does p85α (Dhand *et al.*, 1994). The bound proteins were then used in protein kinase assays. The results shown in Figure 1 demonstrated that, in the absence of p110, neither p85α nor p85β could be phosphorylated *in vitro* (lanes 5 and 6). However, when the insect cells were coinfected with viruses expressing p110, both the p85α and the p85β proteins were found to be phosphorylated when analysed following immuno-

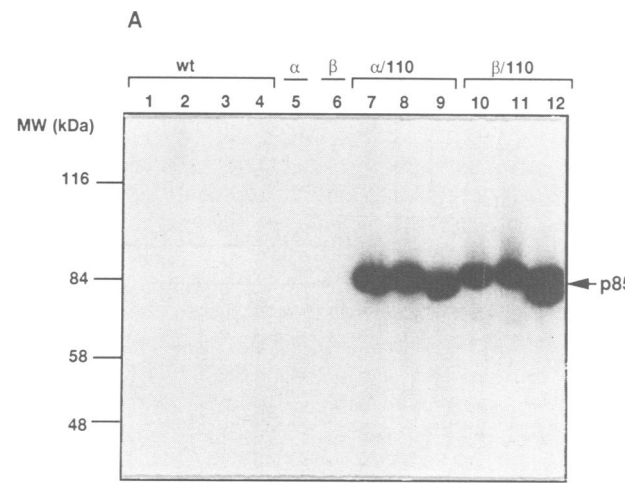

Fig. 1. Both p85α and p85β are substrates for a protein kinase activity. (**A**) Sf9 cells were infected with wild type (wt) virus (lanes 1–4), p85α (lane 5) or p85β (lane 6), or were coinfected with p85α/p110 viruses (lanes 7, 8 and 9) or p85β/p110 viruses (lanes 10, 11 and 12). Lysates of these cells were precipitated using polyclonal, affinity purified antibodies raised against p85α (lanes 1 and 7), monoclonal antibodies raised against p85β (lanes 2 and 10) or polyclonal, affinity purified antibodies raised against p110 (lanes 3, 8 and 11) or bound to an immobilized Y_{751} phosphopeptide (lanes 4–6, 9 and 12). Samples were subjected to *in vitro* protein kinase assays and analysed by SDS–PAGE and autoradiography.

precipitation using antibodies directed to either subunit of the complex (Figure 1, lanes 7, 8, 10 and 11), or after binding to the phosphopeptide column (Figure 1, 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 the phosphopeptide affinity beads from control infected cells (Figure 1, 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+} (data not shown).

The nature of the protein kinase activity associated with the PI 3-kinase complex from Sf9 cells infected with recombinant baculoviruses was investigated further. Phosphoamino acid analysis of the PI 3-kinase complex affinity purified using Y_{751} phosphopeptide and then phosphorylated *in vitro* revealed that the p85α subunit contained exclusively phosphoserine (Figure 2A). The level of phosphate incorporated into 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 p85α- and p110-expressing baculoviruses and metabolically labelled with $^{32}PO_4$. Lysates of these cells were bound to the Y_{751} phosphopeptide beads which were then washed; SDS buffer-eluted proteins were visualized by Coomassie Blue staining after SDS–PAGE. Autoradiography of these gels revealed a much higher level of phosphate incorporated into p85α than into p110 with respect to the amount of protein present which was determined by Coomassie Blue staining (Figure 2B, lanes 1 and 2). Phosphoamino acid analysis revealed that the p85α contained both phosphoserine and phosphothreonine when labelling was carried out *in vivo*, while the p110 subunit contained only phosphoserine (Figure 2C, i and ii).

Anti-p110 immunoprecipitates of p85α/p110 were used to study the kinetics of phosphorylation. The measured K_m (ATP) for the phosphorylation of p85α was ~4 μM. The stoichiometry of phosphorylation of p85α was then measured in the presence of excess ATP (50 μM). Approximately 0.9 mol of phosphate was incorporated into 1 mol of p85α protein.

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.

Mutagenesis of p110 abolishes both kinase activities. Neither of the p85 protein amino acid sequences exhibits any recognizable motifs related to those found in protein kinases (Hanks *et al.*, 1988) or to those of ATP- or GTP-binding domains in other proteins (Saraste *et al.*, 1990). Thus, the p110 subunit, which has redundant 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

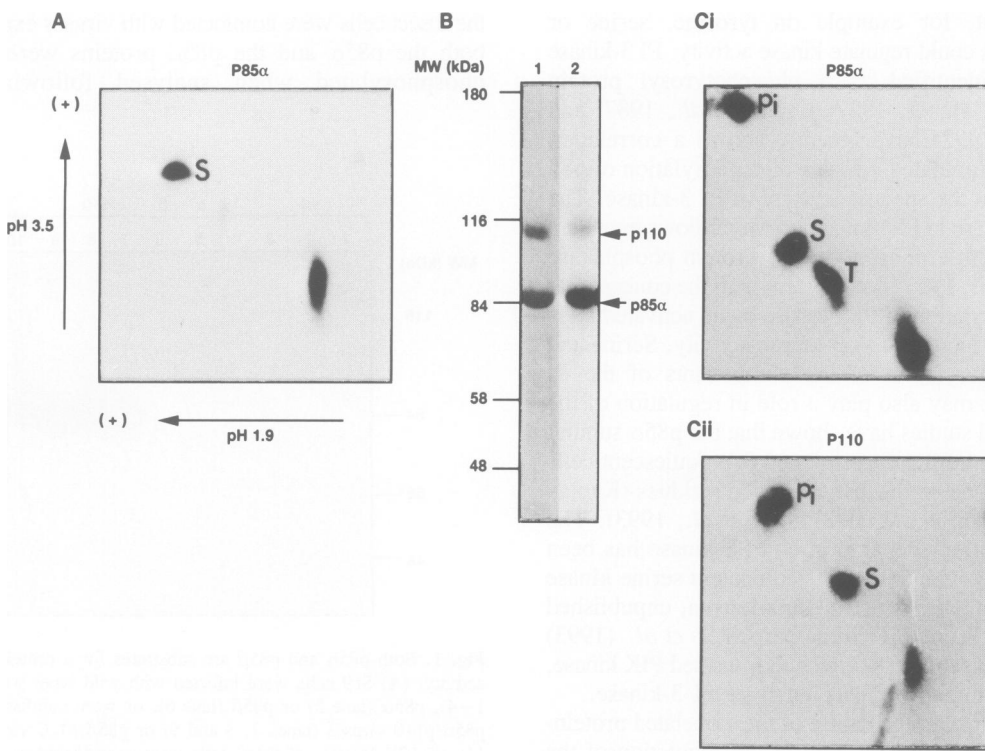

Fig. 2. Phosphoamino acid analysis of PI 3-kinase *in vivo* and *in vitro*. **(A)** Phosphoamino acid analysis of the p85α subunit from p85α/p110 complex phosphorylated *in vitro* using [γ-^{32}P]ATP, after affinity purification using phosphopeptide. **(B)** Insect cells that had been coinfected with p85α/p110 viruses were labelled with $^{32}PO_4$ and bound to Y_{751} phosphopeptide. The beads were then washed and visualized by Coomassie Blue staining of the SDS–polyacrylamide gel (lane 1) and then subjected to autoradiography (lane 2). **(C)** Phosphoamino acid analysis of (i) p85α and (ii) p110 excised from the gel in (B).

