Binding to the Platelet-derived Growth Factor Receptor Transiently Activates the p85α-p110α Phosphoinositide 3-Kinase Complex in Vivo*

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Ligand stimulation of the platelet-derived growth factor (PDGF) receptor results in its association with phosphoinositide 3-kinase activity and a corresponding synthesis of 3-phosphorylated lipids. Early studies that examined this interaction in vivo employed anti-phosphotyrosine antiserum or antiserum against the PDGF receptor. The recent identification of multiple isoforms of both the regulatory and the catalytic subunit of the enzyme have led us to utilize antisera against p85α and p110α to characterize the association of this particular phosphoinositide 3-kinase complex with the PDGF receptor following ligand stimulation of murine fibroblasts. Both the p85α and p110α subunits rapidly associated with the ligand-activated receptor resulting in a transient, 2-fold increase in the total pool of p110α lipid kinase activity. This association was stable for 15 min after initial stimulation. Subsequently, both subunits began to dissociate from the receptor with similar kinetics. By 60 min this process was complete, demonstrating that p85α and p110α both associate with the receptor and dissociate from the receptor as a dimeric complex. At this time, marked PDGF receptor down-regulation was observed. Immunoprecipitation from metabolically labeled cells revealed that p85α is constitutively phosphorylated on serine residues in quiescent cultures. Upon PDGF stimulation, this phosphorylation upon serine residues was maintained in addition to tyrosine phosphorylation of this subunit. No phosphorylation of the p110α subunit was detected in either quiescent or PDGF-stimulated cells. Quantitation of Western blot analysis demonstrated that only 5% of the total pool of p85α associated with the PDGF receptor upon ligand stimulation. The 2-fold increase in the lipid kinase activity measured in immunoprecipitates using either anti-p85α or anti-p110α antiserum therefore reflects a far greater increase in the specific activity of the enzyme upon its association with the PDGF receptor.

Platelet derived growth factor (PDGF) potently stimulates the proliferation of cells of mesenchymal origin. It has been reported to play a role in the physiology of early development, wound healing, and inflammation in addition to the etiology of atherosclerosis, rheumatoid arthritis, and oncogenesis, (1, 2). PDGF consists of a disulfide-linked dimer of two related polypeptide chains assembled either as homodimers or as a heterodimer. The binding of PDGF to its cell surface receptors causes receptor dimerization and transphosphorylation of specific residues along the PDGF receptor polypeptide chain (1, 3, 4). These phosphorylated tyrosine residues then serve as specific attachment sites for many cytoplasmic effector proteins (5). Substrates for the PDGF receptor include phospholipase Cγ, ras GAP, members of the pp60c-src family of protein tyrosine kinases, Nck, Grb2, and the phosphotyrosine phosphatase Syp (6–9). Once bound to the receptor, many of these substrates are themselves phosphorylated on tyrosine residues by the intrinsic receptor kinase activity. In this way, PDGF triggers a diverse array of downstream early signaling events (10–14).

One of the earliest events triggered by PDGF stimulation is an increase in the intracellular level of the 3-phosphorylated inositol lipids, Ptd Ins(3, 4)P2 and Ptd Ins(3, 4, 5)P3 (15, 16). Immunoprecipitates of anti-phosphotyrosine antiserum from PDGF-stimulated cells have been found to contain up to 50-fold more PI 3-kinase activity than those from control cells (17). This interaction between the PDGF receptor and PI 3-kinase was later also demonstrated using antibodies specific for the receptor (18). Because the increase in lipid products following stimulation could be correlated with the mitogenic activity of growth factors and cellular transformation, such studies prompted a series of experiments aimed at characterizing the enzyme responsible (17, 19–21). This work culminated in the purification and cloning of a catalytic subunit from bovine brain termed p110α (22, 23). Biochemical analysis had shown, however, that the purified bovine brain PI 3-kinase was a heterodimer consisting of the catalytic subunit and an adaptor protein that facilitated its interaction with phosphotyrosine residues. Two isoforms of the adapter subunit were originally characterized (24, 25). These proteins termed p85α and p85β link the enzymatically active p110α to receptors with intrinsic tyrosine kinase activity, polyoma middle T antigen, and to complexes formed as a consequence of tyrosine kinase activity. More recently, a related molecule termed p59F1K was cloned on the basis of its association with IRS-1 (26).

