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Interaction of Phosphatidylinositol 3-Kinase-Associated p85 with Epidermal Growth Factor and Platelet-Derived Growth Factor Receptors

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One of the immediate cellular responses to stimulation by various growth factors is the activation of a phosphatidylinositol (PI) 3-kinase. We recently cloned the 85-kDa subunit of PI 3-kinase (p85) from a λgt11 expression library, using the tyrosine-phosphorylated carboxy terminus of the epidermal growth factor (EGF) receptor as a probe (E. Y. Skolnik, B. Margolis, M. Mohammadi, E. Lowenstein, R. Fischer, A. Drepps, A. Ullrich, and J. Schlessinger, Cell 65:83–90, 1991). In this study, we have examined the association of p85 with EGF and platelet-derived growth factor (PDGF) receptors and the tyrosine phosphorylation of p85 in 3T3 (HER14) cells in response to EGF and PDGF treatment. Treatment of cells with EGF or PDGF markedly increased the amount of p85 associated with EGF and PDGF receptors. Binding assays with glutathione *S*-transferase (GST) fusion proteins demonstrated that either Src homology region 2 (SH2) domain of p85 is sufficient for binding to EGF and PDGF receptors and that receptor tyrosine autophosphorylation is required for binding. Binding of a GST fusion protein expressing the N-terminal SH2 domain of p85 (GST-N-SH2) to EGF and PDGF receptors was half-maximally inhibited by 2 and 24 mM phosphotyrosine (P-Tyr), respectively, suggesting that the N-SH2 domain interacts more stably with PDGF receptors than with EGF receptors. The amount of receptor-p85 complex detected in HER14 cells after growth factor treatment and the binding of GST-N-SH2 to activated growth factor receptors in vitro correlated with the amount of PI 3-kinase activity in anti-P-Tyr immunoprecipitates of lysates from HER14 cells treated with EGF or PDGF. Growth factor treatment also increased the amount of p85 found in anti-P-Tyr immunoprecipitates of cell lysates. However, tyrosine-phosphorylated p85 was not detectable in lysates of PDGF-treated HER14 cells, suggesting that the vast majority of p85 in the anti-P-Tyr fraction is receptor associated but not phosphorylated on tyrosine residues. Only upon transient overexpression of p85 and PDGF receptor did p85 become tyrosine phosphorylated. These results are consistent with the hypothesis that p85 functions as an adaptor molecule that targets PI 3-kinase to activated growth factor receptors.

The association of a phosphatidylinositol (PI) kinase activity with the v-*src* and v-*ros* oncoproteins (24, 43) raised the possibility that such an activity might be involved in cell transformation. This possibility was supported by studies on polyomavirus middle-T-antigen mutants that demonstrated a strong correlation between middle-T-antigen-associated PI kinase activity and transforming potential (18, 53). Demonstration that a similar PI kinase activity was induced by platelet-derived growth factor (PDGF) treatment of fibroblasts (2, 17, 52) and associated with activated PDGF receptors (9) implicated this activity as a potential participant in the normal mitogenic signaling pathway activated by PDGF. Biochemical characterization of the product of this PI kinase revealed a phospholipid which is phosphorylated at the 3′ position of the inositol ring (51). This finding suggested that PI 3-kinase was part of a signal transduction pathway distinct from the inositol trisphosphate/diacylglycerol pathway mediated by phospholipase C cleavage of PI-4′,5′-P_2 (reviewed in reference 5). Studies with other mitogenic growth factors have shown that PI 3-kinase is also activated by colony-stimulating factor 1 (36, 39, 47), insulin (11, 37), and epidermal growth factor (EGF) (4). PI 3-kinase activity has also been shown to be associated with the viral oncoproteins v-Abl, v-Crk, and v-Fps (14, 46).

The presence of PI 3-kinase activity in antiphosphotyrosine (anti-P-Tyr) immunoprecipitates of either PDGF-treated or middle-T-antigen-transformed cells has been correlated with the presence of an 85-kDa phosphoprotein (10, 17). Consistent with this observation, association of PI 3-kinase activity with the PDGF receptor correlates with the association of an 85-kDa protein (12). Point mutations in the PDGF receptor that abrogate association of PI kinase activity also block association of an 85-kDa protein that is phosphorylated in in vitro kinase assays (19, 20). These studies suggested that this 85-kDa protein is either a PI 3-kinase or tightly associated with a PI 3-kinase. Purification of PI 3-kinase showed that it is a heterodimer consisting of 85-kDa (p85) and 110-kDa (p110) subunits (6). Our group and others have recently cloned human, murine, and bovine p85, using different cloning strategies (13, 34, 40). The amino acid sequence of p85 contains two Src homology region 2 (SH2) domains and one SH3 domain but no apparent ATP-binding site (13, 34, 40). Efforts to detect PI 3-kinase activity intrinsic to p85 have been unsuccessful (34), suggesting that p110 contains the catalytic activity. Thus, p85 may be a regulatory subunit of PI 3-kinase.

