The catalytic subunit of phosphatidylinositol 3-kinase is a substrate for the activated platelet-derived growth factor receptor, but not for middle-T antigen–pp60^{c-src} complexes

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The interaction of phosphatidylinositol 3-kinase (PI 3-K) with polyoma-virus middle-T antigen–pp60^{c-src} (mT:cSrc) complexes and with the platelet-derived growth factor (PDGF) receptor has been investigated. Firstly, we undertook reconstitution studies, using proteins derived from a baculovirus expression system. The p110 catalytic subunit of the PI 3-K associated with tyrosine kinases only when complexed with the p85 α regulatory subunit. Both p85 α and p110 were substrates of the PDGF receptor. In contrast, only the p85 α subunit was detectably phosphorylated when PI 3-K was associated with mT:cSrc. Secondly, we studied PI 3-K in mammalian cells. In mT-antigen-transformed NIH-3T3 cells neither p85 α nor p110 was phosphorylated on tyrosine residues *in vivo*, even though p85 α was a substrate in kinase assays *in vitro*. In quiescent NIH-3T3 cells, PI 3-K showed detectable activity *in vitro*; PDGF stimulation resulted in a rapid

and transient association of PI 3-K with the receptor, which was correlated with a transient increase in intrinsic PI3-K activity (approx. 2-fold). The activated PDGF receptor phosphorylated p110 *in vitro*, at one major site. *In vivo*, PDGF stimulation induced tyrosine phosphorylation of p110 that persisted for at least 1 h after stimulation. Immunodepletion of the PDGF receptor from stimulated cell lysates showed that p110 was released from the receptor in a tyrosine-phosphorylated form. From these results we conclude that (i) the mT:cSrc complex and the PDGF receptor differ in their association with PI 3-K activity, (ii) PDGF receptor appears to activate PI 3-K *in vivo* both by relocation of the enzyme and by stimulation of its intrinsic activity, and (iii) tyrosine phosphorylation of the p110 subunit by the PDGF receptor may play a role in PI 3-K regulation in some circumstances.

INTRODUCTION

Phosphatidylinositol 3-kinase (PI 3-K) was first identified as an enzyme associated with protein tyrosine kinases of the Src family and with the activated platelet-derived growth factor (PDGF) receptor (Sugimoto et al., 1984; Kaplan et al., 1987). It was subsequently shown to associate with several receptors, including those for epidermal growth factor, nerve growth factor and colony-stimulating factor-1 (CSF-1), as well as with several non-receptor tyrosine kinases such as the polyoma-virus middle-T antigen–pp60^{c-src} (mT:cSrc) complex, and *fms*, *crk* and *abl* oncogene products (reviewed by Cantley et al., 1991). PI 3-K phosphorylates phosphoinositides [including phosphatidylinositol (PI), its 4-phosphate (PI4P) and its 4,5-bisphosphate [PI(4,5)P₂]] at the D-3 position of the inositol ring, leading to the formation of PI3P, PI(3,4)P₂ and PI(3,4,5)P₃ (Whitman et al., 1988). These products are poor substrates for any known member of the phospholipase C family (Lips et al., 1989; Serunian et al., 1989), suggesting that they may not belong to the canonical PI cycle, but that they may form a new class of second messenger (Cantley et al., 1991). In addition, PI 3-K activation and formation of 3-phosphorylated phosphoinositides occur rapidly after growth-factor activation (Auger et al., 1989), and are closely, although not totally, correlated with cell transformation or cell proliferation induced by tyrosine kinases (Courtneidge and Heber, 1987; Kaplan et al., 1987; Talmage et al., 1989; Ulug et al., 1990; Fantl et al., 1992; Kazlauskas et al., 1992); thus PI 3-K activation and the formation of its products could be an important step in mitogenic/oncogenic signal transduction.

PI 3-K has been purified from many tissues (Carpenter et al., 1990; Otsu et al., 1991; Shibasaki et al., 1991); it usually consists of a heterodimer of a 85 kDa subunit and a 110 kDa subunit. Protein sequencing and cDNA cloning revealed two related 85 kDa proteins (called p85 α and p85 β) that could bind and were substrates for both the PDGF receptor and the mT:cSrc complex (Escobedo et al., 1991a,b; Otsu et al., 1991; Skolnik et al., 1991). These proteins lack PI 3-K activity, but are nonetheless components of the PI 3-K, since antibodies specific for them precipitate PI 3-K activity from stimulated cells (Escobedo et al., 1991b; Otsu et al., 1991). The p85 proteins contain one src homology region 3 (SH3) domain, as well as two SH2 domains. The latter have been shown to bind with high specificity and high affinity to tyrosine-phosphorylated sequences (Koch et al., 1991), and indeed, p85 α has been shown to form complexes with activated autophosphorylated growth-factor receptors and the mT:cSrc complex via its SH2 domains (Hu et al., 1992; Klippel et al., 1992; McGlade et al., 1992; Yoakim et al., 1992). From these data the idea has emerged that p85 α subunit serves as a docking protein between the catalytic subunit of the PI 3-K and activated tyrosine kinases.