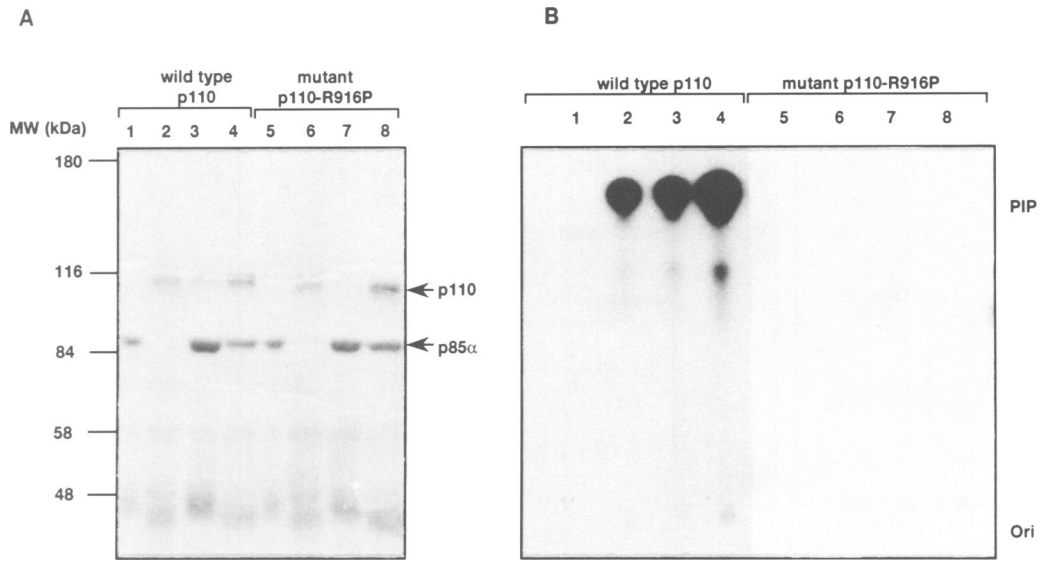

Fig. 3. Mutagenesis defines p110 as the catalytic subunit of the PI 3-kinase. (**A**) Coomassie Blue stain of a 7.5% SDS–polyacrylamide gel of lysates of infected insect cells immunoprecipitated with the described antibody and treated as follows: anti-p85α (lane 1); anti-p110 (lane 2); anti-p85α immunoprecipitate, 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-p110 immunoprecipitate of insect cells infected with p110-R916P (lane 6); anti-p85α immunoprecipitate incubated with mutant p110-R916P containing Sf9 cells *in vitro* (lane 7); anti-p110 immunoprecipitate of insect cells that had been coinfected with p85α/mutant p110-R916P viruses. (**B**) PI 3-kinase assays were performed on immunoprecipitates infected and treated as described in panel (A).

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, R916P, which converted arginine 916 to proline within the DRHNSN motif of bovine p110. This mutant protein, p110-R916P, was then expressed in Sf9 cells using a baculovirus transfer vector.

As the p110 subunit has clearly been shown by itself to express PI 3-kinase activity (Hiles *et al.*, 1992) the effects of this mutation on the intrinsic phosphoinositide kinase activity were first assessed. Insect cells were infected with p85α and p110 viruses either separately or together. Immunoprecipitated p85α and p110 could then be visualized as Coomassie Blue stained proteins following resolution on SDS–polyacrylamide gels (Figure 3A, lanes 1 and 2). The p110 protein was found to form a stable complex with p85α if the two proteins were allowed to associate *in vitro* or if they were coexpressed in insect cells, as can be seen in the SDS–PAGE analysis (Figure 3A, lanes 3 and 4). PI 3-kinase assays of these samples showed that the p110 protein alone was active (Figure 3B, lane 2) and that an active complex was formed when p110 was bound to p85α either *in vitro* or *in vivo* (Figure 3B, lanes 3 and 4). Immunoprecipitated p110-R916P was also visualized by Coomassie Blue staining of SDS–polyacrylamide gels (Figure 3A, lane 6) and was seen to comigrate with the wild type p110 protein (Figure 3A, lane 2). A stable complex was recovered when insect cells coinfected with mutant p110-R916P and p85α were analysed by immunoprecipitation using anti-p110 antibodies (Figure 3A, lane 8), or when mutant immunopurified p110-R916P was allowed to associate with p85α *in vitro* (Figure 3A, lane 7). These immunoprecipitates, which contained the mutant p110, were all found to lack PI 3-kinase activity (Figure 3B, lanes 6, 7 and 8). As the expression and binding capabilities of the mutant p110-R916P were commensurate with those of wild type p110, these data suggest that the mutation has disrupted the catalytic site of the protein without completely disrupting the structure of the p110 protein.

The PI 3-kinase inactive mutant p110-R916P was then employed to study the effect of this mutation on the associated protein-serine kinase activity. Insect cells were coinfected either with p85α and wild type p110 or with p85α 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 p85α subunit was seen to be phosphorylated *in vitro* when in complex with wild type p110 (Figure 4A, lane 1). However, in sharp contrast, the p85 subunit associated with the mutant p110-R916P was not phosphorylated in this assay (Figure 4A, lane 2). Neither the wild type p110 nor the mutant p110-R916P alone was observed to autophosphorylate (Figure 4A, lanes 3 and 4).