Much evidence suggests that SH2 domains mediate interactions with tyrosine-phosphorylated proteins (1, 26, 28, 30, 32, 40). SH2 domains have been found in the v-*crk* oncogene product (29) and cellular tyrosine kinases belonging to the Src family (35, 38) as well as in proteins involved in signal

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transduction such as *ras* GTPase-activating protein (45, 48) and phospholipase C-γ (PLC-γ) (42, 44). Studies on PLC-γ have shown that it associates with activated growth factor receptors and becomes tyrosine phosphorylated in response to EGF and PDGF treatment (22, 27, 31, 49, 50). It was subsequently demonstrated that growth factor-induced tyrosine phosphorylation of PLC-γ is required for activation of enzymatic activity in vitro (33) and for inositol phospholipid hydrolysis in vivo (21). The presence of SH2 domains in p85 and the wealth of evidence suggesting a role for PI 3-kinase in a variety of mitogenic signaling pathways implied that PI 3-kinase may be activated by a mechanism similar to that elucidated for PLC-γ.

In this study, we have examined the association of p85 with activated growth factor receptors and the tyrosine phosphorylation of p85 in response to growth factor treatment. We demonstrate that p85 interacts with activated EGF and PDGF receptors in cell lysates and in vitro. Either SH2 domain of p85 is sufficient for its interaction with EGF and PDGF receptors. Analysis of these interactions suggests that the stability of the interaction of p85 with EGF and PDGF receptors correlates with the levels of PI 3-kinase activity detected in anti-P-Tyr immunoprecipitates of lysates from HER14 cells treated with EGF and PDGF. However, although growth factor treatment increases the amount of p85 in the anti-P-Tyr fraction of HER14 lysates, we have been unable to demonstrate tyrosine phosphorylation of endogenous p85 in response to PDGF. Tyrosine-phosphorylated p85 was detected only upon transient overexpression of p85 and PDGF receptor. The observation that p85 associates with activated growth factor receptors but does not appear to be phosphorylated on tyrosine residues suggests that it may function as a regulatory subunit of PI 3-kinase or as an adaptor molecule that targets PI 3-kinase to growth factor receptor tyrosine kinases.

MATERIALS AND METHODS

Immunoprecipitation and immunoblotting. HER14 cells are NIH 3T3 (clone 2.2) cells that express the wild-type human EGF receptor (16). They were grown as previously described (27). After overnight starvation in Dulbecco modified Eagle medium (DMEM) containing 1% fetal bovine serum, cells were stimulated for 5 min at 37°C with either EGF (250 ng/ml) or PDGF (50 ng/ml) (both from Intergen) in DMEM containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5) and 1 mg of bovine serum albumin per ml. Cell lysis, immunoprecipitation, and immunoblotting were performed as previously described (27) except that *p*-nitrophenylphosphate was omitted from the lysis buffer.

Antibodies. Anti-P-Tyr immunoprecipitations were performed with a monoclonal antibody covalently coupled to agarose beads (Oncogene Science), and anti-P-Tyr immunoblots were performed with a rabbit polyclonal antibody. For anti-p85 immunoprecipitations and immunoblots, we generated rabbit polyclonal antibody Ab21 against a glutathione *S*-transferase (GST)–p85 bacterial fusion protein containing amino acids 265 to 523 of human p85 (40). This antibody recognizes p85 in immunoprecipitations and immunoblots of purified PI 3-kinase (6). Two other antibodies were used for p85 immunoprecipitations. One (Ab51) is directed against a peptide corresponding to amino acids 513 to 531 of human p85 (40), and the other (AbC) is directed against a C-terminal peptide of human p85 (amino acids 711 to 724 [40]). Anti-EGF receptor immunoprecipitations were performed with monoclonal antibody 108 (3), and anti-EGF receptor immunoblots were performed with anti-C (27). The anti-PDGF receptor antibody used in this study is a rabbit antipeptide antibody against the carboxy-terminal 12 amino acids of the mouse PDGF-β receptor (courtesy of A. Zilberstein, Rhone-Poulenc Rorer).