The function of the PI 3-K is currently unknown; however, the recent cloning of the p110 catalytic subunit has provided new insight (Hiles et al., 1992). Surprisingly, p110 displays a remarkable similarity to the *Saccharomyces cerevisiae* protein VPS34, which itself has PI 3-K activity (Schu et al., 1993). This protein has been implicated in targeting of proteins to the yeast vacuole, as well as in vacuole morphogenesis during budding (Herman and Emr, 1990). This homology led to speculation on

Abbreviations used: PI, phosphatidylinositol; PI 3-K, PI 3-kinase; PDGF, platelet-derived growth factor; mT, polyoma-virus middle-T antigen; mT:cSrc, mT–pp60^{c-src} complex.

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the possible role of the PI 3-K in the biological processes involving protein trafficking and vesicular morphogenesis (Panayotou and Waterfield, 1992).

The molecular mechanism by which tyrosine kinases activate PI 3-K is obscure. One possibility is that, by binding to membrane-associated proteins, the PI 3-K is translocated close to its substrates. Indeed, a mutant of the oncogenic Abl protein kinase that does not associate with the plasma membrane is unable to activate PI 3-K and to transform cells (Varticovski et al., 1991). On the other hand, tight binding to receptors in the plasma membrane might also cause activation by an allosteric mechanism. In support of this idea, PI 3-K activity can be increased simply by addition of tyrosine-phosphorylated peptides that correspond to binding sites on the insulin-receptor substrate (IRS1) for PI 3-K (Baker et al., 1992). In addition, a conformational change of the p85 α subunit has been reported when it is associated with phosphorylated peptides that mimic known binding sites to PI 3-K (Panayotou et al., 1992; Shoelson et al., 1993). Tyrosine phosphorylation could also take part in PI 3-K activation. For example, phospholipase C γ is not only recruited to the plasma membrane by binding to the activated PDGF and epidermal-growth factor receptors (Kumjian et al., 1989; Margolis et al., 1989; Meisenhelder et al., 1989; Wahl et al., 1989; Morrison et al., 1990), but is also activated by tyrosine phosphorylation catalysed by the receptors (Nishibe et al., 1990; Kim et al., 1991). Several pieces of data are consistent with this hypothesis in the case of PI 3-K: the p85 α subunit has been described as a substrate of PDGF receptor and mT:cSrc complex *in vitro* (Courtneidge and Heber, 1987; Kaplan et al., 1987), and treatment of purified bovine brain PI 3-K with a tyrosine phosphatase has been shown to decrease its activity (Ruiz-Larrea et al., 1993). However, p85 α is poorly phosphorylated on tyrosine *in vivo* after PDGF stimulation (Hu et al., 1992), and the phosphorylation state of the catalytic subunit after tyrosine kinase activation is still obscure. Firm evidence for activation by phosphorylation in the case of PI 3-K is still lacking and needs clarification. However, these three mechanisms are clearly not mutually exclusive, and could co-operate to achieve full activation. In order to understand more clearly the regulation of the PI 3-K by tyrosine kinases, we describe in this paper an analysis of the molecular association of PI 3-K with the mT:cSrc complex and with the PDGF receptor.

EXPERIMENTAL

Antibodies

Antibodies specific for phosphotyrosine were purchased from Upstate Biochemical Inc. The p110.1 antisera (used for immunoprecipitation) were generated in rabbits to the 16 C-terminal amino acids of bovine brain p110 subunit as previously described (Hiles et al., 1992). The p110.2 antisera (used for immunoblotting) was generated in rabbits to amino acids 776–791 of the bovine brain p110 subunit. Antisera to the PDGF receptor (generated against a peptide corresponding to the C-terminal 13 residues of the human PDGF receptor, PR4) (Kypta et al., 1990), middle-T-antigen-specific antibodies (3C3, mTc) as well as cSrc antibody (327) have also been described previously (Courtneidge and Smith, 1983).

Cell culture

NIH-3T3 cells and middle-T-antigen-transformed derivatives were maintained in Dulbecco's modified Eagle medium (DMEM) (GIBCO) containing 10% foetal-calf serum. For PDGF-stimu-

lation experiments, NIH-3T3 cells were growth-arrested at confluence, incubated for 40–48 h in DMEM supplemented with 0.5% foetal-calf serum, and then stimulated for various times with PDGF BB (12.5–50 ng/ml) (Upstate Biochemical Inc.). *Spodoptera frugiperda* (Sf9) were maintained in IPL-41 medium supplemented with 10% foetal-calf serum.

Baculoviruses and insect-cells infection

Recombinant baculoviruses expressing p110 and p85 α subunits of bovine brain PI 3-K were generated as described previously (Otsu et al., 1991; Hiles et al., 1992). Recombinant baculoviruses expressing human PDGF β receptor, chicken cSrc and middle-T antigen were kindly provided by Debbie Morrison, Joan Brugge and Beverley Griffin respectively. Cells were infected or co-infected for 2 days with corresponding baculoviruses before lysis.