Since p110 is able to bind p85α *in vitro* as well as *in vivo*, the possibility that p110 could exhibit a trans-kinase activity by binding to, and phosphorylating, p85α *in vitro* was examined. p85α from insect cells was bound to phosphopeptide beads which were washed stringently. The beads were then incubated either in lysis buffer or with lysates of insect cells that had been infected with either wild type p110 or mutant p110-R916P. The complexes were washed, phosphorylated *in vitro* and then analysed by SDS–PAGE and autoradiography. The p85α protein expressed alone was not phosphorylated (Figure 4B, lane 3), while the p85α that had been bound *in vitro* to wild type p110 was heavily phosphorylated (Figure 4B, lane 1). In contrast, p85α complexed with mutant p110-R916P was not phosphorylated (Figure 4B, 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 p85α as a glutathione *S*-transferase (GST) fusion protein was employed as an alternative source of this protein. The experiment was then carried out as

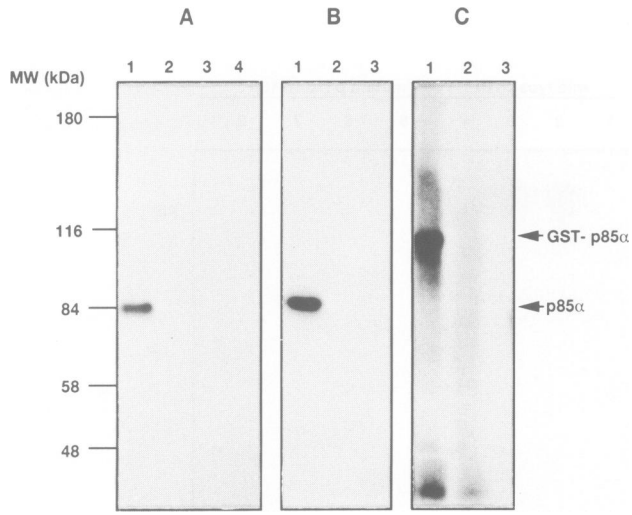

Fig. 4. p110 possesses intrinsic protein-serine kinase activity. (**A**) Insect cells were infected with viruses as follows: p85α and p110 (lane 1); p85α/mutant p110-R916P (lane 2); p110 alone (lane 3); mutant p110-R916P alone (lane 4). Anti-p110 immunoprecipitates of these samples were then subjected to *in vitro* phosphorylation and analysis by autoradiography. (**B**) p85α was affinity purified using the Y_{751} phosphopeptide column from Sf9 cells and then incubated *in vitro* with lysates of Sf9 cells infected with p110 (lane 1) or mutant p110-R916P (lane 2), or wild type virus (lane 3). Washed samples were then subjected to *in vitro* protein kinase assays and then analysed by SDS−PAGE and autoradiography. (**C**) GST-p85α was bound to affinity resin and then treated as described in (B).

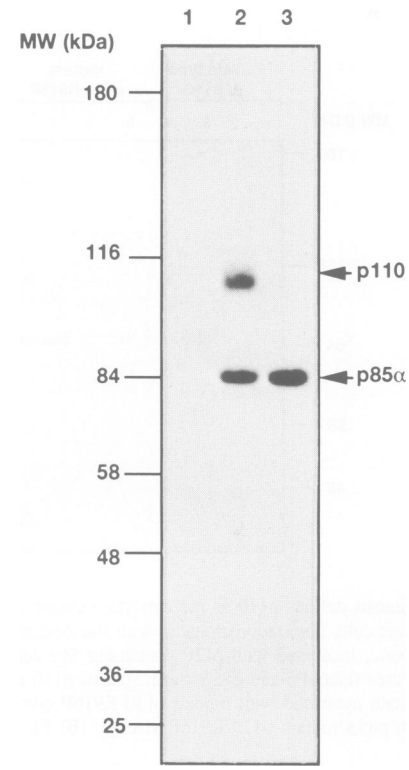

Fig. 5. [^{35}S]methionine labelling of the PI 3-kinase complex. Insect cells infected with wild type virus (lane 1), p85α virus and p110 viruses (lane 2), or p85α virus alone (lane 3) were labelled with [^{35}S]methionine for 16 h. Lysates of these cells were then bound to a Y_{751} phosphopeptide column. After several washes radiolabelled proteins were analysed by SDS−PAGE and autoradiography.

described above with this material. Following binding, *in vitro* kinase assays revealed that the GST-p85α alone had no associated protein kinase activity (Figure 4C, lane 3) but, upon association with wild type p110, this p85α fusion protein became heavily phosphorylated (Figure 4C, lane 1). Again no phosphorylation of GST-p85α bound to the mutant p110-R916P was detected (Figure 4C, lane 2).

No other proteins are detected in association with p85 and p110. To complement the mutagenesis data and to 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 [^{35}S]methionine. Lysates of these cells were then incubated with Y_{751} 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 5 do not reveal any proteins bound to the affinity phosphopeptide from insect cells infected with wild type baculovirus. Furthermore, from cells that had been infected with either p85α alone, or p85α together with p110, no other associated proteins were detected even after a 3 day autoradiographic exposure. It is thus clear that an extrinsic kinase associated with p85α or p110 would have to be present at a very low stoichiometry.

Sucrose gradients can be an efficient alternative method for the fractionation and purification of macromolecules. Thus, lysates of Sf9 cells infected either with p110 virus alone or with p110 and p85 viruses together, were separated on sucrose gradients, and fractions were assayed for PI 3-kinase and protein-serine kinase activity. Analysis of p110 infected cells separated on a sucrose gradient revealed that the bulk of the PI 3-kinase activity (Figure 6A) and p110 protein (data not shown) sedimented with a molecular weight consistent with its migration as a monomer, with a peak in fraction 7. As p110 does not autophosphorylate significantly and hence cannot be detected, p85 was used as an exogenous substrate for protein kinase activity. Immunoprecipitates of each fraction were made and incubated with purified p85α protein (Gout *et al.*, 1992), and these were then subjected to kinase assays. Results shown in panel B reveal a peak of phosphorylated p85, which comigrated precisely with the peak of PI 3-kinase activity. A similar analysis was performed on lysates of insect cells that had been coinfected with p110 and p85α viruses. The PI 3-kinase activity sedimented with a molecular weight consistent with that of a heterodimer of p85 and p110, peaking in fractions 10−12 (panel C). The protein-serine kinase activity that had been immunoprecipitated with antibodies specific for either p110 (panel D) or p85 (data not shown), also comigrated with PI 3-kinase activity and the bulk of the p85 and p110 proteins. These data further support the idea that the protein-serine kinase activity is intrinsic to the PI 3-kinase complex.

