

# Autophosphorylation of p110 $\delta$ phosphoinositide 3-kinase: a new paradigm for the regulation of lipid kinases *in vitro* and *in vivo*

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**Phosphoinositide 3-kinases (PI3Ks) are lipid kinases which also possess an *in vitro* protein kinase activity towards themselves or their adaptor proteins. The physiological relevance of these phosphorylations is unclear at present. Here, the protein kinase activity of the tyrosine kinase-linked PI3K, p110 $\delta$ , is characterized and its functional impact assessed. *In vitro* autophosphorylation of p110 $\delta$  completely down-regulates its lipid kinase activity. The single site of autophosphorylation was mapped to Ser1039 at the C-terminus of p110 $\delta$ . Antisera specific for phospho-Ser1039 revealed a very low level of phosphorylation of this residue in cell lines. However, p110 $\delta$  that is recruited to activated receptors (such as CD28 in T cells) shows a time-dependent increase in Ser1039 phosphorylation and a concomitant decrease in associated lipid kinase activity. Treatment of cells with okadaic acid, an inhibitor of Ser/Thr phosphatases, also dramatically increases the level of Ser1039-phosphorylated p110 $\delta$ . LY294002 and wortmannin blocked these *in vivo* increases in Ser1039 phosphorylation, consistent with the notion that PI3Ks, and possibly p110 $\delta$  itself, are involved in the *in vivo* phosphorylation of p110 $\delta$ . In summary, we show that PI3Ks are subject to regulatory phosphorylations *in vivo* similar to those identified under *in vitro* conditions, identifying a new level of control of these signalling molecules.**

**Keywords:** autophosphorylation/lipid/phosphoinositide 3-kinase/phosphospecific antibodies

## Introduction

Phosphoinositide 3-kinases (PI3Ks) form a large family of evolutionarily conserved enzymes that are involved in a wide variety of biological phenomena such as intracellular vesicular transport, metabolism, growth, proliferation, pro-

tection from apoptosis, differentiation and cytoskeletal rearrangements (reviewed in Stephens *et al.*, 1993; Ward *et al.*, 1996; Toker and Cantley, 1997; Shepherd *et al.*, 1998).

PI3Ks phosphorylate the 3' position of the inositol ring in phosphatidylinositol (PtdIns) lipids. Three classes of PI3Ks can be distinguished based on their structure and *in vitro* lipid substrate specificity (Vanhaesebroeck *et al.*, 1997a; Fruman *et al.*, 1998; Wymann and Pirola, 1998). Class I PI3Ks show a broad lipid substrate specificity and can convert PtdIns, PtdIns(4)P and PtdIns(4,5)P<sub>2</sub> to the corresponding 3'-phosphorylated derivatives. Class I<sub>A</sub> PI3Ks consist of ~110–120 kDa catalytic subunits (p110 $\alpha$ ,  $\beta$  and  $\delta$  in mammals) that associate with adaptor molecules containing two Src homology 2 (SH2) domains (p85 $\alpha$ , p85 $\beta$ , p55 $\gamma$  and their splice variants). The interaction of the adaptor SH2 domains with phosphotyrosines links these PI3Ks to tyrosine kinase signalling pathways. Class I<sub>B</sub> enzymes are activated by the G $\beta\gamma$  subunits of heterotrimeric G proteins. The only class I<sub>B</sub> PI3K identified is p110 $\gamma$ , a 120 kDa catalytic subunit that exists in complex with the putative adaptor protein p101. Class I PI3Ks also interact with the small GTP-binding protein Ras, but the physiological role of this interaction is not entirely clear. It is possible that Ras binding allosterically activates PI3Ks. Alternatively, the interaction of PI3Ks with membrane-bound Ras could contribute to their recruitment to their lipid substrates (Rodriguez-Viciana *et al.*, 1996; Rubio *et al.*, 1997). Class II PI3Ks are enzymes that utilize PtdIns and PtdIns(4)P as *in vitro* substrates; three have been identified so far in mammals (PI3K-C2 $\alpha$ ,  $\beta$  and  $\gamma$ ). They are large molecules (>170 kDa) that are characterized by a C-terminal C2 domain. Class III PI3Ks are enzymes that only use PtdIns as a substrate. They are the homologues of the yeast vps34p PI3K that is involved in vesicular trafficking from the Golgi to the vacuole, the yeast equivalent of mammalian lysosomes. Mammals have one class III PI3K catalytic subunit which, as in yeast, exists in complex with a 150 kDa Ser/Thr protein kinase. Extracellular stimuli that acutely trigger class II and III PI3Ks are unknown.

All PI3Ks contain a kinase domain region that is related to the catalytic domain of protein kinases (Hunter, 1995; Zvelebil *et al.*, 1996; Fruman *et al.*, 1998). As well as the lipid kinase activities described above, class I and III PI3Ks possess an intrinsic protein kinase activity *in vitro* (Hunter, 1995). This kinase activity is directed towards the class I<sub>A</sub> PI3K adaptors or the catalytic subunits themselves. The best documented example is the *in vitro* phosphorylation of the p85 adaptor by the p110 $\alpha$  catalytic subunit, which results in the down-regulation of p110 $\alpha$  lipid kinase activity (Carpenter *et al.*, 1993b; Dhand *et al.*, 1994b). Mammalian p110 $\gamma$  autophosphorylates, but this does not affect its lipid kinase activity (Stoyanova *et al.*,

1997). Yeast vps34p also autophosphorylates, but the functional impact of this phosphorylation has not been investigated (Stack and Emr, 1994).

A region analogous to the kinase domain of PI3Ks has also been identified in a large group of proteins (the so-called PIK-related proteins; Keith and Schreiber, 1995), none of which have been shown to possess lipid kinase activity. Several PIK family members, however, possess protein kinase activity. These include mTOR (mammalian target of rapamycin), DNA-PK (DNA-dependent protein kinase) and the ATM protein (encoded by the gene responsible for the human disorder ataxia telangiectasia). This protein kinase activity is directed towards the catalytic subunits themselves (as in the case of mTOR), as well as against exogenous substrates such as the translational repressor protein PHAS-I for mTOR (Brunn *et al.*, 1997) and p53 for DNA-PK (Lees-Miller *et al.*, 1992; Shieh *et al.*, 1997; Woo *et al.*, 1998) or ATM (Banin *et al.*, 1998; Canman *et al.*, 1998).

Fundamental questions regarding the relevance of PI3K protein kinase activity remain to be answered. At present, it is not clear whether the phosphorylations identified *in vitro* also occur in intact cells. In addition, it is not clear whether these enzymes possess other physiological substrates *in vivo*. There is some evidence to suggest that class I<sub>A</sub> PI3Ks can phosphorylate the IRS-1 adaptor protein, but the involvement of other (contaminating) kinases in these studies is difficult to exclude (Lam *et al.*, 1994; Tanti *et al.*, 1994; Uddin *et al.*, 1997).

Here we investigate the protein kinase activity of p110 $\delta$ , a class I<sub>A</sub> PI3K that possesses an intrinsic *in vitro* autophosphorylation capacity which down-regulates its lipid kinase activity. We have identified Ser1039 in the C-terminal region of the p110 $\delta$  catalytic domain as the unique phosphorylation site on p110 $\delta$ . p110 $\delta$  mutants in which Ser1039 was mutated to acidic residues have a reduced lipid kinase activity. Antisera that specifically recognize the Ser1039-phosphorylated form of p110 $\delta$  were raised and used to investigate the phosphorylation status of this site *in vivo*. The level of Ser1039 phosphorylation in p110 $\delta$  is very low in cell lines but can be increased by treatment of cells with the phosphatase inhibitor okadaic acid or upon recruitment to activated receptor complexes (such as CD28 in T cells). The time-dependent increase in phosphorylation on Ser1039 *in vivo* is accompanied by a down-regulation of the lipid kinase activity of p110 $\delta$ . These observations show that p110 $\delta$  is subject to regulatory phosphorylations *in vivo*, on the same site as that identified *in vitro*. It is anticipated that strategies similar to those described here will be essential to delineate further the relevance of protein kinase events in physiological signalling by PI3Ks.