It is now apparent that p110α is only one member of a family of PI 3-kinases. Another member with marked sequence similarity to p110α has been termed p110β (27). This enzyme can interact with both the p85α and p85β adaptor proteins and associate with the PDGF receptor in vitro (28). A further two related PI 3-kinases have been characterized in mammalian cells. The first also shows sequence similarity to p110α and p110β but does not interact with any of the known adaptor proteins; it is activated by heterotrimeric G-proteins and is present in cells of hematopoietic origin (29, 30). The second has

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§ The abbreviations used are: PDGF, platelet-derived growth factor; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; PI, phosphoinositide; IRS, insulin receptor substrate.
sequence similarity with the yeast protein vps34; it does not translocate to the plasma membrane upon ligand stimulation and has been implicated in intracellular lysosomal enzyme targeting (31).

With the identification of these additional PI 3-kinases, it now seems likely that studies that examined in vivo mitogen-stimulated increases in PI 3-kinase activity using anti-phosphotyrosine, anti-receptor, or anti-p85α antibodies alone may have inadvertently characterized the activation of several PI 3-kinases and not that of p110α catalytic subunit alone. We have therefore used specific antisera raised against the p85α and p110α subunits to examine the activation of the p85α-p110α PI 3-kinase and its association with the PDGF receptor following ligand stimulation of NIH 3T3 fibroblasts. Our results demonstrate that stimulation with PDGF results in a rapid but transient association of the complex with the PDGF receptor, leading to a transient 2-fold increase in total cellular p110α PI 3-kinase activity. Because we find that no more than 5% of the total p85α pool associates with the activated receptor, we conclude that the translocation of p110α and the corresponding tyrosine phosphorylation of the p85α but not the p110α subunit dramatically increases the specific activity of the p110α catalytic subunit in vivo, resulting in the generation of 3′ phosphorylated inositol lipids.

EXPERIMENTAL PROCEDURES

Cell Culture—Stock cultures of NIH 3T3 cells were subcultured in 33- or 90-mm Nunc dishes using DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were incubated in a humidified atmosphere of 10% CO2, 90% air at 37°C for 3 days. After this time, cells were switched to DMEM containing 0.5% fetal bovine serum. The cultures were confluent and quiescent 24–48 h later.

Generation of Antiserum—A synthetic peptide was generated corresponding to amino acids 1054–1068 of p110α. This peptide was coupled to keyhole limpet hemocyanin using gluteraldehyde and antiserum raised to this conjugate (23). Anti-p85α antiserum was obtained by immunization with recombinant p85α produced in Sf9 cells. The purified protein was mixed with Freund’s adjuvant and subcutaneously injected into rabbits (32). When required, affinity purifications were performed using peptide-antigel affinity columns. The anti-p110α antiserum generated did not cross-react with p110β (33), and the anti-p85α antiserum did not recognize p85β (32). Polyclonal antiserum to the PDGF receptor was generated against a peptide corresponding to the C-terminal 13 residues kindly provided by Sara Courtneidge (34). Antiseras specific to the PDGF receptor α and β chains were purchased from Santa Cruz Biotechnology.

Immunoprecipitations—Quiescent cultures of NIH 3T3 cells were washed twice with DMEM and treated with PDGF for various periods of time at 37°C. The cells were lysed at 4°C in 1 ml of 10 mM Tris/Cl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM NaN3VO4, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer). Lysates were clarified by centrifugation at 13,000 × g, and the supernatants were transferred to a fresh tube. Immunoprecipitation of the PDGF receptor, p85α, and p110α was performed for 4 h at 4°C. At the end of this time, the immunocomplexes were bound to protein A–agarose for 1–2 h at 4°C. After this time, the beads were collected by centrifugation, and the proteins were extracted in Laemmli sample buffer.

Western Blotting—Immunoprecipitates were washed three times with lysis buffer, extracted in 2 × sample buffer (200 mM Tris/Cl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8), and analyzed by SDS-page. Immunoprecipitated proteins were then transferred to PVDF membranes. These membranes were blocked using 5% nonfat dried milk in phosphate-buffered saline, pH 7.2, and incubated for 3–5 h with antibody in phosphate-buffered saline containing 3% nonfat milk. Immunoreactive proteins were visualized using ECL or 125I-labeled protein A.

PI Kinase Assays—The assays for PI kinase activity were performed as described previously (19).