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 sulfhydryl-modifying reagents (Hou *et al.*, 1988; Scholz *et al.*, 1991), as are certain other kinases (Muirhead *et al.*, 1986; Maru and Witte, 1991). 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

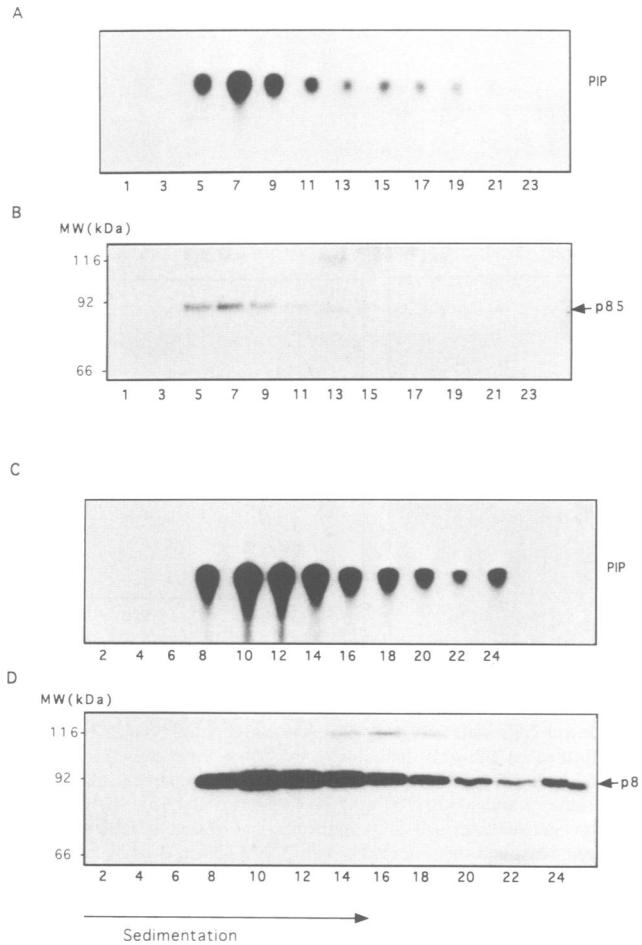

Fig. 6. PI 3-kinase activity co-sediments with serine kinase activity on a sucrose density gradient. Cells expressing p110 alone or coexpressing p110 and p85α were lysed, fractionated on sucrose gradients and harvested. Lysates of these cells were immunoprecipitated with an anti-p110 antibody and assayed for PI 3-kinase or protein kinase activity. Sedimentation was from the left to the right. PI 3-kinase activity immunopurified from fractionated cell lysate expressing p110 (**A**) and p110/p85α (**C**). Serine kinase activity immunopurified from fractionated cell lysates expressing p110 (**B**) and p110/p85α (**D**). In (B) immunoprecipitates were incubated with purified p85α for 1 h before assaying for serine kinase activity. In these gradients marker proteins sedimented as follows: bovine serum albumin (67 kDa), fraction 6; β-amylase (200 kDa), fraction 10; apoferritin (443 kDa), fraction 22.

left untreated (Figure 7A, lanes 1 and 7; Figure 7B, lane 1) or treated with 5,5′-dithio-*bis*[2-nitrobenzoic acid] (Nbs_2) and analysed on SDS gels (Figure 7A, lanes 3−6 and 9−12; Figure 7B, lanes 3−6). Approximately 95% of the PI 3-kinase activity was lost when the samples were incubated with 0.3 mM Nbs_2 (Figure 7A, lanes 3 and 9) and a similar loss of protein-serine kinase activity was observed (Figure 7B, lane 3). Both catalytic activities could be restored by incubating the modified enzyme with increasing concentrations of dithiothreitol (DTT) (Figure 7A, lanes 4−6 and 10−12; Figure 7B, 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 7A, lanes 2 and 8) and the protein-serine kinase activity (Figure 7B, lane 2). Both inactivation by Nbs_2 and reactivation by DTT showed essentially identical dose−response curves for both lipid and protein kinase activities (Figure 7C). It is likely that the loss of activity seen here is due to the formation of a disulfide bond between an essential but as yet unidentified cysteine residue(s) and thionitrobenzoate (Nbs^-) anion(s) in the catalytic domain.

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 if binding of the p85α 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 p85α (Figure 8A) were utilized as potential substrates for the protein-serine kinase 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 become phosphorylated *in vitro*. Phosphorylated proteins were analysed by SDS−PAGE and autoradiography. The inter-SH2 region of p85α has been shown to be responsible for binding to the p110 protein. In particular this interaction requires residues 478−574, since their deletion in a mutant p85αN-CΔ478−514 renders the protein totally unable to bind p110 or PI 3-kinase activity when compared with a