PI 3-kinase assay. PI 3-kinase activity was assayed essentially as described previously (53). Immunoprecipitates from 500 μl of HER14 lysate (~10^7 cells) were washed three times with 1% Nonidet P-40 (NP-40) in phosphate-buffered saline, twice with 0.5 M LiCl in 0.1 M Tris (pH 7.6), twice in TNE (10 mM Tris [pH 7.6], 100 mM NaCl, 1 mM EDTA [pH 8.0]), and twice in a mixture containing 0.03% NP-40, 20 mM HEPES (pH 7.5), 50 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 200 μM sodium orthovanadate, 10 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl sulfate. Anti-P-Tyr immunoprecipitates were eluted with 25 μl of final wash buffer containing 5 mM phenylphosphate and 0.01% ovalbumin for 10 min on ice. The eluate was added to 25 μl of 40 mM Tris (pH 7.6)–150 mM NaCl–20 mM $MgCl_2$–0.4 mg of sonicated PI (Avanti) per ml in 20 mM HEPES (pH 7.5)–20 μM ATP–10 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; New England Nuclear) per sample–200 mM adenosine. Adenosine specifically inhibits PI 4-kinase activity (52). The mixture was incubated at room temperature for 10 min. The reaction was stopped with 100 μl of 1 N HCl. After extraction with 200 μl of chloroform-methanol (1:1), the organic phase was dried, resuspended in 10 μl of chloroform-methanol (1:1), spotted on a thin-layer chromatography plate, and chromatographed in chloroform–methanol–4.0 M NH_4OH (9:7:2) for 1 h. Labeled PI 3-phosphate was visualized by autoradiography.

Generation of GST fusion proteins. Oligonucleotides flanking both SH2 domains of human p85 (40) and containing appropriate restriction sites were synthesized. Polymerase chain reaction was used with p85 cDNA as a template (40) to amplify the appropriate DNA fragments. These fragments were cleaved with *Bam*HI and *Eco*RI and ligated to pGEX3X (41) that had been cleaved with *Bam*HI and *Eco*RI. Competent *Escherichia coli* HB101 cells were transformed, and recombinant clones were screened by restriction enzyme analysis and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of overproduced fusion proteins. The fusion proteins were purified on a large scale by affinity chromatography on glutathione-agarose beads (Sigma) as described previously (41) except that 10 mM dithiothreitol was included in the lysis buffer. The fusion proteins express the following fragments of human p85: N-SH2 (amino acids 321 to 440), C-SH2 (amino acids 614 to 724), and N+C-SH2 (amino acids 321 to 724).

Binding assays. To assay binding of growth factor receptors to GST fusion proteins, 500 μl of HER14 cell lysate was incubated for 90 min at 4°C with approximately 5 μg of fusion protein coupled to glutathione-agarose beads. When inhibitors were added, the HEPES concentration was raised to 200 mM. The beads were washed three times with HNTG (27), and proteins were separated by 8% SDS-PAGE. Bound proteins were transferred to nitrocellulose and blotted with antibodies as described. For quantitation, appropriate bands were excised from filters and counted in an LKB 1282 Compugamma CS counter.

Transient expression assays. 293 cells were transfected as described previously (7) with human p85 (40) and PDGF-β receptor (8, 15) cDNAs subcloned into a cytomegalovirus-based expression vector. Briefly, cells were plated at a density of 10^6 cells per 10-cm dish in DMEM containing 10%

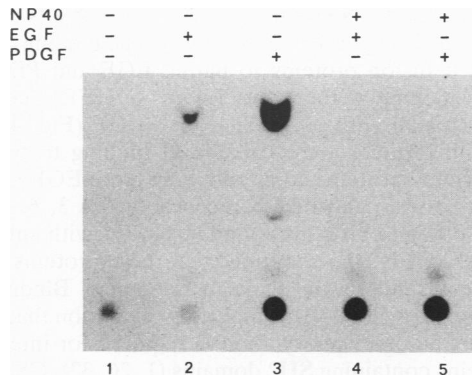

FIG. 1. PI 3-kinase activity in anti-P-Tyr immunoprecipitates of HER14 lysates. HER14 cells were treated with either EGF (250 ng/ml) or PDGF (50 ng/ml) for 5 min at 37°C. Lysate from ~10^7 cells was immunoprecipitated with anti-P-Tyr antibodies and assayed for PI 3-kinase activity as described in Materials and Methods; 0.5% NP-40 was included in the assay buffer as indicated.