Phosphoamino Acid Analysis—Confluent cultures metabolically labeled with [32P]orthophosphate were treated with PDGF (20 ng/ml) for various times. After cell lysis and centrifugation, the samples were immunoprecipitated with affinity purified anti-p110α antisera and protein A–agarose beads. Bound proteins were separated by SDS-PAGE, Western blotted onto PVDF membrane, and visualized by autoradiography. Radiolabeled proteins were excised from the membrane and hydrolyzed with 6 M HCl for 1 h at 60°C, and the products were analyzed by thin layer chromatography using cellulose-coated plates and flat bed electrophoresis as described previously (35).

Assay of Protein Kinase Activity—Immunoprecipitates prepared as above were washed three times with lysis buffer and twice with 50 mM HEPES, pH 7.4, 0.1 mM EDTA, 0.001% Brij, 75 mM NaCl, and 100 μM NaN3VO4 (kinase assay buffer). After this time, the samples were resuspended in 20 μl of kinase buffer, and the reaction was initiated by the addition of 10 mM MnCl2, 5 mM MgCl2, and 100 μM [γ-32P]ATP (10 μCi/ml) in a total volume of 30 μl at 30°C. After a 10-min incubation, immunoprecipitates were washed twice with lysis buffer, and the phosphorylated proteins were analyzed by SDS-PAGE and autoradiography.

Materials—PDGF (c-sis homodimer), [γ-32P]ATP (5000 Ci/mmol), [3H]methionine (>1000 Ci/mmol), and ECL reagent was obtained from Amersham International. Protein A-Sepharose was from Pharmacia Biotech Inc., and goat anti-mouse IgG-Sepharose was from Sigma. PVDF membrane was from the Millipore Corporation, and Actigel was from Sterogene Bioseparations (Arcadia, CA). All other reagents used were of an analytical grade.

RESULTS

PI 3-Kinase Activity Transiently Associates with the PDGF Receptor Following Ligand Stimulation—To examine the kinetics of PDGF receptor association with PI 3-kinase, NIH 3T3 murine fibroblasts were stimulated with PDGF (20 ng/ml) for increasing periods of time. The cells were then washed with phosphate-buffered saline and lysed immediately on ice. After centrifugation, lysates were immunoprecipitated with an excess of anti-PDGF receptor antisera. The resulting immunocomplexes were collected on protein A-Sepharose beads and washed with lysis buffer. These immunoprecipitates were used for cell-free protein kinase assays, Western blotting, and the measurement of associated PI 3-kinase activity. Phosphorylation of the PDGF receptor was observed 30 s after ligand addition, and it remained maximally phosphorylated for up to 7 min after PDGF addition (Fig. 1A). After this time, the level of receptor tyrosine kinase activity slowly decreased. Phosphorylated p85α was seen in these immunoprecipitates 30 s after PDGF addition. The association of p85α with the receptor remained stable for 15 min, after which time it began to decrease such that after 45 min of stimulation it could no longer be seen.

Western blot analysis of the immunoprecipitated receptor using anti-phosphotyrosine antisera demonstrated receptor autophosphorylation in agreement with those results obtained using the cell-free kinase assay (Fig. 1B). No detectable tyrosine phosphorylation of the PDGF receptor was observed in quiescent cells. Upon ligand addition, receptor phosphorylation was observed after 30 s and was maximal by 1 min. This phosphorylation could be seen for up to 120 min. Western blots were also performed using anti-PDGF receptor antisera (Fig. 1B). Following PDGF addition, a marked down-regulation of the receptor was observed such that it was barely detectable by the antisera 240 min after stimulation. Similar results were obtained when anti-PDGF receptor α and β chain-specific antisera were used for immunoprecipitation (data not shown).

Examination of immunoprecipitates for PI 3-kinase activity showed that no lipid kinase activity was associated with the PDGF receptor in quiescent cells (Fig. 1C). Stimulation with ligand resulted in a transient burst of lipid kinase activity. Maximal PI 3-kinase activity was obtained from PDGF recep-
Phosphorylation of the P85 α/PI 3-Kinase Complex and PDGF Receptor Binding