Immunoprecipitation, kinase assay *in vitro* and immunoblotting

Methods for immunoprecipitation of proteins, kinase assay and SDS/PAGE have been described previously (Kypta et al., 1990). Briefly cells were rinsed twice in cold TBS (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM sodium orthovanadate) and then lysed in LB (20 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P40, 1% aprotinin, 20 μ M leupeptin, 0.1 mM sodium orthovanadate, 10 mM NaF) at 4 °C. For some experiments cells were lysed in 0.5% SDS, boiled for 3 min at 95 °C and then diluted in 5-fold-concentrated RIPA buffer (see below) lacking SDS. Lysed cells were transferred to microfuge tubes, vortex-mixed, incubated for 10 min and then centrifuged for 10 min at 10000 *g* to remove insoluble material. The supernatants were then pre-cleared by incubation for 30 min with formalin-fixed *Staphylococcus aureus* (final concn. 10%, v/v) that had been pre-saturated with normal rabbit serum for 30 min and washed three times in lysis buffer. Lysates were incubated with antisera for 60 min and then with 10 μ l of *S. aureus* (for kinase assay or lipid kinase assay) or 10 μ l of Protein A-Sepharose (for immunoblotting experiments) for a further 30 min. All incubations and centrifugations were at 4 °C. Immunoprecipitates were washed four times with LB or RIPA (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1% aprotinin, 20 μ M leupeptin, 100 μ M sodium orthovanadate) as appropriate and once with TBS. Kinase assays were performed in 20 μ l of 20 mM Hepes (pH 7.5)/10 mM MnCl₂ containing 2–10 μ Ci of [γ -³²P]-ATP (3000–5000 Ci/mmol) (Amersham) for 10 min at 30 °C. The samples were then electrophoresed on SDS/polyacrylamide gels and analysed by autoradiography. In some experiments the gels were treated with 1 M KOH at 55 °C to remove the background due to serine phosphorylation. In the reprecipitation experiments, the kinase assays were stopped in TBS, and, after centrifugation, the *S. aureus* pellet was resuspended in 0.5% SDS and boiled for 3 min. *S. aureus* cells were removed by centrifugation, the supernatants were diluted 5-fold in RIPA buffer lacking SDS, and immunoprecipitations were carried out with the indicated antibodies.

For immunoblotting experiments, transfer of proteins to nitrocellulose (BA85; Schleicher and Schuell) was performed by using a semi-dry apparatus according to the manufacturer's (Millipore) instructions. Bound antibodies were detected with a horseradish peroxidase-conjugated anti-mouse IgG for the anti-phosphotyrosine antibodies and a horseradish peroxidase-conjugated anti-Protein A for the anti-PDGF receptor and anti-p110.2 antibodies, followed by ECL detection (Amersham).

For immunodepletion experiments, cell lysates were incubated with 15 μ l of antisera and further incubated with Protein A-

Sephacrose for 30 min, centrifuged, and the supernatant was used for a second round of immunodepletion. After centrifugation, the supernatant was incubated for 30 min with *S. aureus*. Immunoprecipitation was then performed on the immunodepleted cell lysate as described above.

Analysis of labelled proteins

Phosphoamino acid analysis was performed as described by Twamley et al. (1992). Briefly, the labelled proteins were transferred to Immobilon membranes (Millipore), identified by autoradiography, excised, and washed several times in water. The membrane pieces were then incubated for 90 min at 110 °C in 5.7 M HCl in a sealed Eppendorf tube. The insoluble material was removed by centrifugation and the supernatant dried under vacuum. The dried residue was resuspended in pH 3.5 buffer (for 1 litre: 5 ml of pyridine, 50 ml of acetic acid and 945 ml of water) containing unlabelled phosphoamino acids. The sample was loaded on a t.l.c. plate (Kodak) and run at pH 3.5 at 750 V for 50 min.

For tryptic mapping of proteins, pieces of nitrocellulose membrane containing the protein of interest were washed with water and then soaked in 0.5 % polyvinylpyrrolidone (PVP-360; Sigma) in 100 mM acetic acid at 37 °C for 30 min. The membrane pieces were then washed five times with water, then twice with freshly made buffer (0.05 M Na₂CO₃) and then digested with 10 µg of trypsin [treated with tosylphenylalanylchloromethane (TPCK); Boehringer] in 0.15 ml of incubation buffer for 4 h at 37 °C. Another 10 µg of trypsin was added for a further 4 h, and then water was added and the same was dried under vacuum. Trypsin-digested peptides were separated by electrophoresis at pH 8.9, followed by ascending chromatography as described above.