## Results

### *In vitro* protein kinase activity of class I<sub>A</sub> PI3Ks

The class I<sub>A</sub> adaptor proteins p85 $\alpha$  and p85 $\beta$  are phosphorylated by p110 $\alpha$  *in vitro*. In p85 $\alpha$ , this phosphorylation occurs on Ser608 in the inter-SH2 region (Dhand *et al.*, 1994b). We expressed recombinant p110 $\alpha$  and p110 $\delta$  in insect cells and compared their protein kinase activities. In contrast to p110 $\alpha$ , p110 $\delta$  autophosphorylates, and only a very low level of phosphorylation of the associated

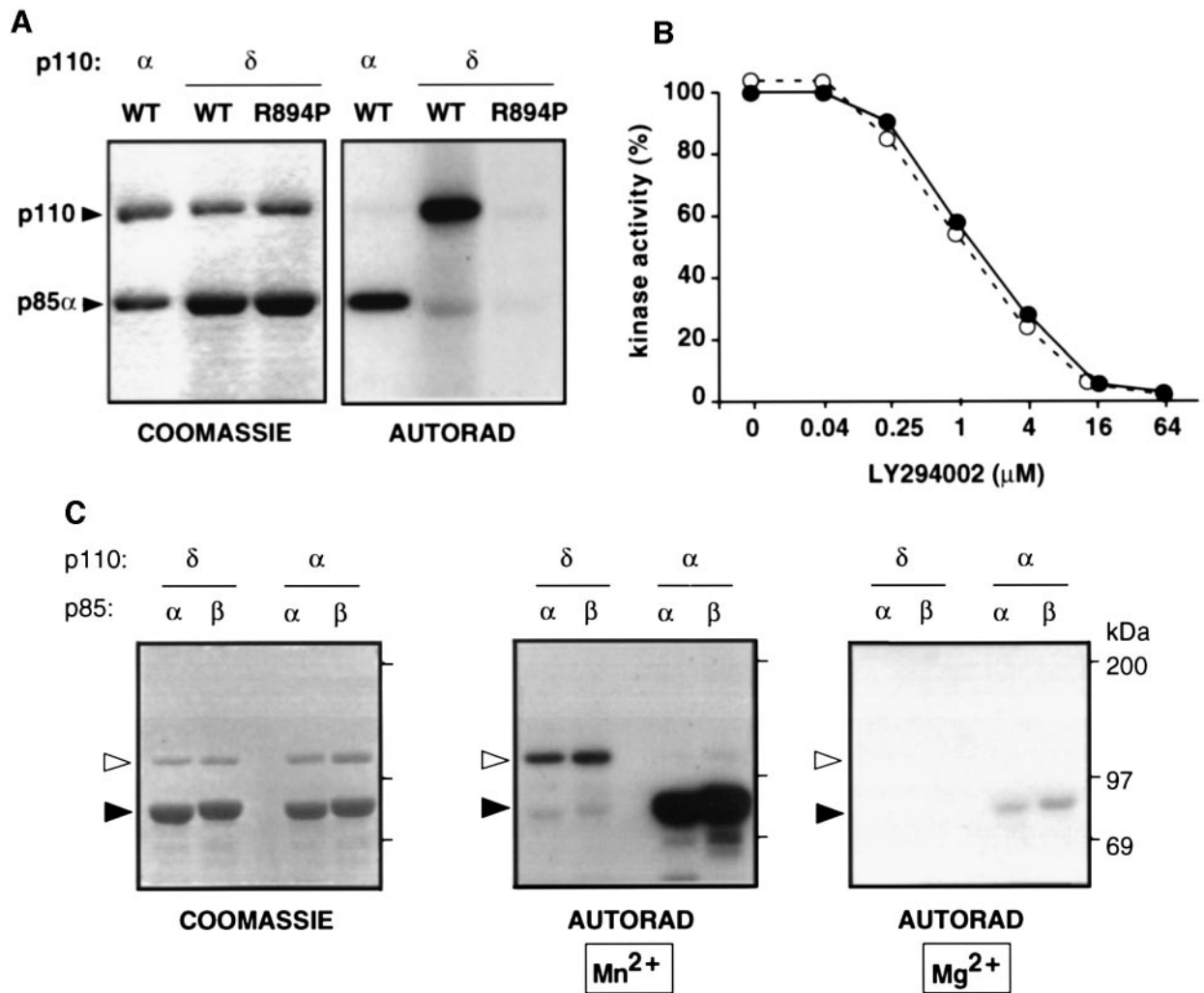
adaptors was observed (Figure 1A). A mutation in the putative ATP-binding site of p110 $\delta$  (R894P mutation) completely abolishes both its *in vitro* lipid and protein kinase activity (Figure 1A), indicating that p110 $\delta$  itself is responsible for this phosphorylation. Moreover, the protein kinase activity of p110 $\delta$  has a similar sensitivity to inhibition by wortmannin and LY294002 as its lipid kinase activity (IC<sub>50</sub> of 5 nM for wortmannin and 0.5–1  $\mu$ M for LY294002; Figure 1B, data for wortmannin are not shown). Like the phosphorylation of p85 by p110 $\alpha$ , *in vitro* autophosphorylation of p110 $\delta$  is largely Mn<sup>2+</sup>-dependent, with *in vitro* phosphorylation levels in the presence of Mg<sup>2+</sup> reaching a maximum of 5% of those observed in the presence of Mn<sup>2+</sup> (Figure 1C).

Class I<sub>A</sub> PI3Ks are highly homologous at the primary sequence level and it is therefore remarkable that, whilst in complex with the same adaptor, these enzymes show such a dramatic difference in protein kinase substrate specificity. One of the structural features that discriminates p110 $\delta$  from the other class I<sub>A</sub> PI3Ks is the presence of a proline-rich region in its N-terminus (amino acids 292–311 in human p110 $\delta$ ; Vanhaesebroeck *et al.*, 1997b). This motif potentially could interact with the SH3 domain of p85 $\alpha/\beta$ , resulting in a configuration of the heterodimeric complex in which p110 $\delta$  cannot phosphorylate p85. This is unlikely to be the case, however, as the p55 $\gamma$  adaptor that lacks an SH3 domain was also not phosphorylated by p110 $\delta$ , despite it being a good substrate for p110 $\alpha$  (Figure 2A).

To exclude the possibility that p110 $\delta$  had already fully phosphorylated the associated adaptors during the *in vivo* co-expression in insect cells, exogenous purified p85 $\alpha$  was added to immobilized GST–p110 $\delta$ . A parallel experiment was carried out using GST–p110 $\alpha$  (wild-type, or kinase-dead = R916P mutant). After washing away the excess p85 $\alpha$ , bound p85 $\alpha$  was phosphorylated efficiently by p110 $\alpha$  but again not by p110 $\delta$  (Figure 2B).

### *p110 $\delta$ autophosphorylates on a single serine at its C-terminus*

p110 $\delta$  was phosphorylated *in vitro*, digested with Lys-C endoprotease and the resulting peptides separated by two rounds of anion exchange and reverse-phase HPLC. The absorbance at 215 nm and radioactivity of each fraction were determined and a single peak of radioactivity was found (Figure 3A). Protein sequencing of this fraction revealed the VNWLAHNVSK sequence which is the equivalent of amino acids 1031–1040 in human p110 $\delta$  (which has 1044 amino acids in total). These observations suggest that Ser1039 of p110 $\delta$  is the site of autophosphorylation. In order to verify this, a p110 $\delta$  mutant in which Ser1039 was changed to alanine (p110 $\delta$  S $\rightarrow$ A) was created, expressed in insect cells and subjected to an *in vitro* protein kinase assay. As is clear from Figure 3B, no phosphate was incorporated into this protein. Taken together with the observation that this S $\rightarrow$ A mutant of p110 $\delta$  is still catalytically active as a lipid kinase (see below), these results demonstrate that Ser1039 is the only site of autophosphorylation in p110 $\delta$ . Alignment of the C-termini of class I<sub>A</sub> catalytic subunits shows that this Ser is unique to p110 $\delta$  amongst the mammalian isoforms (Figure 3C) but is conserved in *Drosophila* p110, a molecule that also autophosphorylates (Weinkove *et al.*,



**Fig. 1.** Protein kinase activity of p110 $\alpha$  and p110 $\delta$ . (A) p110 $\delta$  autophosphorylates. p110–p85 $\alpha$  complexes immobilized on PDGF receptor phosphopeptide beads were subjected to an *in vitro* protein kinase reaction and analysed by SDS–PAGE, Coomassie Blue staining and autoradiography. WT = wild-type, R894P = p110 $\delta$  mutant in which Arg894 was mutated to Pro. (B) Sensitivity of p110 $\delta$  protein kinase (●) and PtdIns lipid kinase (○) activity to LY294002. Lipid kinase and autophosphorylation activity in the absence of inhibitor were taken as 100%. (C) Protein kinase activity of p110 $\delta$  and p110 $\alpha$  in the presence of Mn<sup>2+</sup> and Mg<sup>2+</sup>. Open and closed arrowheads point to p110 and p85 proteins, respectively.

1997). It is also of interest to note that the C-terminus of p110 $\delta$  shows no homology to the region surrounding Ser608 in p85 $\alpha$  that becomes phosphorylated by p110 $\alpha$  (Dhand *et al.*, 1994b).