The P85 α and P110 α Subunits Associate with and Dissociate from the Activated PDGF Receptor as a Dimeric Complex—The association of the P85 α and P110 α subunits with the PDGF receptor was examined by incubating lysates of PDGF-stimulated NIH 3T3 fibroblasts with an excess of specific antisera. The protein and PI 3-kinase activities of the resulting immunoprecipitates were then measured. Our results demonstrate that P85 α immunoprecipitated from lysates of quiescent cells is phosphorylated in a cell-free kinase assay (Fig. 2, A and B). As early as 30 s after ligand addition, the phosphorylation of P85 α was increased and the PDGF receptor co-immunoprecipitated by antisera to both the P85 α and the P110 α subunit. After 15 min, the PDGF receptor had begun to dissociate from the P85 α-P110 α complex, and by 60 min its association was barely detectable. Even after 240 min of stimulation, however, phosphorylated P85 α could still be immunoprecipitated with both the anti-P85 α and the anti-P110 α antisera. A rapidly phosphorylated 120-kDa protein of unknown identity was also transiently immunoprecipitated by both anti-P85 α and anti-P110 α antisera.

Analysis of the PI 3-kinase activity from both anti-P85 α and anti-P110 α immunoprecipitates revealed that PDGF addition stimulated an increase in cellular PI 3-kinase activity (Fig. 2C). This kinase activity was maximal at 3 min when it represented 162 and 182% of the activity immunoprecipitated from quiescent cells by anti-P85 α and anti-P110 α antisera, respectively. Thereafter it rapidly declined, reaching basal levels by 45 min. Immunoprecipitates obtained using anti-P85 α antisera were also used to examine PDGF receptor association by Western blotting using both anti-phosphotyrosine and anti-PDGF receptor antisera. Fig. 2D shows that association of the P85 α-P110 α complex with the activated receptor could be detected by 30 s and that this association could be seen for up to 60 min. These results using anti-P85 α and anti-P110 α antisera complement the data presented in Fig. 1 using the anti-PDGF receptor antibody. We demonstrate in two ways that the association of the P85 α-P110 α complex with the PDGF receptor results in approximately a 2-fold increase in total P110 α PI 3-kinase activity. The subsequent decrease in lipid kinase activity correlated with the dissociation of both the P85 α and P110 α subunits from the PDGF receptor. The kinetics with which both the P85 α and P110 α subunits dissociate from the PDGF receptor together with the constitute immunoprecipitation of P85 α with anti-P110 α antisera demonstrate that loss of PI 3-kinase activity from the PDGF receptor is due to the dissociation of the P85 α-P110 α complex rather than P110 α alone. This dissociation occurs at a time where marked receptor phosphorylation is still observed, although receptor degradation is apparent (Fig. 1).

Ligand Stimulation of the PDGF Receptor Does Not Result in the Phosphorylation of the P110 α Subunit in Intact Cells—From the previous experiment it was not possible to determine if the 120-kDa phosphoprotein seen transiently in anti-P85 α and anti-P110 α immunoprecipitates represented phosphorylated P110 α or an associated protein. Although the in vitro phosphorylation of the catalytic subunit by an activated PDGF receptor tyrosine kinase had been previously reported, phosphorylation of P110 α was not shown to be a transient event, and it only appeared maximally phosphorylated after 30–60 min of ligand stimulation (36). To resolve this issue, confluent and quiescent cultures of NIH 3T3 fibroblasts metabolically labeled with 32P for 16 h were stimulated with PDGF for various times. The cells were washed with phosphate-buffered saline and lysed on ice, and the lysates were immunoprecipitated with affinity purified anti-P110 α antibodies. The resultant immune complexes were analyzed by SDS-PAGE, and the phosphoproteins were transferred onto a PVDF membrane and analyzed by autoradiography (Fig. 3A). These results confirm that P85 α is phosphorylated in quiescent cells, and the addition of PDGF further stimulates its phosphorylation. Phosphoamino acid analysis of the immunoprecipitated P85 α demonstrates that in quiescent fibroblasts, P85 α is phosphorylated only on serine residues (Fig. 3B, left panel). At 3 min after PDGF addition, P85 α was found to be phosphorylated on both
serine and tyrosine residues (Fig. 3B, right panel). At later times, only serine phosphorylation of p85α was detected (data not shown). Over the 60 min of ligand addition, however, no phosphorylation of p110α was observed. As early as 30 s after PDGF addition, phosphorylated PDGF receptor was co-immunoprecipitated with the p85α-p110α complex.