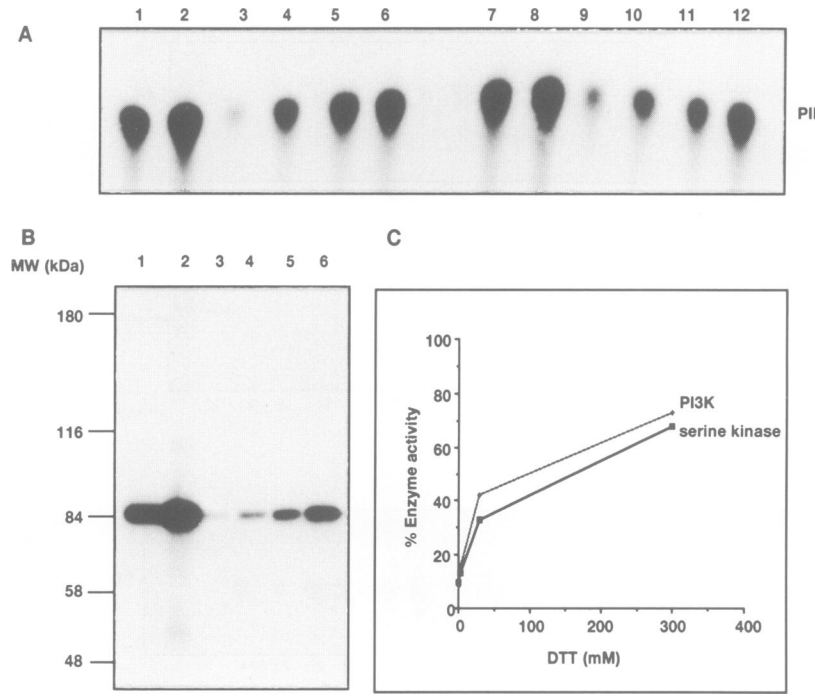

Fig. 7. Thiol requirement for catalysis. (**A**) Insect cells were infected with p85α/p110 viruses (lanes 1–6) or infected with p110 virus alone (lanes 7–12). *In vitro* PI 3-kinase assays were performed on anti-p110 immunoprecipitates of these cells that had been treated as follows: untreated (lanes 1 and 7); 300 mM DTT (lanes 2 and 8); treated with 0.3 mM Nbs_2 (lanes 3 and 9); immunoprecipitates pretreated with 0.3 mM Nbs_2 incubated with 3 mM DTT (lanes 4 and 10), 30 mM DTT (lanes 5 and 11) or 300 mM DTT (lanes 6 and 12). (**B**) Lysates of Sf9 cells that had been coinfected with p85α/p110 baculoviruses were immunoprecipitated with anti-p110 antibodies and treated as follows: untreated (lane 1); 300 mM DTT (lane 2); 0.3 mM Nbs_2 (lane 3); pretreated with 0.3 mM Nbs_2 and then incubated with 3 mM, 30 mM or 300 mM DTT (lanes 4, 5 and 6, respectively). Samples were then subjected to *in vitro* kinase assays and analysis by SDS–PAGE. Phosphorylated proteins were visualized by autoradiography. (**C**) Dose–response curves for inactivation by Nbs_2, and reactivation with increasing concentrations of DTT, for both kinase activities.

similar construct without the deletion p85αN-C (Dhand *et al.*, 1994). Results in Figure 8C 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 p85α and p85αN-C domain which includes the N- and the C-terminal SH2 domains and the region in between. The inter-SH2 domain is known to bind PI 3-kinase activity (Dhand *et al.*, 1994) 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 that the presence of the various unbound subdomains of p85 alone is not sufficient for activation of the kinase and phosphorylation to take place.

Identification of the site of phosphorylation on p85α

Phosphoamino acid analysis of the p85α subunit which was affinity purified from Sf9 cells showed that it contained phosphoserine (Figure 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 Y_{751} 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. First, 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 GST-p85α and GST-p85αN-C were 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 [γ-^{32}P]ATP. These samples were resolved on SDS–polyacrylamide gels and the p85α proteins were identified by autoradiography and excised from the gel. Following trypsin digestion, the p85α protein digests were subjected to analysis by reversed-phase HPLC and the radioactivity in the eluted fractions was analysed by measuring Cerenkov radiation. As can be seen from the results shown in Figure 9, the same major phosphopeptide was detected in maps of p85α from all the different preparations, whether the proteins had been labelled *in vitro* or *in vivo*. As the bacterial GST-p85αN-C, phosphorylated *in vitro* by p110 and digested with trypsin, contains the same phosphopeptide, these data suggest 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 analyses. Automated N-terminal Edman degradation identified the N-terminus of the phosphopeptide and suggested that it had the sequence LNEWLGNENTEDQY-SLVEDDEDLPHHDEK. Mass analysis revealed a

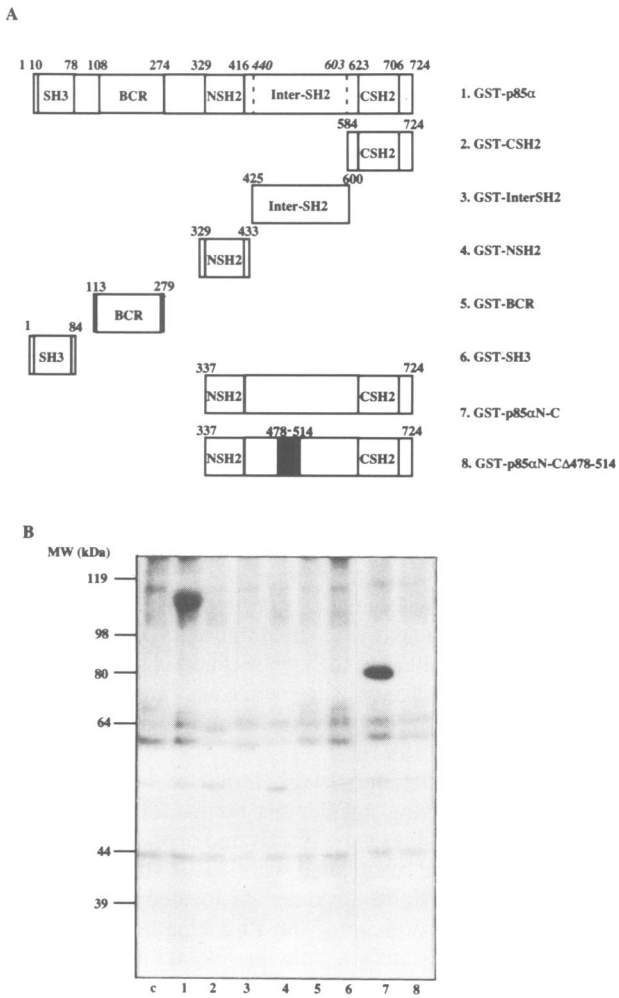

Fig. 8. p85α is only a substrate for p110 when associated in a complex. (**A**) Full-length GST-p85α (1) and deletion mutants (2–8). (**B**) p110 immunoprecipitated from insect cells was incubated with 1 μg of each of the fusion proteins 1–8 described in (A). GST alone incubated with immunoprecipitated p110 was used as a control (lane c). These proteins were then subjected to *in vitro* kinase assays in the presence of [γ-^{32}P]ATP. Phosphoproteins were analysed by SDS–PAGE and visualized by autoradiography.