#### Autophosphorylation of p110 $\delta$ down-regulates its lipid kinase activity

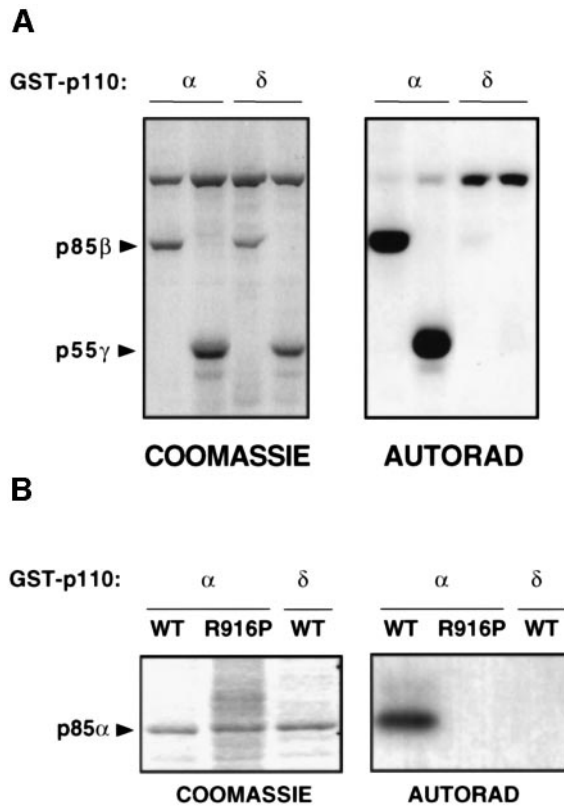
Phosphorylation of the p85 adaptor by p110 $\alpha$  down-regulates the lipid kinase activity of the p85–p110 $\alpha$  complex (Carpenter *et al.*, 1993b; Dhand *et al.*, 1994b). Likewise, the lipid kinase activity of p110 $\delta$  towards PtdIns and PtdIns(4,5)P<sub>2</sub> was almost completely lost upon autophosphorylation (Figure 4), implying an important regulatory role for the phosphorylation status of Ser1039. We therefore investigated the catalytic activity of p110 $\delta$  proteins in which Ser1039 was mutated.

Wild-type p110 $\delta$  and the non-autophosphorylating S→A mutant possess the same lipid kinase activity (Figure 5), showing that Ser1039 is not essential for catalytic activity of p110 $\delta$ . The lipid kinase activity of this p110 $\delta$  S→A

mutant was unaffected by the experimental pre-phosphorylation conditions that down-regulate wild-type p110 $\delta$  (Figures 4A and 5), showing that p110 $\delta$  does not become unstable during the incubation time and conditions used for pre-phosphorylation. We next created mutants of p110 $\delta$  in which Ser1039 was substituted by the negatively charged amino acids aspartic acid (D) or glutamic acid (E) in order to try to mimic the effect of phosphorylation. As expected, p110 $\delta$  S→D/E mutants no longer autophosphorylated (data not shown). The lipid kinase activity of the S→D/E mutants, however, was found to be reduced by a factor 50–75% and thus mimicked to a large extent the effect of p110 $\delta$  autophosphorylation (Figure 5).

#### Generation of an antiserum specific for p110 $\delta$ phosphorylated on Ser1039

In order to investigate whether the results of the *in vitro* experiments described above have any relevance for the *in vivo* regulation of p110 $\delta$ , we raised antisera specific for the Ser1039 phosphorylation site. Rabbits were immunized



**Fig. 2.** p110 $\delta$  does not phosphorylate class I<sub>A</sub> adaptors. (A) p110 $\delta$  does not phosphorylate co-expressed class I<sub>A</sub> adaptors. GST-p110 $\alpha$  and GST-p110 $\delta$ , expressed in insect cells in complex with p85 $\beta$  or p55 $\gamma$ , were purified on glutathione-Sepharose beads, subjected to an *in vitro* protein kinase reaction and analysed further as described in the legend to Figure 1A. (B) p110 $\delta$  does not phosphorylate exogenously added p85 $\alpha$ . Purified GST-p110 $\alpha$  or GST-p110 $\delta$ , immobilized on glutathione-Sepharose beads, were incubated in the presence of 10  $\mu$ g of recombinant p85 $\alpha$  for 20 min, washed, subjected to an *in vitro* protein kinase reaction and analysed further as described in the legend to Figure 1A. WT = wild-type, R916P = mutant in which Arg916 was mutated to Pro.

with a synthetic p110 $\delta$  C-terminal peptide in which the equivalent of Ser1039 was phosphorylated (see Materials and methods). The resulting antiserum (referred to as anti-phospho-p110 $\delta$ ) specifically recognized the autophosphorylated form of p110 $\delta$  on immunoblots (Figure 6A). Antiserum to non-phosphorylated p110 $\delta$  recognized both phosphorylated and non-phosphorylated p110 $\delta$  in these experiments (Figure 6A) and is referred to here as anti-total-p110 $\delta$ .

#### Stoichiometry of p110 $\delta$ Ser1039 phosphorylation *in vitro*

The observation that phosphorylation of Ser1039 in p110 $\delta$  results in an almost complete inactivation of the lipid kinase activity of this enzyme (Figure 4) might suggest that Ser1039 is phosphorylated stoichiometrically at this residue. However, we found that the stoichiometry of *in vitro* phosphorylation was maximally 0.5 mol of phosphate per mol of p110 $\delta$ .

Our finding that the S1039 $\rightarrow$ A mutant of p110 $\delta$  no longer incorporates  $\gamma$ -<sup>32</sup>P (Figure 3B) excludes the possibility that more than one site is autophosphorylated in p110 $\delta$  *in vitro*. This is also suggested by the single peak of radioactivity found in the peptide digest of autophosphoryl-

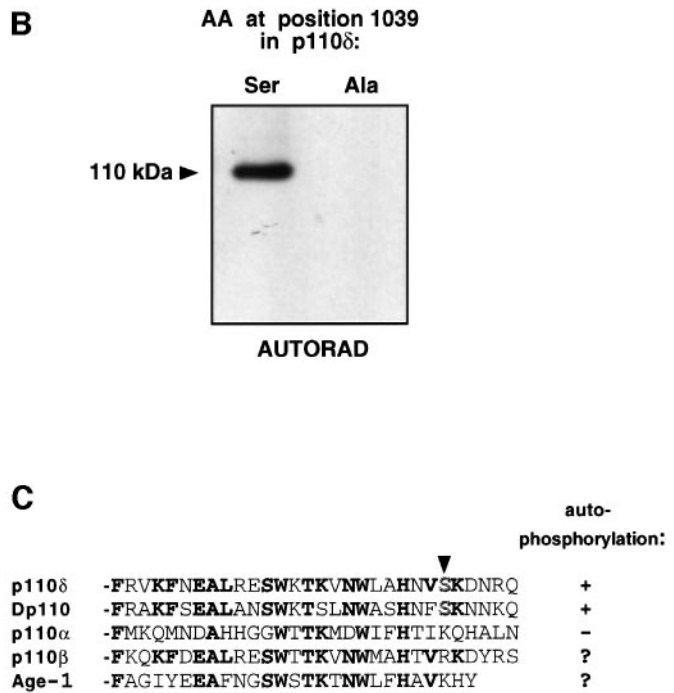
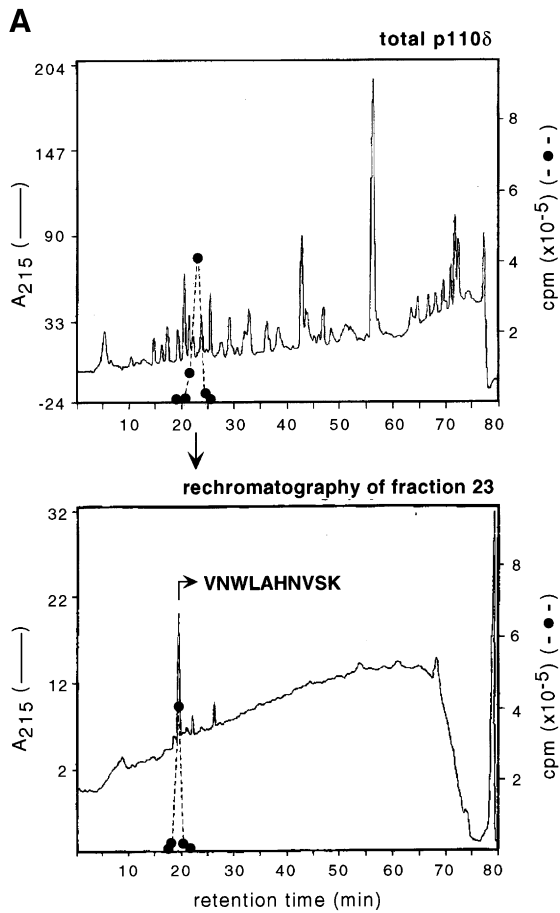
ated p110 $\delta$  (Figure 3A). In addition, anti-phospho-p110 $\delta$  revealed very little or no Ser1039 phosphorylation in recombinant p110 $\delta$  isolated from insect cells (non-phosphorylated control in Figure 6A), indicating that non-100% stoichiometry of *in vitro* autophosphorylation is not due to phosphorylation of Ser1039 in the cells in which p110 $\delta$  is produced. Pre-treatment of p110 $\delta$  with alkaline phosphatase or protein phosphatase 1 or 2A (which did remove 50–80% of the phosphate incorporated in p110 $\delta$  following *in vitro* autophosphorylation; data not shown) failed to increase the stoichiometry of p110 $\delta$  autophosphorylation, making it unlikely that p110 $\delta$  had acquired other inhibitory phosphorylations during its synthesis in insect cells.