PI 3-K assay *in vitro*

PI 3-K assay was performed essentially as described by Courtneidge and Heber (1987) with minor modifications. Briefly, after extensive washing, immune complexes were resuspended in 40 µl of 20 mM Hepes (pH 7.5), 5 mM MgCl₂, 0.5 mg/ml phosphatidylinositol (previously dispersed by sonication for 15 min at 4 °C in 20 mM Hepes, pH 7.5), and 5–10 µCi of [γ -³²P]ATP. After incubation for 10 min at 30 °C, the reaction was stopped by addition of 100 µl of 1 M HCl and 200 µl of chloroform/methanol (1:1, v/v), washed with 80 µl of HCl methanol (1:1, v/v) and the solvent was evaporated. Products were resolved by chromatography on silica-gel 60 plates impregnated with 1 % potassium oxalate in chloroform/methanol/4 M NH₄OH (9:7:2, by vol.) for 1 h. Phosphorylated products were detected by autoradiography and identified by comparison with radiolabelled PI3P generated from immunopurified Sf9-expressed PI 3-K used as a standard. Lipids products were quantified by using Phosphorimager analysis.

Binding assay *in vitro*

This was performed essentially as described by Otsu et al. (1991). Cell lysates were prepared as described above. The mT:cSrc complex formed in co-infected Sf9 cells, or PDGF receptor expressed in infected cells, was precipitated with appropriate antibodies. The immune complexes were washed three times in RIPA buffer and once in TBS buffer and incubated with a second Sf9-cell lysate expressing p85 α , p110 or the complex p85 α :p110, for 30 min at 4 °C. At the end of the incubation, the complexes

were washed three times in RIPA buffer and twice in TBS and then subjected to either phosphorylation *in vitro* or PI 3-K assays as described above.

RESULTS

Association of PI 3-K with mT:cSrc

Reconstitution of PI 3-K with the mT:cSrc complex was performed *in vitro*. The mT:cSrc complex was generated in insect cells by co-infection with recombinant baculoviruses encoding mT and cSrc proteins. We have previously shown that we achieve correct complex-formation of middle-T antigen and cSrc in insect cells (Otsu et al., 1991). The PI 3-K was generated by co-infecting insect cells with baculoviruses expressing p85 α (the regulatory subunit) and p110 (the catalytic subunit): under these conditions a stable p85 α -p110 complex is formed, and immunoprecipitation analysis using specific antisera has shown that the complex has PI 3-K activity (Hiles et al., 1992). The mT:cSrc complex was isolated by using an antiserum specific for mT, and incubated with Sf9 lysates expressing p85 α alone, p110 alone, or both p85 α and p110. As shown in Figure 1(a), no PI 3-K activity was associated with the mT:cSrc complex when it was incubated with lysates expressing either p85 α or p110 alone. Since PI 3-K is active in insect cells infected with the baculovirus encoding p110 alone (Hiles et al., 1992), we concluded that p110 was unable to bind directly to the mT:cSrc complex. In contrast, incubation of mT:cSrc with lysates expressing the p85 α :p110 complex led to the detection of mT-associated PI 3-K, indicating that the mT:cSrc:PI 3-K heterotetramer was generated *in vitro*. Thus we conclude that the association of the PI 3-K activity with the mT:cSrc complex required p85 α . To define further the molecular interaction between the mT:cSrc complex and PI 3-K kinase assays *in vitro* were performed using the various immunocomplexes. As shown in Figure 1(b), p85 α was phosphorylated when associated with mT:cSrc, either on its own, or when co-associated with p110. Phosphoamino acid analysis showed that p85 α was phosphorylated on tyrosine (results not shown). In contrast, no p110 phosphorylation was detected in the mT:cSrc:PI 3-K complex, suggesting that the catalytic subunit was, at best, a poor substrate for cSrc. We next determined whether PI 3-K was a substrate of mT:cSrc *in vivo*. Insect cells were co-infected with the baculovirus expressing the different components of the complex (i.e. mT, cSrc, p85 α and p110). Immunoprecipitates of middle-T antigen or cSrc with their respective antibodies from co-infected cell lysates contained PI 3-K activity (results not shown). Immunoprecipitation of the complex was performed with antibodies specific for mT antigen, cSrc and p110, and tyrosine phosphorylation was assessed by immunoblotting with a monoclonal anti-phosphotyrosine antibody. Immunocomplexes of mT and cSrc showed tyrosine phosphorylation of the respective proteins, but no tyrosine phosphorylation of either subunit of PI 3-K (Figure 1c). Similarly, the p110 immunocomplex contained tyrosine-phosphorylated mT, but no phosphorylation of p110 was observed. A low level of phosphorylation of p85 was seen in the p110 immunoprecipitate.

The interaction of PI 3-K with the mT:cSrc complex was next investigated in the same way in mouse mT-transformed fibroblasts. Immunoprecipitates were made with a serum specific for mT and a polyclonal antibody raised against the C-terminal sequences of the bovine p110 (Hiles et al., 1992). Both immunocomplexes contained PI 3-K activity (Figure 1d). Protein kinase assay *in vitro* of the mT immunocomplex revealed mT, cSrc and the p85 α subunit of the PI 3-K, but no specific phosphorylation of the p110 subunit could be detected (Figure 1e). Protein kinase

Figure 1 Association of PI 3-K with the mT:cSrc complex in Sf9 cells and in mT-transformed NIH-3T3 cells