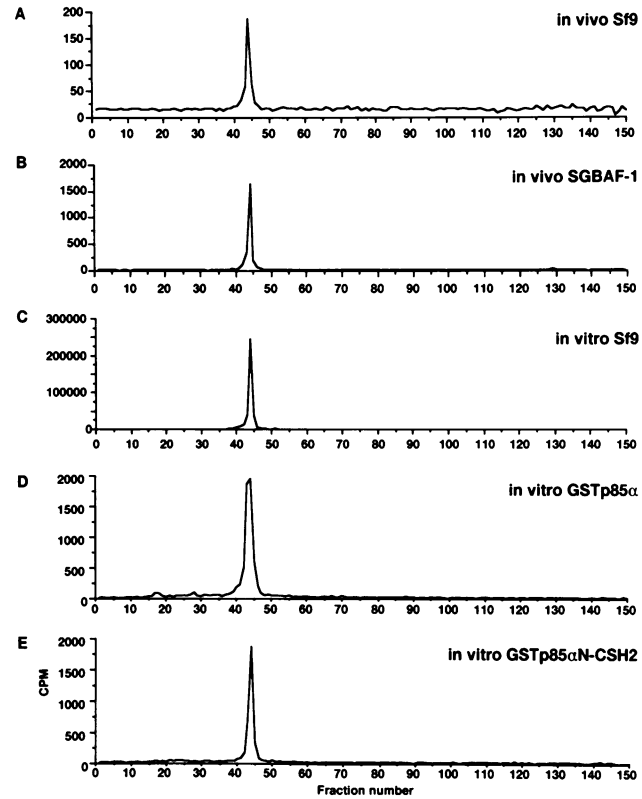

Fig. 9. Phosphopeptide mapping of p85α phosphorylated *in vivo* and *in vitro*. (**A**) Phosphopeptide-purified PI 3-kinase from Sf9 cells coinfected with p85α/p110 viruses and labelled with phosphate *in vivo*. (**B**) PI 3-kinase treated as above from SGBAF-1 cells labelled with phosphate *in vivo*. (**C**) Phosphopeptide-purified PI 3-kinase from Sf9 cells coinfected with p85α/p110 viruses phosphorylated *in vitro* in the presence of [γ-^{32}P]ATP. GST-p85α (**D**) or GST-p85αN-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-^{32}P]ATP. All samples were resolved on SDS–PAGE and the p85 protein was identified by autoradiography and excised from the gel. Following trypsin digestion, the p85 proteins were subjected to analysis by reversed-phase HPLC as described in Materials and methods, and eluted fractions were counted for Cerenkov radiation.

mass of 3583 Da (data not shown) which was consistent 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 Ser608 is the major site of phosphorylation. Phosphothreonine was also detected in the phosphoamino acid analysis of Sf9 cells labelled only *in vivo* (Figure 2); however, it was not possible to recover a peptide containing a phosphothreonine residue(s) from cells that had been metabollically labelled with phosphate *in vivo* (Figure 9A). The presence of a phosphothreonine on the sequenced peptide phosphorylated *in vitro* can be excluded, as this would change the migration characteristics of the *in vivo* phosphosphorylated peptide on the HPLC. We can only conclude that the phosphothreonine modified peptide was not detectable using the present system of analysis.

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 [γ-^{32}P]ATP for increasing periods of time (Figure 10A, 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 $MnCl_2$. These immunocomplexes were then divided into two; half was used for analysis of proteins by SDS–PAGE and autoradiography while the remainder was subjected to PI 3-kinase assay. Results in Figure 10B 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 incubating the enzyme for 20 min in the presence of $MnCl_2$ and [γ-^{32}P]ATP, ~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 10C show that treatment of the serine phosphorylated enzyme (lane 2) with either phosphoprotein phosphatase 2A (lane 3) or alkaline phosphatase (lane 4), removed [γ-^{32}P]ATP from the p85α subunit. Parallel PI 3-kinase assays of these phosphatase-treated samples revealed

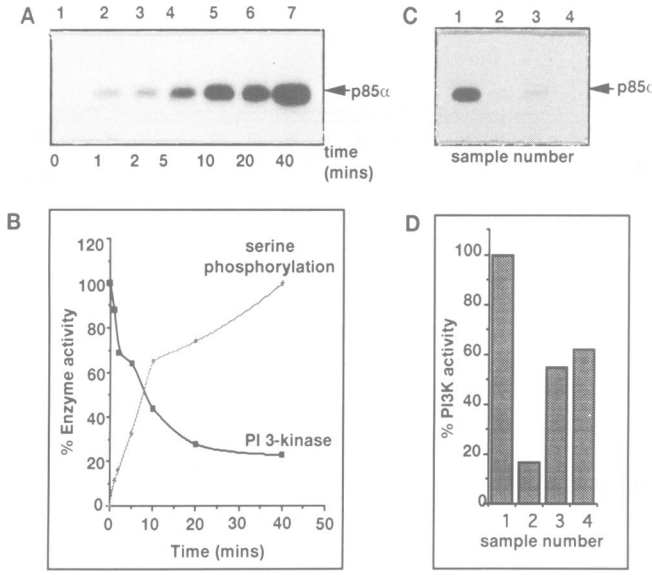

Fig. 10. Serine phosphorylation of the p85α inhibits PI 3-kinase activity. Anti-p110 immunoprecipitates of insect cells that had been coinfected with p85α/p110 viruses were subjected to *in vitro* phosphorylation in the presence of [γ-^{32}P]ATP for 0, 1, 2, 5, 10, 20 or 40 min (lanes 1–7 respectively). After each time period, reactions were stopped by extensive washing with lysis buffer, containing 10 mM EDTA to chelate $MnCl_2$. Samples were then split into two; half was subjected to analysis on SDS–PAGE and autoradiography (**A**) and the remainder subjected to *in vitro* PI 3-kinase assays (**B**). (**C**) Effect of phosphatase treatment. PI 3-kinase was immunoprecipitated from insect cells that had been coinfected with p85α/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 was subjected to analysis on SDS–PAGE and autoradiography and the remainder assayed for PI 3-kinase activity as shown in panel D. (**D**) Lane 1, unphosphorylated PI 3-kinase; lane 2, [γ-^{32}P]ATP phosphorylated PI 3-kinase; lane 3, protein phosphatase 2A treatment of [γ-^{32}P]ATP phosphorylated PI 3-kinase; lane 4, alkaline phosphatase treatment of [γ-^{32}P]ATP phosphorylated PI 3-kinase.

restoration of activity (Figure 10D, lanes 1–4). Untreated PI 3-kinase was also incubated with either alkaline phosphatase or phosphoprotein phosphatase 2A, but no significant change in PI 3-kinase activity was observed (data not shown).