To demonstrate that the binding of p85α to p110α did not alter as a result of PDGF stimulation, NIH 3T3 cells were metabolically labeled with [35S]methionine. The cultures were then stimulated with PDGF, and the lysates were incubated with either preimmune or anti-p85α antiserum (Fig. 3C). The resulting immunoprecipitates were collected on protein A-Sepharose beads, and after several washes, radiolabeled proteins were fractionated by SDS-PAGE and detected by autoradiography. The results show that the total amount of p85α and p110α immunoprecipitated by this antiserum remained unchanged over the course of ligand stimulation as did their relative ratios. Immunoprecipitations performed using the anti-p110α antibody revealed identical results (data not shown).

Phosphorylation of the p110α Subunit Immunoprecipitated from PDGF-stimulated Cell Lysates—Failure to detect receptor-stimulated tyrosine phosphorylation of the p110α subunit in metabolically labeled cells led us to investigate the identity of the 120-kDa protein, which was immunoprecipitated with anti-p85α and anti-p110α antibodies from PDGF-stimulated cell lysates and phosphorylated in the cell-free assay. Quiescent cultures were incubated in the absence or the presence of PDGF for 3 min, and lysates were prepared that were immunoprecipitated with either anti-p85α or anti-p110α antibodies. The resultant immunocomplexes were used for a cell-free phosphorylation assay, and the products were fractionated by SDS-PAGE. After transfer onto PVDF membrane, 32P-labeled phosphoproteins were visualized by autoradiography. The membrane was Western blotted with anti-p110α antibody and visualized with the ECL detection system. The results were precisely superimposed and shown in Fig. 4.

When antiserum to either p85α or p110α was used for immunoprecipitation, the p85α protein was phosphorylated in immunoprecipitates of lysates from both quiescent and PDGF-stimulated cells. Ligand stimulation resulted in the association of the p85α-p110α complex with the phosphorylated PDGF receptor, and consequently it was immunoprecipitated with antiserum to both the p85α and the p110α subunit. In addition, both antisera immunoprecipitated a phosphorylated protein of approximately 120 kDa. Western blotting with the anti-p110α antiserum, however, revealed two immunoreactive bands. The first was a major band of immunoreactivity (labeled a in Fig. 4), which migrated slightly ahead of a minor component of the anti-p110α immunoblot (labeled b in Fig. 4). Direct alignment of the anti-p110α immunoblot with the 32P-labeled phosphoproteins showed that the 120-kDa phosphoprotein was superimposed upon the minor band visualized with the anti-p110α antiserum. These results are consistent with the 120-kDa phosphoprotein representing phosphorylated p110α. Although precise quantitation of the ECL signal poses technical problems, it is clear that this minor band represents only a very small proportion of the total p110α present in the lysate. We propose that the conditions used for the cell-free kinase assay lead to the artificial phosphorylation of p110α. The immunoprecipitation brings together the strongly stimulated receptor with the p110α subunit, which is a potential but not physiologically utilized substrate of the receptor tyrosine kinase. Because we obtain identical results using both anti-p85α antiserum and anti-p110α antiserum, we can exclude the possibility that the minor band represents p110β.

Only a Small Pool of p85α Associates with the PDGF Receptor upon Ligand Stimulation—To determine the proportion of p85α-p110α complexes that associates with the PDGF receptor upon ligand stimulation, NIH 3T3 cells were treated with PDGF for various times and lysates were prepared. These
PDGF stimulates the phosphorylation of p85<sup>α</sup> but not p110<sup>α</sup> in metabolically labeled cells. A, confluent and near quiescent NIH 3T3 cells were labeled for 16 h with 32P (50 mCi/ml). At the end of this time, the cells were stimulated with PDGF (20 ng/ml) for the times indicated. The monolayers were then lysed, immunoprecipitates were prepared with affinity purified anti-p110<sup>α</sup> antiserum, and the proteins were analyzed by SDS-PAGE. The fractionated proteins were Western blotted onto PVDF membrane and visualized by autoradiography. B, the p85<sup>α</sup> bands were excised, and the proteins were hydrolyzed with 6 M HCl for 1 h at 65 °C. At the end of this time, samples were mixed with phosphopeptide standards, subjected to flat bed electrophoresis on cellulose plates, and visualized by autoradiography. C, cells were also labeled with [35S]methionine (100 μCi/ml) for 16 h and stimulated with PDGF at 37 °C. Lysates were prepared, and proteins were immunoprecipitated using anti-p85<sup>α</sup> antiserum and protein A-Sepharose beads. The resultant pellets were washed three times with lysis buffer, and the radiolabeled proteins were analyzed by SDS-PAGE and autoradiography. The results obtained using preimmune serum are shown on the left, and the results obtained using anti-p85<sup>α</sup> antiserum are shown on the right.