(a) Sf9 cells expressing mT and cSrc immunoprecipitated with normal rat serum (lanes 1, 3 and 5) or with anti-mT antiserum (3C3 serum) (lanes 2, 4 and 6) were incubated with p85 α -expressing Sf9 lysate (lanes 1 and 2), p110-expressing Sf9 lysate (lanes 3 and 4), or cell lysate expressing p85–p110 complex (lanes 5 and 6), and a PI 3-K assay was performed. The position of PI3P is shown. (b) As in (a), except that immunoprecipitates were assayed by protein kinase assay *in vitro*. The positions of p85 α , Src (pp60) and middle-T antigen (mT), as well as size markers (in kDa) are shown. (c) Anti-phosphotyrosine immunoblotting. Immunoprecipitations were made from Sf9 cells co-expressing mT:cSrc and p85 α –p110 complexes, using normal rabbit serum (lane 1), anti-mT serum (lane 2), anti-src antibody (lane 3) and anti-p110 serum (lane 4). The positions of the size markers, as well as the heavy chain (H chain) of immunoglobulin, are marked on the right. (d) PI 3-K assay. Immunoprecipitations were made from mT-transformed NIH-3T3 cell lysates, using normal rat serum (lane 1), anti-mT serum (mtc) (lane 2), preimmune serum (lane 3), or p110 serum (lane 4). (e) Immunoprecipitates using mT-transformed NIH-3T3 cells and normal rat serum (lane 1) or 3C3 serum (lane 2), p110 preimmune serum (lane 3) or p110 serum (lane 4) were assayed *in vitro* for associated kinase activity. The positions of the size markers (in kDa) are shown. (f) Anti-phosphotyrosine immunoblotting. Lanes as in panel (d).

assay of the p110 immunocomplex revealed three proteins of molecular masses 110, 85 and 55 kDa: re-precipitation of the denatured immunocomplexes with p110 and p85 antibodies revealed that the 110 kDa band was the catalytic subunit of the PI 3-K and the 85 kDa protein was p85 α , and partial proteolytic mapping identified the 55 kDa protein as mT (results not shown). Phosphoamino acid analysis showed that p110 was phosphorylated exclusively on serine and to a lesser extent on threonine, suggesting the existence of a serine/threonine kinase associated with PI 3-K (results not shown). However, no tyrosine phosphorylation was detected, suggesting that p110 was not a substrate of cSrc *in vitro*. Tyrosine phosphorylation of PI 3-K was investigated in mouse middle-T-transformed fibroblasts by anti-phosphotyrosine immunoblotting. As shown in Figure 1(f), the mT immunocomplex revealed tyrosine-phosphorylated mT, but no tyrosine phosphorylation of proteins of 85 or 110 kDa was observed, suggesting that the PI-3 K associated with the mT:cSrc complex was not phosphorylated on tyrosine *in vivo*. [The two subunits of PI 3-K could be detected by immunoblotting with their specific antibodies (results not shown).] In the p110 immunocomplex, a phosphotyrosine-containing protein of 55 kDa was observed which is probably mT, but again no tyrosine phosphorylation of either subunit of PI 3-K was

detected. These results strongly suggest that the PI 3-K is at best a poor substrate of the mT:cSrc complex in murine fibroblasts.

Association of PI 3-K with the PDGF receptor

The molecular association between the PI 3-K and the activated PDGF receptor was investigated. PDGF receptor was generated in insect cells by infection with a recombinant baculovirus encoding the human PDGF receptor: when expressed in this way, the PDGF receptor is active, presumably as at high concentration in the insect cells it is already dimerized and autophosphorylated (Morrison et al., 1989). PI 3-K was only retained by the PDGF receptor when it was mixed with a cell lysate containing the p85 α and p110 complex (Figure 2a). Protein kinase assays *in vitro* of the various immunocomplexes were then performed. As shown in Figure 2(b), p85 α was phosphorylated when associated with the PDGF receptor, both when present alone and when complexed with p110. Phosphoamino acid analysis confirmed tyrosine phosphorylation of p85 α (results not shown). No phosphorylation of p110 was detected in PDGF-receptor immunoprecipitates mixed with p110 alone (Figure 2b, lane 4); however, phosphorylation of an approx. 110 kDa protein was detected in the PDGF-receptor–PI 3-K complex. The identity

Figure 2 Association *in vitro* of PI 3-K with PDGF receptor

(a) Sf9 cells expressing human PDGF receptor were immunoprecipitated with normal rabbit serum (lanes 1, 3 and 5) or with anti-PR4 (lanes 2, 4 and 6), and incubated with a p85 α -expressing Sf9 lysate (lanes 1 and 2), p110-expressing Sf9 lysate (lanes 3 and 4) or a cell lysate expressing the p85–p110 complex (lanes 5 and 6), and a PI 3-K assay was performed. (b) As in panel (a), except assayed for protein kinase activity. (c) Re-precipitation analysis of the products detected by kinase assay of anti-PR4 immunocomplexes incubated with cell lysates expressing the p85 α –p110 complex. Immune complexes similar to those shown in lane 6 were dissociated with SDS, and then subjected to a second round of immunoprecipitation using either pre-immune serum (P-I) or antibodies specific for p110, as shown (Ab, antibody). (d) Phosphoamino acid analysis of the 110 kDa band detected in panel (b), lane 6.