Discussion

Many laboratories have observed a protein-serine kinase activity associated with PI 3-kinase isolated from a variety of sources (Kaplan *et al.*, 1987; Cohen *et al.*, 1990; Carpenter *et al.*, 1993b; Reif *et al.*, 1993). The expression of recombinant subunits of the PI 3-kinase complex in our laboratory has permitted a comprehensive investigation of the origin of the activity. Our earlier analysis of the 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 consensus 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 consensus 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 examine further the nature of the kinase activity intrinsic to p110, we mutated the DRHNSN sequence (R916P) and demonstrated that, although the resultant p110 protein still binds to the p85 subunit, the mutation results in the concomitant loss of both PI 3-kinase and protein-serine kinase activity, strongly suggesting that this protein can act as both a PI kinase and a protein-serine kinase. Further, a mutation at the DFG motif (F934S) introduced into p110 generates a protein which retains both a 3% PI 3-kinase activity, and a 3% protein-serine kinase activity compared with wild type (data not shown). 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.* 1993b). This difference may be because the preparation of Carpenter *et al.* (1993b) contains two distinct types of p110 which are phosphorylated to varying degrees. We are currently unable to relate these two forms to our cloned bovine 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 sulfhydryl modifying agents; the bovine PI 3-kinase and serine kinase activities were inactivated upon treatment with Nbs_2 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 sulfhydryl 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 (data not shown). A thiol requirement for catalysis has been observed in some but not all kinases (Hou *et al.*, 1988; Scholz *et al.*, 1991). Crystal structure data 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 interesting that the BCR protein is not only a Mn^{2+}-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 Mg^{2+} causes seryl phosphorylation of substrate and Mn^{2+} 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). Since the phosphoinositide lipid and the protein-serine kinase activities of p110 appear to have a common thiol requirement for catalysis, and since the kinetics of inactivation/reactivation appear identical, it appears plausible to suggest that the protein-serine kinase activity is intrinsic to the PI 3-kinase complex and that a single catalytic site on this protein may display a dual substrate specificity.

In our hands, the p85 proteins are the only 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 could arise because 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.* (1993b), who demonstrated that casein was also a substrate for purified rat liver PIK kinase. Analysis of 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 p85α from insect, bacterial and bovine cells, both *in vivo* and *in vitro*. These data strongly suggest that the results observed during *in vitro* analysis, are relevant to the *in vivo* systems. Detailed protein chemical analyses have identified Ser608 in the inter-SH2 region as the major phosphorylation site *in vitro*. A serine residue is present at an analogous position in p85β and, 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. Tyr607 on p85α 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.

The novel observation shown here, that PI 3-kinase 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 exclusively on serine and threonine residues. Only following stimulation of the cells with a growth factor does the p85 subunit become phosphorylated on tyrosine residues with a corresponding increase in total cellular PI 3-kinase activity (R.Dhand and S.Roche, unpublished observations). Further, in polyoma virus middle T-transformed 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$^{c\text{-}src}$ complex and is activated by dephosphorylation of the exposed Ser608 by protein phosphatase 2A. Results presented here define a Mn^{2+}-dependent protein-serine kinase activity that phosphorylates the p85 subunit and causes down-regulation of PI 3-kinase *in vitro*. Together these data suggest the following model for the regulation of the PI 3-kinase: the intrinsic serine kinase activity maintains PI 3-kinase at a low basal state in quiescent cells by phosphorylation on Ser608. Growth factor stimulation causes translocation of the PI 3-kinase to the membrane and association with activated and tyrosine phosphorylated PTK through its SH2 domains (reviewed in Pawson, 1992). A combination of events would then cause activation of the PI 3-kinase at the membrane, including access to substrates (Susa *et al.*, 1992; Zhang *et al.*, 1992), induced conformational changes (Panayotou *et al.*, 1992b; Carpenter *et al.*, 1993b) or access of Ser608 on the p85 subunit to a serine phosphatase. Additional phosphorylation at tyrosine by PTKs may also increase the activation state of the PI 3-kinase (Hayashi *et al.*, 1992) or may alter the affinity of the SH2 domains for phosphotyrosine residues and allow dissociation of the PI 3-kinase from the PTK.

At present it is difficult to determine the exact role of this dual specificity kinase *in vivo*. However, the observed use of the Ser608 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, suggest that it may have physiological relevance *in vivo*. Site-directed mutagenesis of Ser608 on the p85 protein will ultimately be required to determine the relevance *in vivo*. These studies are currently in progress. Further structural studies of the p110 subunit of PI 3-kinase and the identification of other forms will lead to a better understanding of the mechanism of catalysis and regulation of this enzyme.

Materials and methods

Materials

Restriction enzymes and DNA modification enzymes were obtained from standard commercial sources and used according to the manufacturers' recommendations. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer and used directly in all subsequent procedures.

Cells and viruses

The SGBAF-1 cell line was established by transfection of bovine adrenal cortex zona faciculata cells with pSV3neo as previously described for other cell types (Whitley *et al.*, 1987). SGBAF-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 10 IU of penicillin/ml and 10 μg of streptomycin/ml. Maintenance of insect cell (Sf9) culture was carried out as described by Summers and Smith (1987). Both p85α and p85β proteins were expressed in insect cells using baculovirus vectors as described previously (Otsu *et al.*, 1991; Gout *et al.*, 1992). Recombinant baculovirus expressing p110 was described in Hiles *et al.* (1992).

Recombinant DNA methods

Construction of p110 mutant. Arginine 916 of p110 was changed to proline by oligonucleotide mediated site-directed mutagenesis. Briefly, the oligonucleotide 5'-TGGGAATTGGGGATCCTCACAATAGTA-3' was synthesized (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 also introduces a novel *Bam*HI site by means of silent codon changes. The sequence of a 802 bp *Pst*I–*Hin*dIII cartridge containing the R916P mutation was verified by DNA sequence analysis. For expression in Sf9 cells, a 903 bp *Pst*I–*Kpn*I cartridge from the baculovirus transfer vector, p36C-P110 (Hiles *et al.*, 1992), 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. Recombinant virus stocks were generated as described in Summers and Smith (1987).

Construction of GST fusion proteins. Sequences corresponding to the SH3 domain (amino acids 1–86), the BCR homology region (amino acids 125–322) and the helical inter-SH2 domain (amino acids 425–600) of the bovine p85α subunit of the PI 3-kinase (Otsu *et al.*, 1991) were amplified by PCR and cloned into the pGEX-2T expression vector (Pharmacia). *Bam*HI and *Eco*RI sites were included at the N- and the C-termini of oligonucleotides respectively to facilitate cloning. Stop codons were introduced at the end of each of the cloned sequences, 5' to the *Eco*RI site. PCR fragments were verified using the Sequenase system (US Biochemicals). The full-length p85α (amino acids 1–724) was subcloned into pGEX-2T by digesting pGEX-2T-SH3 with *Bgl*II and *Eco*RI and replacing this fragment with a similarly cut cartridge from p85α in the BlueScript vector (Otsu *et al.*, 1991) which contained the remaining coding region of the p85α protein. The C- and N-terminal SH2 domains of p85α and the p85αN-C construct are described in Yonezawa *et al.* (1992). The p85αN-CΔ478–514 construct is described in Dhand *et al.* (1994). pGEX-2T constructs were transferred into

Escherichia coli XL1-Blue (Stratagene) and expression of GST fusion proteins was carried out as described previously (Smith and Johnson, 1988).