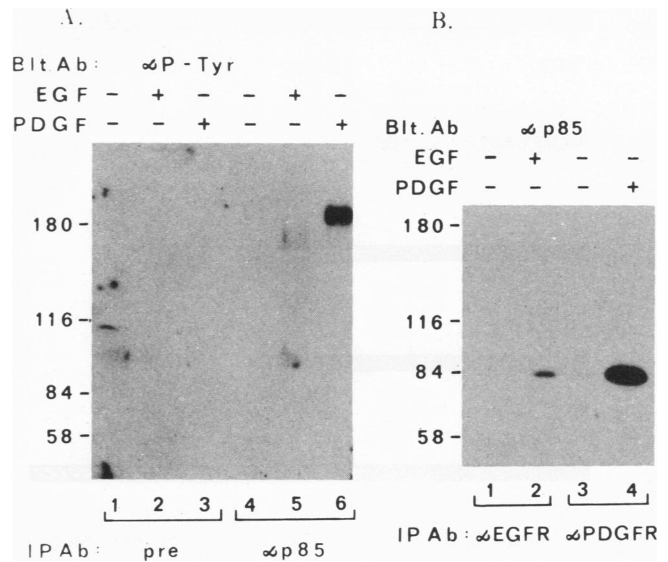

FIG. 2. Association of p85 with EGF and PDGF receptors in HER14 lysates. HER14 cells were treated as described for Fig. 1. (A) Lysate from ~10^7 cells was immunoprecipitated with either preimmune serum or anti-p85 antiserum (Ab21). Immunoprecipitates were subjected to 8% SDS-PAGE, transferred to nitrocellulose, and blotted with anti-P-Tyr antibodies. Antibodies were detected by incubation with ^{125}I-protein A followed by autoradiography. Immunoblots were exposed for 12 h at −70°C. The EGF receptor migrates slightly faster and the PDGF receptor migrates slightly slower than does the 180,000-molecular weight marker. (B) Immunoprecipitation and immunoblotting were carried out as described for panel A. Immunoblots were exposed for 14 h at −70°C. Positions of molecular weight markers are indicated in thousands.

fetal bovine serum and grown overnight at 37°C. One hour prior to transfection, the cells were incubated in the presence of 3% CO_2. Forty micrograms of plasmid DNA (1 μg/μl) was mixed with 100 μl of 2.5 M $CaCl_2$ and 1 ml of 2× BES-buffered saline (50 mM BES [N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] [pH 6.95], 280 mM NaCl, 1.5 mM Na_2HPO_4) in a final volume of 2 ml; 20 μg of p85 cDNA and 3 μg of PDGF-β receptor cDNA were used for each transfection. After 15 min of incubation at room temperature, 1 ml of this mixture was added dropwise to each dish of cells, and the cells were incubated overnight in the presence of 3% CO_2. Cells were washed once with phosphate-buffered saline, starved overnight in DMEM containing 1% fetal bovine serum, and treated with PDGF (50 ng/ml) for 2 min at 37°C. Cells were lysed as previously described (27). The plasmid used to overexpress a p85 fragment consisting of both SH2 domains expresses amino acid residues 326 to 724 of human p85 (40).

RESULTS

EGF and PDGF increase the amount of PI 3-kinase activity in anti-P-Tyr immunoprecipitates of HER14 cell lysates. We have recently developed a method for cloning targets of growth factor receptors based on the ability of the tyrosine-phosphorylated carboxy terminus of the EGF receptor to bind to protein sequences expressed in a λgt11 library. The first molecule that we cloned by using this approach was the PI 3-kinase-associated p85 (40). We wished to further examine this interaction between p85 and the EGF receptor, because studies examining the ability of EGF to stimulate PI 3-kinase have yielded conflicting results (4, 17, 34). We chose to compare the effects of EGF with those of PDGF, a known strong stimulator of PI 3-kinase. For these studies, we used NIH 3T3 cells which have been transfected with human EGF receptor cDNA (HER14; 16) and express approximately 50,000 endogenous mouse PDGF receptors and 400,000 human EGF receptors per cell.