Stoichiometry estimations are based on total protein content (see Materials and methods) and do not take into account whether or not these proteins are active. At present, we cannot exclude that only a fraction of the p110 $\delta$  that is isolated from insect cells is in an active configuration, a phenomenon that may also be induced by the immobilization of p110 $\delta$  via its adaptor on tyrosine-phosphorylated peptide coupled to a solid support.

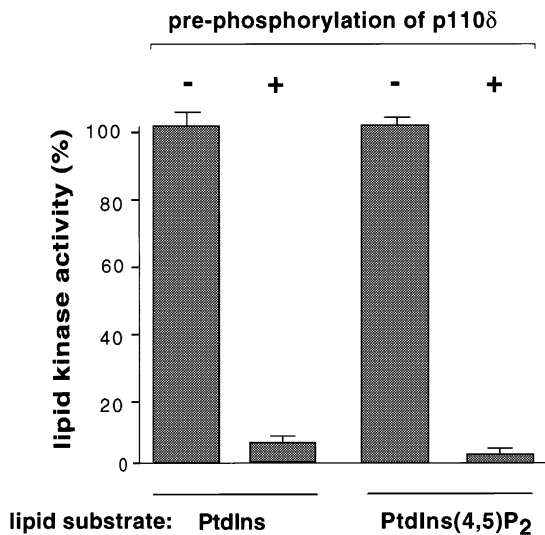
#### Phosphorylation status of p110 $\delta$ Ser1039 in unstimulated cell lines

When total lysates or p110 $\delta$ /p85 immunoprecipitates from unstimulated Jurkat T cells were immunoblotted with anti-phospho-p110 $\delta$ , very little or no Ser1039 phosphorylation could be detected (Figure 6B). Pre-treatment of cells with okadaic acid, an inhibitor of Ser/Thr protein phosphatases, was found dramatically to enhance the level of p110 $\delta$  Ser1039-phosphorylation (Figure 6B). Similar observations were made for the cytokine-dependent cell lines BaF/3 (pre-B cell), FD-6 (myeloid progenitor-like cell) and MC/9 (mast cell) (data not shown). A minimum concentration of 10 nM okadaic acid was required to enhance Ser1039 phosphorylation, with a maximal effect seen at doses  $\geq$ 500 nM (Figure 6C). Short incubation periods (5 min) with 500 nM okadaic acid led to a substantial increase in Ser1039 phosphorylation, with maximum effects reached after 60 min (data not shown). At present, it is unclear whether OA inhibits phospho-Ser1039 phosphatases, or activates kinases that phosphorylate this site by interfering with their phosphorylation/dephosphorylation-mediated control mechanisms (Evans and Hemmings, 1998).

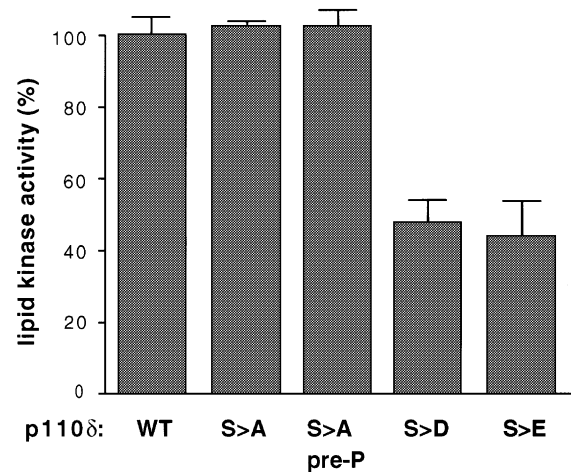
We next investigated the effect of the PI3K inhibitor LY294002 on the Ser1039 phosphorylation induced by okadaic acid. As is clear from Figure 6D, LY294002 was very effective in blocking the okadaic acid-mediated increase in p110 $\delta$  Ser1039 phosphorylation *in vivo*, with a dose-response curve similar to that for inhibition of the protein kinase activity of p110 $\delta$  (Figure 1B) and other PI3Ks (Woscholski *et al.*, 1994; Stoyanova *et al.*, 1997; Withers *et al.*, 1997). Also wortmannin, a structurally unrelated inhibitor of PI3Ks, was extremely potent in inhibiting p110 $\delta$ -Ser1039 phosphorylation *in vivo*, with an IC<sub>50</sub> of 5 nM (data not shown). Taken together, these observations indicate that PI3Ks, and possibly p110 $\delta$  itself are responsible for the *in vivo* phosphorylation of p110 $\delta$ . They could either directly phosphorylate p110 $\delta$  or control Ser/Thr kinases that phosphorylate p110 $\delta$ .



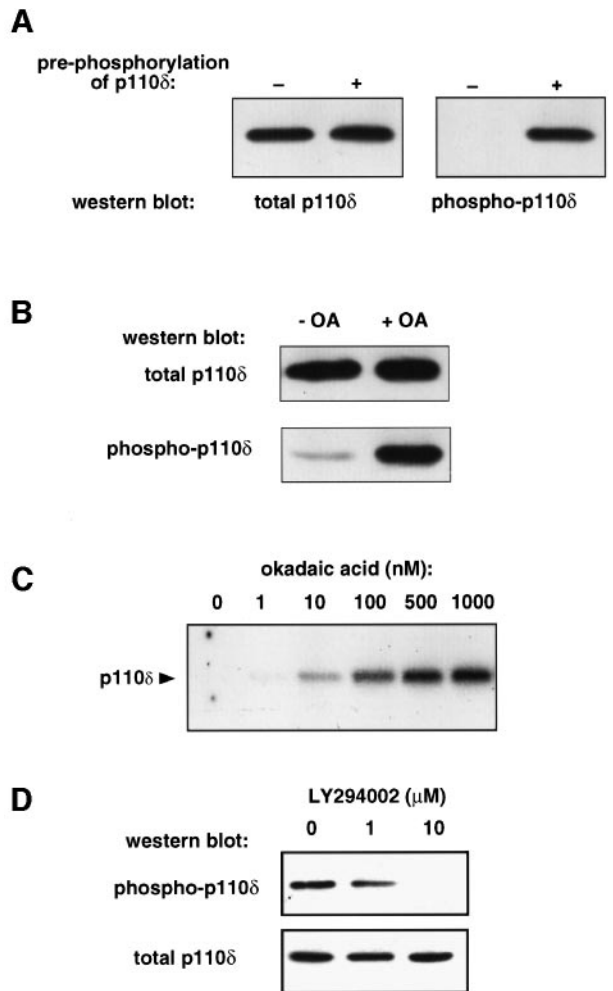
**Fig. 3.** Mapping of the p110 $\delta$  autophosphorylation site to Ser1039. (A) p110 $\delta$ -p85 $\alpha$  was subjected to an *in vitro* protein kinase assay, separated by SDS-PAGE, excised, digested with Lys-C endoprotease and the resulting peptides separated chromatographically. The absorbance at 215 nm (solid curve) and radioactivity (stippled curve) of each fraction were determined. The upper panel shows the profiles obtained from digestion of total p110 $\delta$ . The fraction that contained the peak radioactivity (fraction 23) was then re-chromatographed (lower panel). (B) p110 $\delta$  in which Ser1039 is mutated to Ala does not autophosphorylate. Complexes of p110 $\delta$ -p85 $\alpha$  were incubated with lipids as described in Materials and methods, followed by a protein kinase assay and further analysis as described in the legend to Figure 1A. (C) Alignment of the C-termini of class I<sub>A</sub> PI3Ks. Dp110, *Drosophila* p110; Age-1, *Caenorhabditis elegans* p110. The arrowhead indicates the Ser1039 in p110 $\delta$  and the equivalent site in Dp110.