Figure 3 Detection of p110 in the PDGF-receptor immunocomplex from PDGF-stimulated NIH-3T3 cells

(a) Immunoprecipitation of the PDGF receptor was from NIH-3T3 cells stimulated for 5 min with PDGF (12.5 ng/ml) with normal rabbit serum (lane 1) or anti-PR4 serum (lane 2). The associated proteins were revealed by kinase assay, and products were resolved on a 7.5%-acrylamide gel. (b) Re-precipitation analysis of the 110 kDa products detected by kinase assay in anti-PR4 serum immunocomplexes, with either pre-immune serum (P-I) or antibodies specific for p110. (c) Phosphoamino acid analysis of the 110 kDa band detected in the anti-PR4 immunocomplex by kinase assay *in vitro*. The position of the PDGF receptor (p180) and associated phosphoproteins (p150, p120, etc.) are marked.

of this protein as p110 was confirmed by re-precipitation analysis (Figure 2c), and phosphoamino acid analysis showed that p110 was phosphorylated on tyrosine residues (Figure 2d). Other analyses demonstrated that p110 was also phosphorylated on tyrosine when insect cells were co-infected with PDGF receptor and PI 3-K (results not shown). Thus we conclude that, in contrast with the mT:cSrc complex, both p85 α and p110 subunits were substrates for the PDGF receptor *in vitro*.

We next tested whether PI 3-K was a substrate *in vitro* of the receptor in PDGF-stimulated NIH-3T3-cell lysates. PDGF receptor was immunoprecipitated from quiescent cells stimulated for 5 min with PDGF, and the co-associated proteins were detected by protein kinase assay *in vitro*. This assay revealed several proteins associated with the activated receptor, including proteins of 150, 120, 110, 85, 70 and 60 kDa (Figure 3a). Most of these proteins have already been identified, as phospholipase

Figure 4 Tryptic mapping of tyrosine-phosphorylated p110

(a) Tryptic peptides produced from p110 labelled *in vitro* by the PDGF receptor produced in Sf9 cells. (b) Tryptic peptides produced from p110 labelled *in vitro* by the PDGF receptor from NIH-3T3 cells stimulated with PDGF. (c) Mixture of (a) and (b). Tryptic peptides were separated by electrophoresis at pH 8.9, followed by ascending chromatography. The positions of the major peptides are marked with arrows, and the origin is marked with O.

C γ , GTPase activating protein, p85 α , the tyrosine phosphatase Syp and tyrosine kinases of the Src family. Re-precipitation experiments demonstrated that the 110 kDa protein was indeed p110 (Figure 3b), and phosphoamino acid analysis indicated that it was predominantly phosphorylated on tyrosine residues, although a low level of serine phosphorylation (5% of the total phosphorylation) was also detected (Figure 3c).

Tyrosine phosphorylation *in vitro* of the insect cells expressing bovine p110 was then compared with p110 phosphorylation obtained from PDGF-stimulated fibroblasts. Proteins labelled in kinase assays were digested with trypsin, and the fragments were resolved by electrophoresis at pH 8.9 followed by chromatography. One major labelled fragment was detected, suggesting the existence of a major tyrosine-phosphorylation site in bovine p110 (Figure 4a). One major spot with a very similar migration was also observed in the case of the labelled p110 subunit obtained from PDGF-stimulated NIH-3T3-cell lysates (Figure 4b). The mixing experiment shown in Figure 4(c) confirmed that the phosphopeptides were identical. We conclude that association of p110 with the activated PDGF receptor results in its tyrosine phosphorylation at one major site.

The kinetics of the interaction between PI 3-K and the PDGF receptor was further investigated in fibroblasts stimulated with PDGF. The p110 subunit of PI 3-K was immunoprecipitated from quiescent cells stimulated for various times with PDGF, and the association of PI 3-K with the PDGF receptor was determined by immunoblotting with antibodies raised against the receptor. PDGF stimulation induced a rapid and transient association of PI 3-K with the activated receptor: this complex was detectable within 1 min, maximal after 5 min, and then decreased at later time points, although it was still detectable 1 h

Figure 5 Time course of association of PI 3-K with the PDGF receptor in PDGF-stimulated NIH-3T3 cells

(a) Immunoprecipitates were made by using anti-p110 serum and lysates of quiescent NIH-3T3 cell lysates stimulated with PDGF (50 ng/ml) for the times shown, resolved by SDS/PAGE, transferred to nitrocellulose and probed with antibodies specific for the PDGF receptor (PDGFR). (b) As (a), except that the blot was probed with antibodies specific for p110. (c) Lysates and immunoprecipitation were as in (a), followed by PI 3-K assay. (d) Quantification of the data shown in (c). PI 3-K activity was determined by measurement of labelled PI3P by Phosphorimager quantification. Data are derived from four independent experiments and are expressed as mean $\pm$ S.D.

after stimulation (Figure 5a). Up to 15% of the PDGF receptor was co-immunoprecipitated with p110 (at 5 min after stimulation with 50 ng/ml PDGF; results not shown). PI 3-K activity was present in p110 immunocomplexes from the unstimulated cell lysate; however, a transient increase in activity was observed after growth-factor stimulation, which correlated with the association of the receptor (Figures 5c and 5d). This activation was attributed to an increase in intrinsic activity, since the level of p110 subunits in immunocomplexes was constant (Figure 5b).