Figure 1 depicts the increase seen in PI 3-kinase activity in the anti-P-Tyr fraction of HER14 cells after 5 min of treatment with either EGF (lane 2) or PDGF (lane 3). EGF induces PI 3-kinase activity to a much lower degree than does PDGF, despite the 8- to 10-fold-higher expression of EGF receptors in this cell line. NP-40 (0.5%) completely inhibited PI kinase activity in these immunoprecipitates (lanes 4 and 5), demonstrating that the assay is specifically measuring PI 3-kinase activity (52).

p85 associates with activated EGF and PDGF receptors in vivo. We decided to determine whether the disparity between the effects of EGF and PDGF on PI 3-kinase activity in the anti-P-Tyr fraction could be explained by differences in the interaction between p85 and EGF and PDGF receptors. We examined the association of p85 with these growth factor receptors in HER14 cell lysates by assaying for p85-receptor complex formation. An anti-p85 antibody (Ab21) coimmunoprecipitates tyrosine-phosphorylated proteins that comigrate with activated EGF and PDGF receptors (Fig. 2A, lanes 5 and 6). No associated proteins were observed in untreated cells or in immunoprecipitations with preimmune sera (Fig. 2A, lanes 1 to 4). Consistent with this finding is the observation that antireceptor antibodies coimmunoprecipitate an 85-kDa protein recognized by anti-p85 antibodies in an immunoblot (Fig. 2B). The amount of coprecipitated p85 increases upon growth factor treatment (compare lanes 2 and 4 with lanes 1 and 3). In both coprecipitation experiments, the quantity of p85-receptor complex was much greater in lysates of PDGF-treated cells than in lysates of EGF-treated cells (Fig. 2A, lanes 5 and 6; Fig. 2B, lanes 2 and 4), despite the presence of nearly 10-fold-more EGF receptors than PDGF receptors in these cells. This disparity in complex formation correlates with the difference in levels of anti-P-Tyr-associated PI 3-kinase

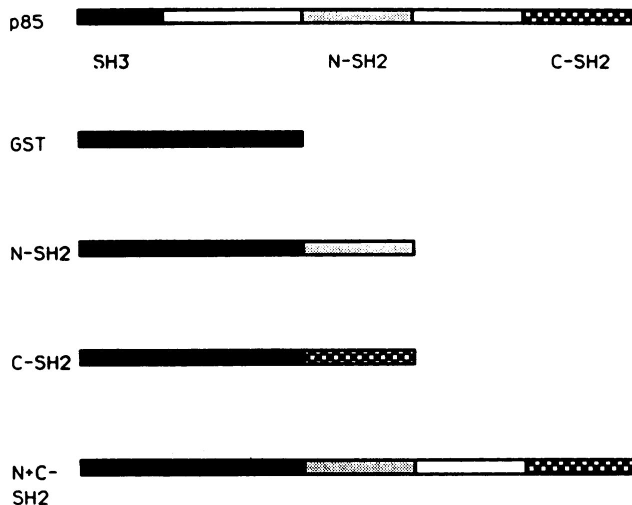

FIG. 3. Schematic diagram of GST-p85 fusion proteins generated for binding studies. Oligonucleotide primers flanking both SH2 domains of p85 were synthesized. Appropriate DNA fragments were amplified from a p85 cDNA template by polymerase chain reaction and subcloned into pGEX3X. Fusion proteins were analyzed by 10% SDS-PAGE and purified by affinity chromatography on glutathione-agarose beads. Portions of p85 expressed in each fusion protein are denoted in Materials and Methods.

activity observed in cells treated with EGF and PDGF (Fig. 1).

Either SH2 domain of p85 can bind to activated EGF and PDGF receptors in vitro. To determine whether the differences observed in EGF- and PDGF-induced p85-receptor complex formation could be explained by differential binding of the SH2 domains of p85 to EGF and PDGF receptors, we constructed three GST fusion proteins expressing individual SH2 domains of p85 (N-SH2 and C-SH2) and both SH2 domains together (N+C-SH2). These constructs are depicted schematically in Fig. 3. We tested the binding of these immobilized fusion proteins to native EGF and PDGF receptors by incubating them with lysates of HER14 cells that had been treated with either EGF or PDGF (Fig. 4A). All three fusion proteins were capable of binding to tyrosine-phosphorylated proteins comigrating with the EGF receptor (lanes 2, 5, and 8) and PDGF receptor (lanes 3, 6, and 9). Immunoblotting of proteins bound to N-SH2 with antireceptor antibodies (Fig. 4B) confirmed that these proteins are the EGF (lane 2) and PDGF (lane 4) receptors. Binding was ligand dependent (Fig. 4B), supporting the notion that receptor tyrosine autophosphorylation is required for interaction with proteins containing SH2 domains (1, 26, 32). GST alone did not bind to any tyrosine-phosphorylated proteins under these assay conditions (data not shown).