Antibodies and immunoprecipitation

The p110 C-terminal antiserum was raised as described by Hiles *et al.* (1992). Affinity purified polyclonal antiserum raised against SDS−PAGE purified p85α was described in Gout *et al.* (1992). Monoclonal antibodies directed to the p85α and to the p85β proteins are described in End *et al.* (1993). Immunoprecipitations were carried out as described previously (Otsu *et al.*, 1991).

PI 3-kinase assay

The assay for PI 3-kinase activity was carried out as described by Whitman *et al.* (1985).

Protein kinase assay

Protein kinase assays were performed as described in Hiles *et al.* (1992), except that the kinase buffer used contained 50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10 mM $MnCl_2$, 0.02% Triton X-100 and 10% glycerol.

Association assays

Association of p110 with p85α and p85β. Sf9 cells were infected with appropriate baculoviruses as described by Gout *et al.* (1992) and Hiles *et al.* (1992). Two days post-infection, cells were harvested and lysed in 50 mM Tris−HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 500 μM sodium orthovanadate, 2 mM PMSF and 100 kallikrein inhibitor units of aprotinin. Lysates were then either immunoprecipitated using appropriate antibodies or applied to a phosphopeptide affinity column derived from sequence surrounding Tyr751 of the human PDGF β-receptor to which PI 3-kinase has been shown to bind (Otsu *et al.*, 1991; Fry *et al.*, 1992). Affinity columns were washed as described. Enzyme complexes were either analysed by 7.5% SDS−PAGE or subjected to *in vitro* kinase assays as described above.

In vitro association assay. In vitro complex formation of the p85α subunit with either wild type p110 or the mutant p110-R916P was examined by immunoprecipitating p85α 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% SDS−polyacrylamide gels or assayed directly for associated PI 3-kinase and protein kinase activities. *In vitro* binding of p110 to p85α domains was performed in essentially the same manner. GST fusion proteins were immobilized 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.

Density gradient sedimentation

Sf9 cells infected with baculoviruses expressing p110 alone or p85α and p110 were lysed and the cell extract fractionated on sucrose gradients as described by Courtneidge and Smith (1983). Briefly, cell extracts were fractionated on a 5 ml continuous gradient of 5−20% (w/v) sucrose in lysis buffer and centrifuged in a Beckman SW55 rotor at 54 000 r.p.m. for 6 h at 4°C. Twenty-four fractions were collected and alternate fractions were immunoprecipitated with affinity purified p110 antibody and assayed for PI 3-kinase or protein-serine kinase activity. To measure protein kinase activity from p110-expressing cell lysates, immunoprecipitates were incubated with purified p85α from Sf9 cells (Gout *et al.*, 1992) for 1 h at 4°C, then washed extensively and assayed for protein kinase activity as described above.

Inhibition of kinase activities 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 by 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 Nbs_2 (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 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.

Phosphatase treatment

PI 3-kinase was immunoprecipitated using anti-p110 antibodies from lysates of Sf9 cells. Immunocomplexes were washed twice in lysis buffer and twice in protein kinase buffer and then phosphorylated for 30 min at 22°C in the presence of [γ-^{32}P]ATP and 15 μM 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 $MgCl_2$, 1 mM DTT] or protein-serine phosphatase buffer [10 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM HEPES (pH 7.4), 1 mM DTT]. Samples were incubated for 20 min at 22°C with either 10 U of alkaline phosphatase (Pharmacia) or 50 U of protein phosphatase 2A. After several washes samples were subjected to PI 3-kinase assays or visualized on SDS−polyacrylamide gels.

Biosynthetic labelling of proteins

Twenty-four hours post-infection, Sf9 cells were transferred to methionine-free Grace's medium (Gibco/BRL) in the absence of serum. Cells were labelled with 100 μCi/ml [^{35}S]methionine (Amersham) for 15 h cells and then lysed and applied to phosphopeptide affinity beads for 2 h at 4°C with rotation. The bound complexes were washed and analysed on 7.5% SDS−polyacrylamide gels.

Phosphate labelling, phosphoamino acid analysis and phosphopeptide mapping

Infections of Sf9 cells were allowed to proceed for 24 h before cells were transferred to phosphate-free Grace's insect cell medium (Gibco/BRL) in the absence of serum. $^{32}PO_4$ (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 quiescent for 16 h were incubated with phosphate-free DMEM and 10% dialysed fetal calf serum. $^{32}PO_4$ (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. Lysates were incubated with phosphopeptide affinity beads for 1 h at 4°C with rotation after which beads were washed extensively, boiled in Laemmli buffer and resolved by 7.5% SDS−PAGE. The phosphorylated proteins were visualized by autoradiography and then excised from the gel. Phosphoamino acid analysis was performed on amino acids liberated by partial acid hydrolysis. Separation of phosphoserine, phosphothreonine and phosphotyrosine residues was performed according to Cooper *et al.* (1983). Phosphoamino acid analysis of the p85α protein phosphorylated *in vitro* was performed on phosphopeptide-purified recombinant PI 3-kinase complex, phosphorylated in the presence of [γ-^{32}P]ATP. For phosphopeptide mapping, gel-excised p85α was washed twice for 20 min in 1 ml of water, once for 12 min in 10 mM Tris−HCl (pH 8.0), 50% acetonitrile and finally for 5 min 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 a trypsin to protein ratio of 1:100 by weight, 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 three times for 20 min each in 10 mM Tris−HCl (pH 8.0), 50% acetonitrile. The combined supernatants were then filtered through a Millipore 0.22 μm filter unit and analysed by reversed-phase HPLC. A 100 mm × 2.1 mm Aquapore OD 300 column was used on a Hewlett Packard HP1090 HPLC system and equilibrated in buffer A [0.08% trifluoroacetic acid (TFA), 1% acetonitrile] at a flow rate of 0.2 ml/min. A gradient with buffer B (0.08% TFA, 90% 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 Cerenkov counting.

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