We next investigated whether tyrosine phosphorylation of PI 3-K could play a role in the interaction of PI 3-K with the PDGF receptor. Lysates were made from cells stimulated for various times with PDGF, p110 was immunoprecipitated, and tyrosine-

Figure 6 Time-course of tyrosine phosphorylation *in vivo* of p110 in PDGF-stimulated NIH-3T3 cells

(a) Immunoprecipitates were made with anti-p110 serum and lysates of quiescent NIH-3T3 cells stimulated with PDGF (50 ng/ml) for the times indicated. Immunocomplexes were resolved by SDS/PAGE, transferred to nitrocellulose, and probed with anti-phosphotyrosine antibodies. (b) Anti-phosphotyrosine immunoblot of immunoprecipitates made using pre-immune serum (lane 1) or p110 serum (lane 2) and cells stimulated with PDGF for 5 min, lysed under denaturing conditions.

Figure 7 Time course of tyrosine phosphorylation *in vivo* of p110 released from the PDGF receptor

Quiescent NIH-3T3 cell lysates were stimulated with PDGF for the times indicated, lysed, and the lysates were immunodepleted of PDGF receptors. p110 was then immunoprecipitated from the depleted extracts. Immunocomplexes were resolved by SDS/PAGE, transferred to nitrocellulose, and probed with either anti-p110 antibodies (a) or anti-phosphotyrosine antibodies (b). The positions of the p110 subunit, the size markers (in kDa) and the time of PDGF stimulation are shown.

phosphorylated proteins were analysed by anti-phosphotyrosine immunoblotting. No phosphotyrosine-containing proteins were detected in p110 immunocomplexes derived from unstimulated cells (Figure 6a). However, PDGF stimulation induced tyrosine phosphorylation of two major proteins in immunoprecipitates: a band of 180 kDa identified as the receptor, and a band of 110 kDa (Figure 6a). Immunoprecipitation from SDS-denatured lysates confirmed the identity of the 110 kDa protein as the catalytic subunit of PI 3-K (Figure 6b). From these results we conclude that p110 was phosphorylated on tyrosine in PDGF-stimulated fibroblasts *in vivo*. The tyrosine phosphorylation of

p110 was detectable after 1 min of stimulation and increased up to 1 h of growth-factor exposure (Figure 6a). Indeed, substantial tyrosine phosphorylation was detected at times late after stimulation, when the association of PI 3-K with the receptor was declining (compare Figures 5 and 6). These results suggested that p110 was perhaps released from the receptor in a tyrosine-phosphorylated form. To address this question, PDGF-stimulated cell lysates were immunodepleted of PDGF receptors, then p110 was immunoprecipitated and its phosphotyrosine content measured. The level of free p110 subunit was strongly and transiently decreased during PDGF stimulation (Figure 7a), in agreement with the transient association of PI 3-K with the receptor shown in Figure 5(a). However, a tyrosine-phosphorylated form of p110 was detected by these analyses that increased during the time of PDGF stimulation (Figure 7b). A band of 85 kDa was also detected after longer times of PDGF stimulation, suggesting the existence of tyrosine phosphorylation of the p85 α subunit. We conclude that p110 was released from the activated receptor in a tyrosine-phosphorylated form.

DISCUSSION

In this paper, we describe the molecular interaction of PI 3-K with the mT:cSrc complex and with the PDGF receptor. We first took advantage of the baculovirus-expression system in order to reconstitute the association of PI 3-K with both tyrosine kinases. In these assays, PI 3-K required the p85 α regulatory subunit to associate with both the PDGF receptor and the mT:cSrc complex: a similar interaction has also been reported for the CSF-1 receptor (Hiles et al., 1992). Such an interaction appears also to occur *in vivo*, as over-expression of p85 α in fibroblasts inhibits the formation of the PI 3-K-PDGF-receptor complex (Hu et al., 1992). Thus p85 α serves as a docking protein for binding the p110 catalytic subunit to activated tyrosine kinases.