Lysates were immunoprecipitated with an excess of antiserum, which recognized either the PDGF receptor or p85<sup>α</sup>. The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and Western blotted with antibodies against either p85<sup>α</sup> or the PDGF receptor. Bound antibodies were detected with 125I-protein A and visualized by autoradiography. The results shown in Fig. 5 reveal the amount of PDGF receptor co-immunoprecipitated by anti-p85<sup>α</sup> antiserum upon PDGF stimulation (panel A) as a proportion of the total present in the lysate (panel B). Similarly, the results displayed in Fig. 5D show the amount of p85<sup>α</sup> associated with the PDGF receptor after PDGF stimulation as a proportion of the total amount of p85<sup>α</sup> present in the cell (Fig. 5C). Quantitation of the autoradiograms by scanning densitometry (Bio-Rad) revealed that no more than 5% of the total PDGF receptor population associated with the p85<sup>α</sup> protein. This agreed with the percentage of the total p85<sup>α</sup> pool that associated with the
PDGF receptor upon ligand stimulation. These findings indicate that the 2-fold increase in PI 3-kinase activity obtained from either anti-p85α or anti-p110α immunoprecipitates of PDGF-stimulated cell lysates reflects of a far greater increase in the specific activity of the enzyme when it associates with the PDGF receptor.

To determine the efficiency of the immunoprecipitations used above, cell lysates were immunoprecipitated with either anti-p85α (Fig. 5E) or anti-PDGFR receptor antisemur (Fig. 5F). After collecting the immunoprecipitates on protein A-Sepha-rose beads, fresh antiserum was added to the lysates, and the immunoprecipitation was repeated. The immunoprecipitates were fractionated by SDS-PAGE and Western blotted with the corresponding antisemur. The first immunoprecipitations are shown in lanes 1, and the second are shown in lanes 2. These results demonstrate that under the conditions used, the immunoprecipitation of both p85α and the PDGF receptor was complete after the first incubation with antisemur.

**DISCUSSION**

Ligand stimulation of the PDGF receptor rapidly activates its intrinsic protein tyrosine kinase activity and elicits a cascade of biochemical responses that culminate in cell division or differentiation. The association of PI 3-kinase activity with activated receptor tyrosine kinases was first demonstrated by immunoprecipitation from PDGF-treated cells with anti-phosphotyrosine antisemur (17). Since then, the interaction of PI 3-kinase with many other receptors has been described and many mechanisms have been proposed for the activation of lipid kinase activity (37–39). Mutagenesis of sites on the PDGF receptor subunit that bind p85α and studies employing the microinjection of anti-p110α subunits have confirmed the dependence of the PDGF mitogenic response upon the activation of PI 3-kinase activity (33, 40). However, despite the identification of novel members of the PI 3-kinase family, most biochemical characterization of PI 3-kinase association with activated receptors has been undertaken using either anti-phosphotyrosine antisemur, anti-receptor antisemur, or antisemur raised against the p85 adaptors alone. In the present study we have utilized antisemur against both the regulatory p85α and the catalytic p110α subunits. These reagents have allowed us to examine the specific interaction of p85α-p110α PI 3-kinase with the activated PDGF receptor in vivo and to define the consequence of this association upon its enzymatic activity.