All three fusion proteins also bind to numerous other tyrosine-phosphorylated proteins in lysates from stimulated cells (Fig. 4A). On the basis of binding experiments with purified EGF receptor kinase (data not shown), it is likely that N-SH2 binds directly to these other tyrosine-phosphorylated proteins. However, at this time we cannot rule out the possibility that some of these proteins bind to N-SH2 indirectly by virtue of their association with activated EGF or PDGF receptors. N+C-SH2 binds to the EGF receptor and other tyrosine-phosphorylated proteins more efficiently than does N-SH2 by itself (Fig. 4A). This may be a consequence of the fact that N+C-SH2 contains twice as many binding sites for tyrosine-phosphorylated proteins as does either N-SH2 or C-SH2 alone; alternatively, the conformation of N+C-SH2 may differ from those of N-SH2 and C-SH2 such that it interacts more favorably with tyrosine-phosphorylated proteins.

Phosphotyrosine specifically and differentially inhibits receptor-SH2 interactions. Since interaction of various SH2-containing proteins with growth factor receptors is dependent on receptor tyrosine phosphorylation (1, 12, 25, 26, 32), we reasoned that P-Tyr might competitively inhibit receptor-

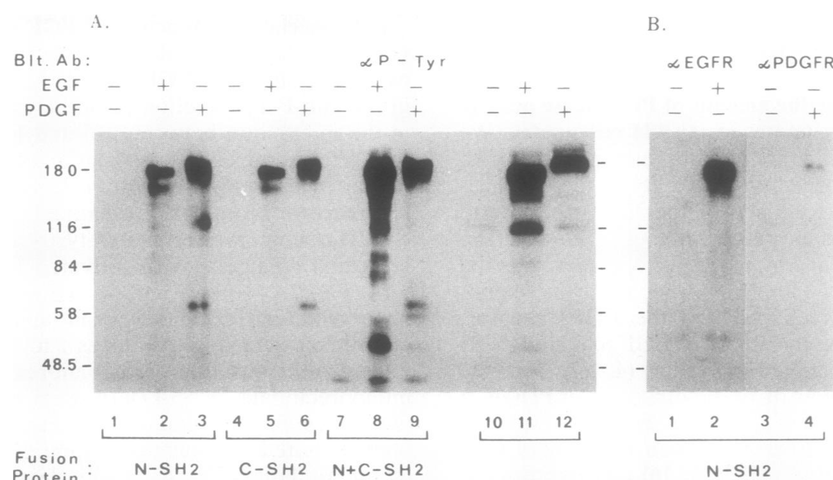

FIG. 4. Binding of GST-p85 fusion proteins to activated EGF and PDGF receptors in HER14 lysates. (A) Binding of GST-p85 fusion proteins to tyrosine-phosphorylated proteins. (B) Binding of N-SH2 to EGF and PDGF receptors. HER14 cells were treated as described for Fig. 1. Lysate from $\sim 7 \times 10^6$ cells was incubated with equal amounts of fusion proteins (~ 5 μg) immobilized on glutathione-agarose beads. Bound proteins were subjected to 8% SDS-PAGE and immunoblotted as described for Fig. 2. Thirty microliters of HER14 lysate was analyzed in lanes 10 to 12. In panel B, it should be noted that the anti-EGF receptor antibodies are much more efficient in immunoblotting than are the anti-PDGF receptor antibodies, resulting in a much stronger signal in the anti-EGF receptor immunoblot. Immunoblots were exposed for 12 h at −70°C. Positions of molecular weight standards are indicated in thousands.

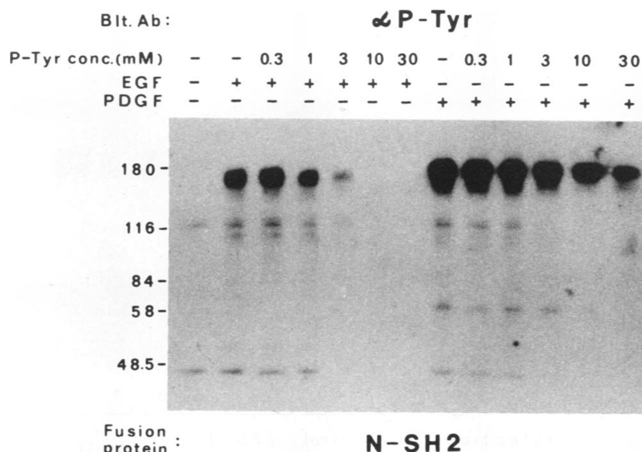

FIG. 5. Dose-response curve of inhibition of N-SH2 binding to EGF and PDGF receptors by P-Tyr. Binding assays were performed as described for Fig. 4 in the presence of increasing concentrations of P-Tyr. Immunoblots were exposed for 16 h at −70°C. Positions of molecular weight markers are indicated in thousands.