**Fig. 4.** p110 $\delta$  autophosphorylation results in down-regulation of its lipid kinase activity. p110 $\delta$ -p85 $\alpha$  complexes, immobilized on PDGF receptor phosphopeptide beads, were subjected to an *in vitro* protein kinase reaction in the absence or presence of Mn<sup>2+</sup>, followed by a lipid kinase assay using PtdIns or PtdIns(4,5)P<sub>2</sub> as a substrate. Lipid kinase activity of p110 $\delta$ -p85 $\alpha$  pre-treated in the absence of Mn<sup>2+</sup> was taken as 100%.



**Fig. 5.** Lipid kinase activity of p110 $\delta$  Ser1039 mutants. Equal amounts of p110 $\delta$  proteins were tested in a lipid kinase assay using PtdIns as a substrate. The activity of the mutants is expressed relative to that of wild-type (WT) p110 $\delta$  = 100%. S→A pre-P: p110 $\delta$  S→A mutant was subjected to an *in vitro* pre-phosphorylation reaction in the presence of Mn<sup>2+</sup> (as shown in Figure 4), followed by a lipid kinase assay using PtdIns as a substrate.

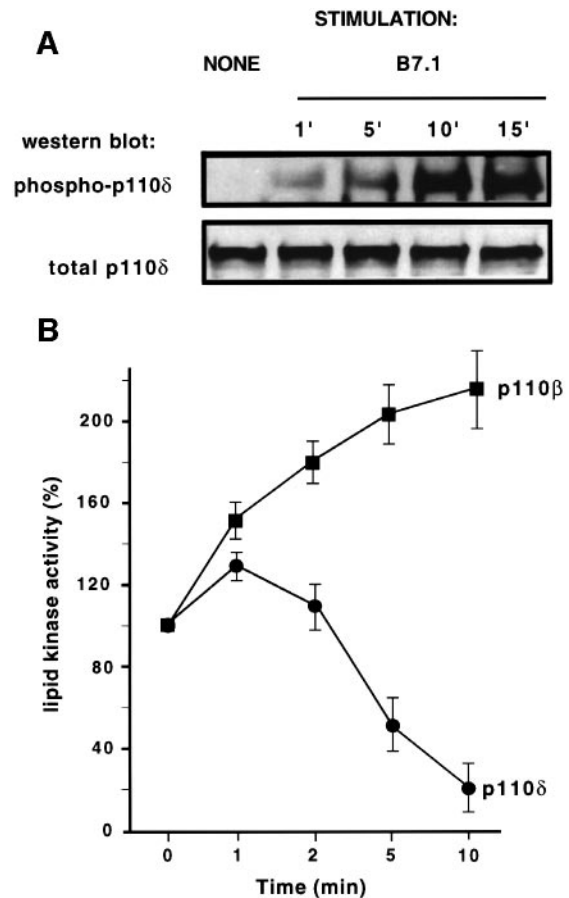


**Fig. 6.** (A) Generation of an antiserum specific for p110 $\delta$  phosphorylated on Ser1039. Recombinant p110 $\delta$ -p85 $\alpha$  was subjected to an *in vitro* protein kinase assay with or without Mn<sup>2+</sup>, separated by SDS-PAGE and immunoblotted with anti-phospho-p110 $\delta$  or anti-total-p110 $\delta$ . (B) Effect of okadaic acid on the level of Ser1039-phosphorylated p110 $\delta$  in Jurkat T cells. Cells ( $2 \times 10^7$  per point) were lysed in the presence of 50 mM NaF, incubated with PDGF receptor phosphopeptide beads, washed and Western blotted using either anti-phospho-p110 $\delta$  or anti-total-p110 $\delta$ . + OA = cells treated for 2 h with 500 nM okadaic acid. (C) Dose dependence of okadaic acid effect on Ser1039 phosphorylation. Cells were treated for 2 h with the indicated dose of okadaic acid, and processed further as described in (B). (D) Effect of LY294002 on the okadaic acid-induced increase in p110 $\delta$  Ser1039 phosphorylation. Jurkat cells were treated for 2 h with 500 nM okadaic acid with or without the indicated doses of LY294002, and then processed further as described in (B). LY294002 was applied 15 min before the addition of okadaic acid.

### Regulation of the phosphorylation status of p110 $\delta$ Ser1039 in cell signalling

Having demonstrated that p110 $\delta$ -Ser1039 can be phosphorylated *in vivo*, we next investigated whether this phosphorylation is regulated by extracellular stimuli.

As a model system, we used Jurkat T cells stimulated via CD28, a co-stimulatory molecule in T cell activation. CD28 is a homodimeric cell surface protein that, upon binding its counter-receptor B7 on neighbouring cells, induces PI3K activity and a long-term association (typically >15 min) of p85 with the intracellular portion of CD28 in Jurkat cells (Ueda *et al.*, 1995). Immunoprecipitates were made using anti-total-p110 $\delta$  and then immuno-



**Fig. 7.** CD28 stimulation of Jurkat T cells induces p110 $\delta$  Ser1039 phosphorylation *in vivo* and a down-regulation of associated lipid kinase activity. Cells were triggered with CD28 ligand (B7.1) for different time points, lysed and immunoprecipitated with anti-total-p110 $\delta$  or antiserum to p110 $\beta$ . Immune complexes were then (A) Western blotted with anti-phospho-p110 $\delta$  (upper panel) or anti-total-p110 $\delta$  (lower panel), or (B) tested for lipid kinase activity towards PtdIns. The lipid kinase activity is expressed relative to that found in immunoprecipitates from unstimulated cells = 100%. The data points are the mean  $\pm$  SEM of four separate experiments.

blotted with anti-phospho-p110 $\delta$  or anti-total-p110 $\delta$ . A time-dependent increase in Ser1039 phosphorylation was observed in these immunoprecipitates (Figure 7A) with a concomitant time-dependent decrease in lipid kinase activity (Figure 7B). This indicates that Ser1039 phosphorylation *in vivo* has the same impact on the regulation of p110 $\delta$  lipid kinase activity as was demonstrated *in vitro*. Remarkably, the lipid kinase activity of p110 $\beta$  in p110 $\beta$  immunoprecipitates was found to be increased upon CD28 ligation, clearly indicating differential regulation of p110 $\beta$  and  $\delta$  *in vivo* in response to the same stimulus.

So far, however, we have failed to find consistent changes in Ser1039 phosphorylation in p85 or p110 $\delta$  immunoprecipitates made from cytokine-stimulated cells [interleukin-3 (IL-3) in BaF/3 and stem cell factor (SCF) in MC/9 cells]. This might be explained by the very transient stimulation of PI3K activity by these molecules (peak typically at 2–5 min) and the low numbers of receptors per cell (typically <1000 IL-3 receptors per cell for BaF/3, 5000 c-kit receptors per cell for MC/9, compared with 40 000–50 000 CD28 molecules per cell in Jurkat), resulting in very transient Ser1039 changes in only a small

proportion of p110 $\delta$  molecules. It cannot be excluded, however, that the observed phenomena are specific for co-stimulatory molecules, such as CD28, for which a long-term association with PI3K might be essential for the co-stimulatory function to feed into other signalling pathways.

## Discussion

Here we demonstrate that protein phosphorylation is an important *in vivo* regulatory mechanism for the catalytic activity of the class I<sub>A</sub> PI3K p110 $\delta$ . This adds a further level of control for class I<sub>A</sub> PI3Ks besides translocation to membranes and interaction with SH2 domain-containing adaptor proteins and Ras.

Phosphorylation of p85 $\alpha$  by p110 $\alpha$  was the first regulatory PI3K phosphorylation to be described (Carpenter *et al.*, 1993b; Dhand *et al.*, 1994b). Since its discovery several years ago, however, no evidence has been presented that this *in vitro* phenomenon also plays a role in the regulation of p110 $\alpha$  *in vivo*. The work of Carpenter and co-workers indicated that the inter-SH2 region of p85 is phosphorylated in cells (Carpenter *et al.*, 1993b), but the residues at which this occurs have not been identified. In all other cases where p85 $\alpha$  phosphorylation has been investigated, the level of serine phosphorylation does not seem to change upon ligand stimulation (Kaplan *et al.*, 1987; Cohen *et al.*, 1990; Carpenter *et al.*, 1993b; Reif *et al.*, 1993; Domin *et al.*, 1996). These studies are compromised, however, by a high constitutive Ser phosphorylation of p85 $\alpha$  *in vivo* and the low fraction (~5%) of total cellular p85 $\alpha$  that seems to become recruited to receptors (Domin *et al.*, 1996). These observations made it clear that other strategies had to be followed in order to address the question of the occurrence and relevance of PI3K phosphorylations *in vivo*.