Several reports have shown that, when associated with the mT:cSrc complex or the PDGF receptor, PI 3-K becomes a substrate for these tyrosine kinases (reviewed by Cantley et al., 1991). Indeed, when expressed in insect cells, p85 α was found to be a substrate of both mT:cSrc and the PDGF receptor. In contrast, p110 was not a substrate of the mT:cSrc complex *in vitro* or *in vivo*, whereas it was a substrate of the PDGF receptor both *in vitro* and *in vivo*. These observations point to distinct differences in the interaction of PI 3-K with the mT:cSrc complex and with the PDGF receptor, and suggest that these tyrosine kinases may regulate PI 3-K in different ways. It should be noted that reconstitution of the PI 3-K:mT:cSrc complex was previously described when a purified fraction of rat liver PI 3-K was used (Auger et al., 1992); under these conditions, tyrosine phosphorylation of a 110 kDa protein was observed, suggesting that the p110 subunit could be a substrate of the mT:cSrc complex, although the stoichiometry of phosphorylation was low. The discrepancy between these results and our observations might be due to species differences (we used bovine brain PI 3-K). However, our observations are also consistent with the absence of detectable phosphotyrosine-phosphorylated p110 in murine mT-transformed fibroblasts. Thus p110 appears to be, if at all, a poor substrate of the mT:cSrc complex.

In mT-transformed NIH-3T3 cells, neither p110 nor p85 α appeared to be substrates of cSrc *in vivo*. It is unlikely that the phosphorylated form was not detected by our antibodies, since the complex was immunopurified by using sera specific for several components of the complex [including a serum raised against p85 α (results not shown)], yet in no case was tyrosine phosphorylation detected. This strongly suggests that tyrosine phosphorylation of PI 3-K by the mT:cSrc complex must not be

relevant in fibroblasts. Such a conclusion raises the question of how PI 3-K is regulated by the mT:cSrc complex. It could be activated in an allosteric manner when complexed with mT, as proposed with IRS-1 (Baker et al., 1992). Indeed, binding of a peptide that mimics the binding site of PI 3-K to mT was shown to activate its enzymic activity *in vitro* (Carpenter et al., 1993a). On the other hand, a serine/threonine kinase activity has been found associated with PI 3-K in different cell types (Carpenter et al., 1993b; the present paper), which may decrease its activity after phosphorylation (Carpenter et al., 1993b). We have recently shown that the cloned bovine p110 subunit possesses dual serine protein kinase and lipid kinase activity (Dhand et al., 1994). Interestingly, no serine kinase activity was detected when PI 3-K was bound to the mT:cSrc complex in middle-T-transformed fibroblasts or when expressed in insect cells (results not shown). This suggests that the association of PI 3-K with mT:cSrc could inhibit the p110 serine kinase activity. Alternatively, serine-phosphorylated PI 3-K could be a substrate of the serine/threonine phosphatase, PP2A, that is associated with mT antigen (Pallas et al., 1990; Walter et al., 1990; Ulug et al., 1992), and serine dephosphorylation of PI 3-K may contribute to its activation. The analysis of mutated forms of mT antigen that can associate with cSrc and PI 3-K, but not with PP2A, will be informative in this regard.

In quiescent NIH-3T3 cells, PI 3-K has measurable activity *in vitro*, so that the very low activity observed *in vivo* (Auger et al., 1989) suggests that its subcellular localization prevents substrate accessibility. When stimulated by PDGF, PI 3-K became rapidly associated with the activated receptor. We also detected, under the assay conditions used, an increase in intrinsic PI 3-K activity. This effect was not specific to phosphatidylinositol, since a similar increase was observed when PIP₂ was used as a substrate (results not shown). The activation was observed under standard assay conditions. Although these conditions may not reflect the situation *in vivo*, our observations are nevertheless consistent with the increase in total PI 3-K activity *in vitro* reported recently (Susa et al., 1992) as well as the transient increase in PIP₃ levels observed *in vivo* (Auger et al., 1989). Thus, activation of PI 3-K by the PDGF receptor *in vivo* may be due to both a recruitment to the membrane and also to an increase in its enzymic activity. Several mechanisms could be responsible for this activation. It could be due to an allosteric change of PI 3-K conformation induced by binding to the receptor, leading to a change of activity, as previously proposed (Panayotou et al., 1992; Shoelson et al., 1993). The close correlation observed between the binding of PI 3-K to the receptor and the increase in lipid kinase activity *in vitro* is in agreement with such a hypothesis.

Tyrosine phosphorylation of PI 3-K may also influence its activity. Indeed, PI 3-K became rapidly phosphorylated on tyrosine when associated with the receptor. Although both p85 α and p110 were substrates of the receptor *in vitro*, tyrosine phosphorylation of PI 3-K occurred mainly on p110 *in vivo*. Tyrosine phosphorylation of p85 α was hardly detected under our conditions, in keeping the results of others (Hu et al., 1992). Close inspection of the kinetics of activation and tyrosine phosphorylation of p110 make it unlikely that it is involved in PI 3-K activation. Our results strongly suggest that p110 is released from the receptor in a tyrosine-phosphorylated form. Thus one possibility could be that such a phosphorylation would trigger the dissociation of the complex. However, preliminary experiments performed in insect cells suggests that this may not be the case (Roche, S., unpublished work). Analysis *in vitro* of the role of the phosphotyrosine by, for example, the use of phosphatases, is complicated by the fact that tyrosine phosphorylation of the PDGF receptor is required for PI 3-K

association. To test the hypothesis, the generation of a mutated form of p110 that cannot be tyrosine-phosphorylated will be required.

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