SH2 interactions. It has recently been shown that P-Tyr can partially inhibit the binding of the *c-abl* SH2 domain to cellular tyrosine-phosphorylated proteins (30). We tested this hypothesis by performing fusion protein binding assays in the presence of increasing concentrations of P-Tyr (Fig. 5). Quantitation revealed that the half-maximal inhibitory concentration of P-Tyr for PDGF receptor binding is approximately 24 mM, or about 12 times greater than that for EGF receptor binding (Fig. 6). This inhibition is specific to P-Tyr, as tyrosine, phosphoserine, and phosphothreonine had no effect on binding (data not shown). We also noted that the binding of the EGF receptor to N-SH2 was more sensitive to increasing salt concentration. For example, 500 mM LiCl completely inhibited the binding of N-SH2 to EGF receptors but reduced N-SH2 binding to PDGF receptors by only 50% (data not shown). These results suggest that p85 itself binds to tyrosine-phosphorylated EGF receptors with lower affinity than it binds to tyrosine-phosphorylated PDGF receptors.

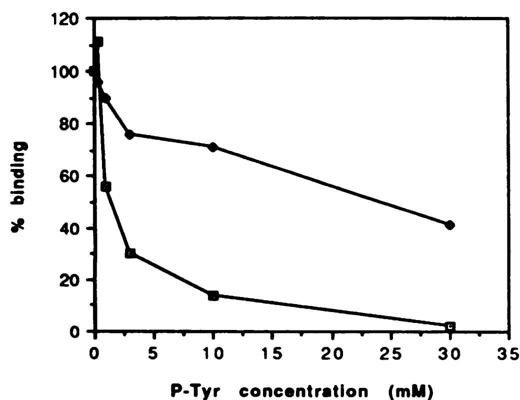

FIG. 6. Graphic representation of quantitative data from Fig. 5. Bands corresponding to EGF (⊡) and PDGF (◆) receptors were excised from nitrocellulose filters and counted in a gamma counter. Binding was normalized to binding in the absence of inhibitor.

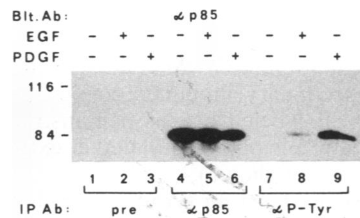

FIG. 7. Recruitment of p85 to the anti-P-Tyr fraction in response to EGF and PDGF treatment of HER14 cells. HER14 cells were treated as described for Fig. 1. Immunoprecipitation and immunoblotting were carried out as described for Figure 2. Immunoblots were exposed for 12 h at −70°C. Positions of molecular weight markers are indicated in thousands.

p85 is associated with the anti-P-Tyr fraction in lysates of HER14 cells treated with PDGF. To determine whether PI 3-kinase-associated p85 was present in the anti-P-Tyr fraction of HER14 cell lysates, we immunoblotted anti-P-Tyr immunoprecipitates with anti-p85 antibodies. EGF and PDGF treatment of HER14 cells increased the amount of p85 present in the anti-P-Tyr fraction (Fig. 7, lanes 7 to 9). PDGF did so to a much greater extent than did EGF, consistent with the magnitude of PDGF and EGF effects on anti-P-Tyr-associated PI 3-kinase activity (Fig. 1).

Tyrosine-phosphorylated p85 is not detectable in lysates of HER14 cells treated with PDGF. In view of the relatively weak effects of EGF on receptor-associated PI 3-kinase activity (Fig. 1) and receptor-p85 complex formation (Fig. 2), we chose to focus on the effect of PDGF on p85 phosphorylation. Anti-P-Tyr immunoblotting of anti-p85 immunoprecipitates demonstrated association of the activated PDGF receptor with p85 but failed to detect tyrosine-phosphorylated p85 (Fig. 2A). Similar results were obtained with three different polyclonal rabbit antibodies against p85; all of these antibodies were able to coimmunoprecipitate tyrosine-phosphorylated PDGF receptor, but none was able to immunoprecipitate tyrosine-phosphorylated p85 detectable in an anti-P-Tyr immunoblot (Fig. 8, lanes 1 to 6). Immunoprecipitation of $^{32}P_i$-labeled HER14 cell lysates with an anti-p85