As a model system, we have studied the autophosphorylation of p110 $\delta$ . We identify Ser1039 in the C-terminus of p110 $\delta$  as the unique site of phosphorylation. Like p85 $\alpha$  phosphorylation by p110 $\alpha$ , *in vitro* phosphorylation of Ser1039 results in a down-regulation of the lipid kinase activity of the complex. In order to assess the p110 $\delta$  Ser1039 phosphorylation status *in vivo*, we raised antisera specific for this phosphorylation site. In all cell lines investigated, the overall level of Ser1039 phosphorylation was very low. Phosphorylation of Ser1039 in p110 $\delta$  is induced *in vivo* upon its recruitment to the CD28 signalling molecule, an event which correlates with a decrease in the p110 $\delta$ -associated lipid kinase activity. Likewise, short-term treatment of cells with okadaic acid, an inhibitor of Ser/Thr phosphatases, also induced a substantial increase in Ser1039 phosphorylation. The observation that okadaic acid does not up-regulate Ser1039 phosphorylation in cells treated with LY294002 or wortmannin implies a role for p110 $\delta$  itself in its *in vivo* phosphorylation. However, an involvement of other PI3Ks or PI3K-related enzymes in this phenomenon cannot be excluded, as their protein kinase activity has a similar sensitivity to PI3K inhibitors as that of p110 $\delta$  (Woscholski *et al.*, 1994; Brunn *et al.*, 1996; Stoyanova *et al.*, 1997; Withers *et al.*, 1997). At present, it is not clear whether LY294002/wortmannin-sensitive kinases directly phosphorylate p110 $\delta$  or whether they control Ser/Thr kinases that phosphorylate p110 $\delta$ .

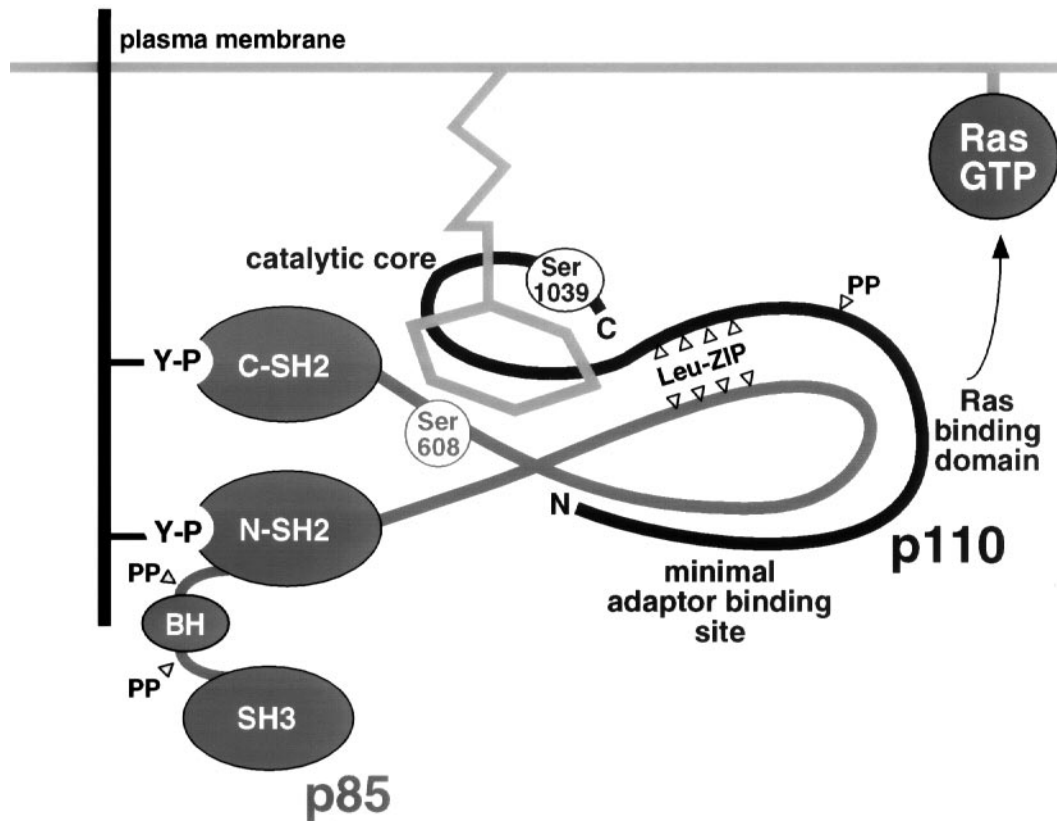
The Mn<sup>2+</sup> dependence of the *in vitro* protein kinase activity of p110 $\delta$  is intriguing. It is well known that the *in vitro* activity of many tyrosine kinases is similar or greater in the presence of Mn<sup>2+</sup> than with Mg<sup>2+</sup>. An example is ZAP70, a human tyrosine kinase involved in T-cell signalling that is exclusively dependent upon Mn<sup>2+</sup> for its *in vitro* protein kinase activity (Isakov *et al.*, 1996). Ample evidence is available for *bona fide* protein kinase activity of ZAP70 in cells where Mg<sup>2+</sup> is prevalent and levels of Mn<sup>2+</sup> negligible. *In vitro*, Ser/Thr kinases usually use Mg<sup>2+</sup> more effectively than Mn<sup>2+</sup>. However, several Ser/Thr kinases with preference for Mn<sup>2+</sup> over Mg<sup>2+</sup> have been isolated recently, some of mammalian origin (such as the human mst-3 Ser/Thr kinase; Schinkmann and Blenis, 1997) and many of viral or plant origin (such as the cytomegalovirus UL97 protein kinase; He *et al.*, 1997). The reason for an *in vitro* preference of certain kinases for Mn<sup>2+</sup> over Mg<sup>2+</sup> is unclear at the moment.

A model that integrates the currently identified control mechanisms of PI3Ks is shown in Figure 8. It depicts the p85–p110 complex that has translocated from the cytosol to the membrane into contact with its lipid substrates by interaction of its SH2 domains with phosphorylated tyrosine residues in a receptor.

The presence of the adaptor itself also affects the catalytic activity of the p110 subunit. This phenomenon has only been studied in detail for p85 $\alpha$ –p110 $\alpha$ , where p85 $\alpha$  conformationally stabilizes p110 $\alpha$  and inhibits its lipid kinase activity (Yu *et al.*, 1998). Activation of the lipid kinase activity by interaction with phosphotyrosine-containing proteins may result from a release of inhibition of the heterodimer (Backer *et al.*, 1992; Carpenter *et al.*, 1993a; Yu *et al.*, 1998). Removal of p85 $\alpha$  inhibitory action may also underly the lipid kinase activation of p110 $\alpha$  complexed to an oncogenic form of p85 $\alpha$  that lacks the last 153 residues, including the C-terminal SH2 domain and the Ser608 phosphorylation site (Jimenez *et al.*, 1998). In addition to its tandem SH2 domains, p85 $\alpha$  has an N-terminal SH3 domain, two proline-rich regions and a BCR-homology (BH) domain (Figure 8). Interaction of these N-terminal extensions with proline-rich proteins and SH3 domains of Src-related kinases has been reported to affect the lipid kinase activity of p85–p110 complexes (Pleiman *et al.*, 1994). It is clear that adaptors that lack these N-terminal extensions (such as the product of the p55 $\gamma$  gene and splice variants of p85 $\alpha$ ) could form complexes with p110 that are not subject to this form of regulation. It is also intriguing that some splice variants of p85 $\alpha$  contain different putative phosphorylation sites flanking the Ser608 in the inter-SH2 region (Antonetti *et al.*, 1996), opening perspectives for a further differential regulation of p110s upon interaction with different class I<sub>A</sub> adaptors.

The interaction of PI3Ks with membrane-localized Ras is an additional point of regulation, the impact of which is not fully clear at present. It is possible that Ras binding allosterically activates PI3Ks and/or helps to recruit PI3Ks to their lipid substrates (Rodriguez-Viciano *et al.*, 1996).