Our results confirm that PI 3-kinase activity is not associated with the PDGF receptor in quiescent cultures of murine NIH 3T3 cells (Fig. 1). Under these conditions, however, p85α and p110α exist as a heterodimer because p85α could be immunoprecipitated using antisemur specific to p110α (Fig. 2). Upon stimulation with PDGF, association of PI 3-kinase activity with the receptor is rapid, becoming maximal by 1 min. After this time, the lipid kinase activity gradually declines such that after 45 min of stimulation, receptor associated PI 3-kinase activity was only 22% of the maximal level, and by 120 min, the activity was reduced to that seen in quiescent cells. These kinetics of receptor association are in agreement with previous studies (15, 17, 18). Using anti-p85α and anti-p110α antisemur we demonstrated that both subunits associate with the receptor and dissociate from the receptor with similar kinetics. The association and dissociation of PI 3-kinase activity with the activated receptor correlates with the kinetics of association and dissociation of the p85α-p110α complex (Fig. 2, A and B). Furthermore, we confirm that the loss of PI 3-kinase activity from the PDGF receptor is not the consequence of heterodimer disruption and a selective dissociation of the p110α subunit. Our results demonstrate that PDGF receptor phosphorylation results in the association and stimulation of p85α-p110α PI 3-kinase activity. The subsequent dissociation of the p85α-p110α complex and the loss of lipid kinase activity does not, however, appear to correlate with a decrease in the phosphorylation state of the receptor. Although it is possible that a certain threshold of receptor tyrosine phosphorylation needs to be surpassed before the association of PI 3-kinase with PDGF receptor can occur and be maintained, other studies have demonstrated that of the many second messengers that associate with the PDGF receptor upon its activation, PI 3-kinase has the least requirement for receptor phosphorylation (41). Western blotting of the immunoprecipitated PDGF receptor from lysates of stimulated fibroblasts showed a time-dependent receptor down-regulation (Fig. 1). The kinetics of receptor degradation are consistent with those observed in HepG2 cells (42) and porcine aortic cells (43) expressing recombinant receptor. Site-directed mutagenesis of receptor phosphotyrosine sites (42) and treatment with wortmannin (44) has demonstrated a role for PI 3-kinase activity in receptor trafficking and degradation following ligand stimulation.

The recent demonstration that phosphatidylinositol 3,4,5-trisphosphate interacts with SH2 domains and modulates the association of p85α-p110α with tyrosine phosphorylated proteins suggests a possible mechanism through which such dissociation may occur (45). The rapid kinetics with which PI 3-kinase activity associates with activated tyrosine kinase receptors is well documented (15, 17, 18, 37, 38). In PC12 cells however, nerve growth factor elicits a more delayed interaction of PI 3-kinase activity with its receptor (39). Following ligand stimulation, association of PI 3-kinase activity with the nerve growth factor receptor occurs only after 5 min but is maintained for 2 h. Such temporal differences in receptor association may be of physiological relevance because nerve growth factor initiates neuronal differentiation in PC12 cells, whereas epidermal growth factor causes cellular proliferation.

Association of the p85α-p110α complex with the PDGF receptor was found to result in a 1.6–1.8-fold stimulation of total cellular p110α PI 3-kinase activity (Fig. 2C). This stimulation was both rapid and transient. The maximal increase in cellular PI 3-kinase activity was achieved by 3 min, but 7 min after ligand addition this stimulation was already decreasing. After 15 min, the total cellular lipid kinase activity was almost equal to that of quiescent cells. This degree of stimulation is entirely consistent with the reported 1.4-fold activation of cellular PI 3-kinase activity determined using Nonidet P-40 extracts of PDGF-stimulated NIH fibroblasts (46) and the 1.5–2-fold activation observed upon phophopeptide binding to the purified enzyme in vivo (47). Other studies obtained a 2–3-fold activation of PI 3-kinase activity following insulin stimulation of Fao cells and a 5-fold activation upon colony-stimulating factor-1 stimulation of NIH 3T3 cells (48, 49). We have shown that association of the p85α-p110α complex with the activated PDGF receptor correlates with the association of PI 3-kinase activity. Similarly the loss of receptor associated PI 3-kinase activity correlated well with the dissociation of p85α-p110α; however, we cannot exclude the possibility that a mechanism exists to inactivate the lipid kinase prior to its dissociation from the receptor.