As documented here, protein phosphorylation adds to the mechanisms for regulation of PI3Ks *in vivo*. Mechanistically, phosphorylation of PI3K adaptor or catalytic subunits might affect the lipid kinase activity in several ways. These include the induction of structural/



**Fig. 8.** Model for p110–p85 $\alpha$  and the impact of phosphorylation on Ser608 in p85 $\alpha$  and Ser1039 in p110 $\delta$  on the lipid kinase activity of class I<sub>A</sub> PI3Ks. The two SH2 domains of p85 $\alpha$  are shown bound to the phosphotyrosines (Y<sub>P</sub>) of a receptor. The phosphoinositide inositol ring is represented by a hexagon that contacts the C-terminus of p110 and the inter-SH2 region of p85. Further support for this model not mentioned in the text is as follows. The inter-SH2 region of p85, predicted to form a coiled-coil, is known to interact with the N-terminus of p110 $\alpha$  and to have a stabilizing effect on full-length p110 (Dhand *et al.*, 1994a; Holt *et al.*, 1994; Klippel *et al.*, 1994; Hu *et al.*, 1995; Yu *et al.*, 1998). The N- and C-termini of p110 are shown in close proximity to each other. This is based on our finding that deletion of >150 amino acids at the N-terminus of p110 $\alpha$  generates molecules that lack lipid and protein kinase activity (B.Vanhaesebroeck, unpublished results), indicating that the N-terminus of p110 $\alpha$  is likely to be important for the folding and/or the activity of its C-terminal catalytic domain. The figure further shows a speculative interaction of a leucine zipper region found in all catalytic subunits (Vanhaesebroeck *et al.*, 1997b) and class I<sub>A</sub> adaptors (amino acid 524–545 in bovine p85 $\alpha$ ). BH, BCR-homology region; PP, proline-rich region (present in p110 $\delta$ , not in p110 $\alpha$  and  $\beta$ ).

conformational changes of the complex, an impact on the phospho-transfer reaction itself or on substrate (ATP/lipid) interaction. In the case of p110 $\alpha$ –p85 $\alpha$ , binding of ATP analogues is unaffected by pre-phosphorylation of p85 $\alpha$  by p110 $\alpha$  (Wymann *et al.*, 1996). It is possible that the C-terminus of p110 $\delta$  and the inter-SH2 region of p85 $\alpha$  both contribute to the binding of the lipid inositol head group, as is shown in the model in Figure 8. According to this model, addition of a negatively charged phosphate group to either Ser608 in p85 $\alpha$  or Ser1039 in p110 $\delta$  is expected to hinder the binding of the negatively charged lipid substrate, and to adversely affect the lipid kinase activity of the p110–p85 complex. Indeed, artificial creation of a negative charge at the C-terminus of p110 $\delta$  (by mutation of Ser1039 to D or E) results in p110 $\delta$  mutants with reduced lipid kinase activity. Analogous mutations have not been described for Ser608 in p85 $\alpha$ , but evidence for the presence of a lipid-binding site in the inter-SH2 region of this molecule has been presented (End *et al.*, 1993). A detailed biochemical and mutational analysis to gain more evidence for this model is in progress.

Taken together, our data show that a regulatory phosphorylation site of p110 $\delta$  *in vitro* is also targeted under physiological circumstances, and leads to a down-regulation of the lipid kinase activity of p110 $\delta$  *in vivo*. Such a

down-regulation of PI3Ks could have important implications, for example by suppressing the anti-apoptotic action of PI3Ks in cancer cells (Franke *et al.*, 1997). The key question that remains, however, is whether this is a down-regulation event that merely serves to switch off this enzyme or whether it leaves the protein kinase activity of p110 $\delta$  intact. In other words, upon being down-regulated as a lipid kinase, does p110 $\delta$  function as a ‘protein kinase only’? Unfortunately, it has not been possible to test this hypothesis directly for class I<sub>A</sub> PI3Ks by adding substrate to pre-phosphorylated p110 $\alpha$  or p110 $\delta$ : neither p110 $\alpha$  nor p110 $\delta$  recognize and convert their protein kinase substrates when presented in solution. These include peptides corresponding to the p85 $\alpha$  or p110 $\delta$  phosphorylation sites, or p85 $\alpha$  presented in solution to an N-terminally truncated p110 $\alpha$  that is still fully competent for lipid conversion but can no longer interact with p85 (B.Vanhaesebroeck, unpublished results). These observations indicate that PI3K protein kinase reactions only occur under conditions of very specific protein–protein interaction such as, for example, those occurring when PI3Ks are recruited to receptor complexes.

Micro-injection studies of p110 isoform-specific antisera show that class I<sub>A</sub> PI3Ks possess non-redundant functions (B.Vanhaesebroeck, G.E.Jones, W.E.Allen, D.Zicha,

R. Hooshmand-Rad, C. Sawyer, C. Wells, M. D. Waterfield and A. J. Ridley, submitted). The distinct protein kinase activities of class I<sub>A</sub> PI3Ks might contribute to these selective functions. Alternatively, the ability of p110 isoforms to go into a protein kinase-only mode of action could itself produce a distinct cellular response, even if the actual protein kinase substrates of the different p110 isoforms were the same. Thus, the cellular response to protein plus lipid kinase activation of PI3Ks might be different from the response to protein kinase activation alone. Answering the question as to whether PI3Ks possess protein kinase activity *in vivo* is one of the main challenges in this research field. Evidence is accumulating that the conserved PI3K-like kinase domain in the PIK-related proteins supports *bona fide* protein kinase reactions, and it is therefore conceivable that this domain also fulfils such a role in PI3Ks (Brunn *et al.*, 1997; Shieh *et al.*, 1997; Banin *et al.*, 1998; Canman *et al.*, 1998; Woo *et al.*, 1998). There is evidence to suggest that the IRS-1 adaptor protein becomes phosphorylated by p85/p110 $\alpha$  upon stimulation with insulin or interferon- $\alpha$ , but it is difficult to exclude the implication of other kinases in this phenomenon (Lam *et al.*, 1994; Tanti *et al.*, 1994; Uddin *et al.*, 1997). Most recently, Wymann and co-workers showed that 'protein kinase-only' mutants of the G protein-linked PI3K p110 $\gamma$  no longer activated protein kinase B (a lipid-dependent phenomenon) but activated the mitogen-activated protein kinase pathways in cells (Bondeva *et al.*, 1998). These findings clearly demonstrate that the protein kinase activity of PI3Ks can function in a cellular context.

We favour the view that the protein kinase activity of PI3Ks is an integral part of their physiological signalling. PI3K protein kinase reactions are likely to be strictly regulated in time and space, governed by localization and translocation of these enzymes. Such specific substrate interactions are likely to be induced by recruitment of PI3Ks to activated signalling complexes. Experiments to unveil PI3K protein kinase targets in these locations are in progress.

## Materials and methods

### General reagents

LY294002 and okadaic acid were purchased from Calbiochem; wortmannin was from Sigma.

### Cell culture

The leukaemic T cell line Jurkat was grown in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. CHO cells transfected with B7.1 cDNA were established and maintained as described previously (Ward *et al.*, 1993). BaF/3, FD-6 and MC/9 cell lines have been described elsewhere (Welham *et al.*, 1994; Vanhaesebroeck *et al.*, 1997b).

### Recombinant proteins

PI3K catalytic subunits (p110s) were expressed in Sf9 insect cells either alone or in combination with class I<sub>A</sub> adaptor proteins (i.e. bovine p85 $\alpha$ ,  $\beta$  or  $\gamma$ ). The p110 proteins were either untagged or tagged with an N-terminal GST tag (Vanhaesebroeck *et al.*, 1997b). GST-p110 proteins were purified using glutathione-Sepharose (Pharmacia). Complexes of untagged p110s with class I<sub>A</sub> adaptor proteins were purified using Actigel (Sterogene) coated with the tyrosine-phosphorylated platelet-derived growth factor (PDGF) receptor peptide Y<sub>p</sub>VPMLG (Y<sub>p</sub> = phosphotyrosine) that binds the co-expressed class I<sub>A</sub> adaptor proteins. All assays were performed using Actigel-bound, untagged p110-p85 $\alpha$  complexes, unless otherwise indicated. The relative amounts of p110 $\delta$  mutant proteins (see below) were determined by quantitative Western blotting

using non-C-terminal antibodies to p110 $\delta$  in combination with [<sup>125</sup>I]protein A.

### Lipid and protein kinase assay

Unless otherwise stated, assays were performed using proteins immobilized on glutathione or PDGF receptor phosphopeptide beads. Lipid kinase assays were for 10 min at 37°C in 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.5 mM EGTA, 1 mM ATP, 50  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP/ml, 2 mM MgCl<sub>2</sub> and sonicated lipids at 150  $\mu$ g/ml.

Unless otherwise stated, *in vitro* protein kinase assays were performed for 30 min at 37°C in 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM MnCl<sub>2</sub>, 40  $\mu$ M ATP and 50  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP/ml.