Translocation of secondary messengers to receptor protein tyrosine kinases often leads to their phosphorylation and modification of enzymatic activity (40). Our data demonstrate that PDGF stimulation of NIH 3T3 cells results in the phosphorylation of p85α on tyrosine residues at times where a corresponding increase in PI 3-kinase activity is observed (Figs. 1 and 2). Although it had been previously reported that tyrosine phosphorylation of the p85α subunit only occurred when both the
PGF receptor and p85α were overexpressed (50), others have also shown a ligand-dependent phosphorylation of p85α (15, 17, 51, 52). Three sites of tyrosine phosphorylation have been mapped to residues 368, 580, and 607 of p85α (53, 54). Consistent with the hypothesis that tyrosine phosphorylation of PI 3-kinase leads to an increase in its enzymatic activity is the report that a decrease in lipid kinase activity follows treatment of the purified bovine brain enzyme with a phosphatidylinositol protein phosphatase (55). Mutagenesis of these sites of tyrosine phosphorylation sites will be required, however, to precisely define their role in the activation of PI 3-kinase activity. We have shown that p85α immunoprecipitated from quiescent fibroblasts is constitutively phosphorylated on serine residues (Fig. 3) in agreement with previous studies (17, 56–58). This serine phosphorylation was maintained upon PDGF stimulation. Much effort was made to identify the kinase responsible for this phosphorylation until it was realized that p110α is a dual specificity enzyme (58, 59). In contrast to its lipid kinase activity, the serine protein kinase activity of p110α in vitro has a preference for Mn2+ ions and leads to the phosphorylation of p85α on residue 608. Serine phosphorylation of p85α has also been postulated as a mechanism for regulating the lipid kinase activity of p110α (60). Experiments with the recombinant protein demonstrated that serine phosphorylation decreased the specific activity of p110α by up to 80% (58, 59). In contrast, insulin-stimulated tyrosine phosphorylation of p85α increased PI-3 kinase activity (60). It remains possible that our failure to detect an inverse relationship in the phosphorylation of tyrosine and serine residues on p85α upon PDGF stimulation in fibroblasts may reflect the low proportion (5%) of total cellular p85α that associates with the PDGF receptor upon ligand stimulation (Fig. 5). The low stoichiometry of binding is in agreement with the results of others who also concluded that only a small fraction of PI 3-kinase (3–10%) directly associates with activated insulin, colony-stimulating factor-1, or ErbB2 receptors in vivo (37, 61, 62). In contrast, between 25 and 70% of the total PI 3-kinase activity has been reported to associate with the IRS-1 protein upon insulin stimulation (63, 64). This increased efficiency of association may reflect the much greater number of phosphorylation sites available for binding PI 3-kinase on the IRS-1 protein compared with tyrosine kinase receptors.

Upon receptor stimulation, phosphorylation of the p110α subunit has been proposed as a further mechanism for regulating lipid kinase activity (36, 52, 65). Using cell-free kinase assays we observed a transiently phosphorylated 120-kDa protein in lysates of PDGF-stimulated cells using both anti-p85α and anti-p110α antisera (Fig. 2). A combination of the cell-free kinase assay and Western blot analysis demonstrated that this phosphoprotein was indeed p110α (Fig. 4). The phosphorylated form of p110α, however, represented only a minor proportion of the total p110α because the phosphorylated and nonphosphorylated forms of the protein could be distinguished by differences in their electrophoretic mobility on SDS-PAGE. A contribution of p110β to this phosphorylated band, co-immunoprecipitated via its association with the PDGF receptor cannot, however, be completely excluded. Immunoprecipitations from lysates of PDGF-stimulated cells metabolically labeled with [32P]orthophosphate, however, revealed no evidence of p110α phosphorylation (Fig. 3), suggesting that the phosphorylation is an artifact of the cell-free assay. No tyrosine phosphorylation of the catalytic subunit was also noted upon association of PI 3-kinase with the insulin receptor and the IRS-1 protein in vivo (48, 66). In those studies, the increase in inositol lipid kinase activity correlated more closely with the degree to which IRS-1 became tyrosine phosphorylated than to the phosphorylation of PI 3-kinase. This is in agreement with experiments demonstrating that tyrosine phosphopeptides correspond to the p85 binding sites on the insulin receptor, polyoma middle T antigen, and the PDGF receptor directly stimulate PI 3-kinase activity in vitro (47, 67, 68). Furthermore, doubly phosphorylated peptides containing two PI 3-kinase binding sites are even more efficient at activating PI 3-kinase, suggesting that both SH2 domains need to be occupied for optimal activation to occur (69).

The quest to determine the biological role of PI 3-kinase has revealed multiple forms of both the catalytic and regulatory subunits of this enzyme. Consequently, great care is now required when ascribing the consequence of PI 3-kinase activity in vivo to a particular molecular form. The results of the present study confirm the involvement of the p85α-p110α PI 3-kinase complex in the PDGF-stimulated increase of 3′inositol phosphorylated lipids in vivo. Although association of this complex with receptor results in an approximately 2-fold increase in cellular p110α PI 3-kinase activity, this reflects a far greater increase in specific activity of the catalytic subunit upon binding. The kinetics of p85α-p110α association and dissociation together with the degree of enzyme activation upon ligand addition correlate so well with PDGF receptor-associated PI 3-kinase activity that we feel that at least in fibroblasts, this complex is the major form of the enzyme to associate with the PDGF receptor. Further work is now required to define the biological significance of other closely related forms of the catalytic subunit such as p110β in receptor-mediated signal transduction.

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