To test the effect of pre-phosphorylation of p110 $\delta$  on its lipid kinase activity, a protein kinase assay was carried out for 1 h at 37°C in the presence of 1 mM MnCl<sub>2</sub> with or without 1 mM ATP. The reaction mixtures were then shifted to ice, equally reconstituted with ATP immediately followed by the addition of 4 mM EGTA (to chelate the Mn<sup>2+</sup>). After a 5 min incubation at room temperature, a mix of lipids, [ $\gamma$ -<sup>32</sup>P]ATP and MgCl<sub>2</sub> was added (to a final concentration of 150  $\mu$ g/ml, 50  $\mu$ Ci/ml and 1.5 mM, respectively). Lipid kinase activity assay was then for 10 min at 37°C.

The stoichiometry of p110 $\delta$  autophosphorylation was determined in a 1 h protein kinase assay in the presence of 0.1 mM ATP and calculated based on the specific activity of the [ $\gamma$ -<sup>32</sup>P]ATP, the counts incorporated into p110 $\delta$  (determined by Cherenkov counting of the p110 $\delta$  band cut from Coomassie Blue-stained SDS-PAGE gel) and the amount of p110 $\delta$  [estimated from Coomassie Blue-stained SDS-PAGE gels by comparison with stained bovine serum albumin standards].

### Inhibitor studies

p110 $\delta$ -p85 $\alpha$  complexes immobilized on PDGF receptor phosphopeptide beads were pre-treated with a titration series of wortmannin or LY294002 in 50 mM Tris-HCl pH 7.4, 100 mM NaCl, immediately followed by addition of 1.5 mM MnCl<sub>2</sub>, 20  $\mu$ M ATP and 50  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP/ml for 30 min at 37°C (for autophosphorylation assay), or 1.5 mM MgCl<sub>2</sub>, 150  $\mu$ g/ml PtdIns, 1 mM ATP and 50  $\mu$ Ci/ml [ $\gamma$ -<sup>32</sup>P]ATP for 10 min at 37°C (for lipid kinase assay). Quantitation of phosphorylation was performed using a phosphoimager.

### Determination of the autophosphorylation site in p110 $\delta$

Gel-excised autophosphorylated p110 $\delta$  was washed twice for 60 min in 50 ml of isopropanol to remove SDS, twice for 20 min in 50 ml of HPLC grade water and once for 20 min in 10 mM Tris-HCl pH 8.0. The gel slice was then crushed and incubated for 24 h at 37°C in 10 mM Tris-HCl pH 8.0 containing Lys-C endoproteinase (Boehringer Mannheim) to a protein ratio of 1:200 by weight, followed by the addition of the same amount of Lys-C for another 24 h at 37°C. The supernatant was collected and the gel fragments were washed twice with 100  $\mu$ l of 20% acetonitrile and once with 100  $\mu$ l of 50% acetonitrile for 10 min each. The combined supernatants were then filtered through a Millipore 0.22  $\mu$ m filter unit, dried down, resuspended in 1% acetonitrile/0.08% trifluoroacetic acid and applied onto a tandem 2.1 $\times$ 30 mm AX-300 anion exchange pre-column coupled to a C18 Aquapore OD 300 (2.1 $\times$ 100 mm) column equilibrated in buffer A [25 mM NaAcOH pH 5.5, 1% acetonitrile] at a flow rate of 0.2 ml/min. A 1-42% CH<sub>3</sub>CN gradient with buffer B [25 mM NaAcOH, 70% acetonitrile] was then applied for 60 min. Fractions were collected every 0.5 min and their radioactivity determined by Cherenkov counting. The fractions containing the peak <sup>32</sup>P counts were pooled and rechromatographed on a 1 $\times$ 100 mm C18 ODS HPLC column equilibrated in buffer A (0.08% trifluoroacetic acid, 1% acetonitrile) and eluted over a 60 min period by application of a 1-54% acetonitrile gradient using buffer B (0.08% trifluoroacetic, 90% acetonitrile). The amino acid sequence of the peptide in the fraction containing the peak radioactivity was then determined using an ABI Procise system.

### Mutagenesis and expression of p110 $\delta$ in insect cells

Baculovirus transfer vectors (InVitrogen) for PI3Ks were co-transfected with BaculoGold DNA (Pharming) in Sf9 insect cells using Lipofectin reagent (Gibco-BRL). Construction of expression vectors for GST-tagged and untagged kinase-dead p110 $\delta$  (p110 $\delta$ -R894P) mutant have been described (Volinia *et al.*, 1995; Vanhaesebroeck *et al.*, 1997b). The same PCR mutagenesis and cloning strategy as that used for the derivation of p110 $\delta$ -R894P was followed in order to create the S1039A, S1039D and S1039E mutants of p110 $\delta$ , with the exception that the latter PCR products were cleaved with *Nde*I only and subcloned sticky-blunt into the *Xho*I-opened, Klenow-blunted and then *Nde*I-cleaved pBluescript-

p110 $\delta$ -EcoII (Vanhaesebroeck *et al.*, 1997b). Oligonucleotides used to create the S1039 mutants were: sense primer 3 (Vanhaesebroeck *et al.*, 1997b) combined with the following antisense mutagenic primers (mutagenic residues are underlined, the stop codons is in bold): primer 5, 5'-CCCCCTCGAGAATTCTACTGCCTGTTGTCTTTGGCCACGTTGTGGCCAGCC-3' (for S1039A); primer 6, 5'-CCCCCTCGAGAATTCTACTGCCTGTTGTCTTTGGCCAGCC-3' (for S1039D); primer 7, 5'-CCCCCTCGAGAATTCTACTGCCTGTTGTCTTTTCCACGTTGTGGCCAGCC-3' (for S1039E).

### Antisera and immunoblotting

Antiserum to the C-terminus of p110 $\beta$  (S-19) was purchased from Santa Cruz. The generation of rabbit polyclonal antiserum against a synthetic peptide based on the C-terminus of p110 $\delta$  (C-KVNWLAHNVSKDNRQ) has been described (Vanhaesebroeck *et al.*, 1997b). This antiserum was found to recognize p110 $\delta$  independently of its autophosphorylation status. For the generation of an antiserum specific for p110 $\delta$  phosphorylated on Ser1039, rabbits were immunized with the synthetic peptide KTKVNWLAHNVSpKDNRQ [Sp = phosphoserine] coupled via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester to BSA. The resulting antiserum was then purified as follows. First, the antibodies reactive with the non-phosphorylated peptide were depleted using the C-KVNWLAHNVSKDNRQ peptide coupled to Actigel (Sterogene). Non-retained antibodies were then affinity purified over a C-KVNWLAHNVSpKDNRQ-Actigel column [purification was performed in phosphate-buffered saline (PBS) supplemented with 30 mM NaF and 0.1 mM NaVO<sub>3</sub>]. The resulting antiserum was found to recognize specifically the autophosphorylated form of p110 $\delta$  in immunoblot experiments [at 0.1  $\mu$ g/ml in an overnight incubation at room temperature in 5% (w/v) skimmed milk/0.2% (v/v) Tween-20/0.05% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> in PBS]. Routinely, an excess of unphosphorylated C-KVNWLAHNVSKDNRQ peptide was added (10  $\mu$ g of peptide per 1–5  $\mu$ g of antiserum) during the immunoblot development in order to exclude any potential residual reactivity with non-phosphorylated p110 $\delta$ . Inclusion of phosphorylated C-KVNWLAHNVSpKDNRQ peptide (20  $\mu$ g/ml) during the Western blot completely wiped out the reactivity of the phospho-Ser1039 antiserum (data not shown). Cell lysis buffer has been described elsewhere (Vanhaesebroeck *et al.*, 1997b). In experiments where cells were pre-treated with okadaic acid, this lysis buffer was supplemented with 50 mM NaF. Western blots were developed using horseradish peroxidase conjugated secondary antibody and chemiluminescent substrates (Amersham ECL or Pierce SuperSignal Ultra).

### CD28 studies

A total of  $3 \times 10^7$  Jurkat cells were co-sedimented gently with  $10^7$  B7.1-expressing CHO cells (B7 is a CD28 ligand) in a volume of 1 ml at 200 g for 10 s, incubated at 37°C in RPMI1640 for the times indicated and the pellets lysed in 1 ml of lysis buffer (1% NP-40, 100 mM NaCl, 20 mM Tris-HCl pH 7.4, 10 mM iodoacetamide, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml antipain, 1  $\mu$ g/ml chymostatin, 1  $\mu$ g/ml pepstatin A, 1 mM sodium orthovanadate, 10  $\mu$ g/ml TLCK and 10  $\mu$ g/ml di-isofluorophosphate). Lysates were pre-cleared and immunoprecipitations performed for 2 h at 4°C as described (Ward *et al.*, 1992) using either anti-CD28 monoclonal antibody 9.3 (1  $\mu$ g/ml lysate) or anti-p110 C-terminal antisera bound to protein A-Sepharose beads (Pharmacia).

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