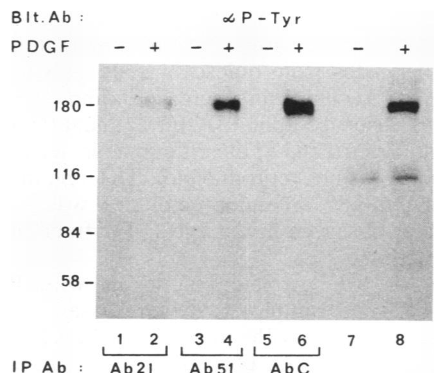

FIG. 8. Tyrosine-phosphorylated proteins in anti-p85 immunoprecipitates of HER14 cells. HER14 cells were treated with PDGF (50 ng/ml) for 5 min at 37°C. Lysate from ~10^7 cells was immunoprecipitated with three different anti-p85 antibodies and immunoblotted with anti-P-Tyr antibodies as described for Fig. 2. Thirty micrograms of lysate was analyzed in lanes 7 and 8. Immunoblots were exposed for 16 h at −70°C. Positions of molecular weight markers are indicated in thousands.

antibody (Ab51) also failed to reveal the presence of tyrosine-phosphorylated p85, although low levels of serine and threonine phosphorylation were detected (data not shown). In addition, secondary immunoprecipitation of anti-P-Tyr eluates from $^{32}P_i$-labeled lysates demonstrated that anti-P-Tyr-associated p85 is phosphorylated at extremely low stoichiometry (data not shown).

p85 is phosphorylated on tyrosine in cells that transiently overexpress p85 and PDGF receptor. To investigate the effect of overexpression on p85 tyrosine phosphorylation, we coexpressed p85 and PDGF receptor transiently in 293 cells. Anti-P-Tyr blotting of lysates and anti-p85 blotting of anti-p85 immunoprecipitates demonstrated PDGF receptor and p85 expression, respectively (Fig. 9A and C). No tyrosine-phosphorylated proteins were detected in anti-p85 immunoprecipitates of control lysates or lysates from cells overexpressing p85 alone (Fig. 9B, lanes 1 to 4). Anti-p85 antibodies coprecipitated PDGF receptor from lysates of cells overexpressing PDGF receptor alone; however, no endogenous tyrosine-phosphorylated p85 was detected (lanes 5 and 6). Upon overexpression of both p85 and PDGF receptor, tyrosine-phosphorylated p85 was detected in anti-p85 immunoprecipitates (lanes 7 and 8).

Transient overexpression of p85 abrogates anti-P-Tyr-associated PI 3-kinase activity. To assess the effect of p85 overexpression on anti-P-Tyr-associated PI 3-kinase activity, we performed PI 3-kinase assays on anti-P-Tyr immunoprecipitates of lysates from 293 cells overexpressing either PDGF receptor alone or both PDGF receptor and p85 (Fig. 10). The anti-P-Tyr fraction from cells transfected with vector alone did not contain significant amounts of PDGF-inducible PI 3-kinase activity (lanes 1 and 2). Upon overexpression of PDGF receptor, ligand-dependent induction of anti-P-Tyr-associated activity was observed (lanes 3 and 4). Concomitant overexpression of PDGF receptor and p85 reduced PI 3-kinase activity in the anti-P-Tyr fraction to levels observed in control transfections (lanes 5 and 6), as did coexpression of PDGF receptor with a fragment of p85 consisting of both SH2 domains (lanes 7 and 8).

Anti-p85 antibodies immunoprecipitate PI 3-kinase activity. To demonstrate that our anti-p85 antibodies recognize PI 3-kinase and not just free p85, we assayed HER14 anti-p85 immunoprecipitates for PI 3-kinase activity. The anti-p85 antibodies Ab51 and AbC both precipitated PI 3-kinase activity (Fig. 11A, lanes 5 to 8), whereas preimmune serum did not (lanes 3 and 4). Activity was present in immunoprecipitates of lysates from quiescent cells as well as PDGF-treated cells. No significant increase in immunoprecipitable activity was observed upon PDGF treatment (compare lanes 5 and 7 with lanes 6 and 8; the difference in activity between lanes 5 and 6 was not reproducible). The amount of activity present in anti-p85 immunoprecipitates was considerably less than that observed in the anti-P-Tyr fraction of PDGF-treated cells (lane 2).

In an effort to correlate p85 levels with PI 3-kinase activity, the same samples that were assayed for activity were immunoblotted with anti-p85 antibodies (Fig. 11B). Surprisingly, much more p85 was detected in anti-p85 immunoprecipitates than in the anti-P-Tyr fraction (compare lanes 5 to 8 with lane 2), despite the fact that the anti-P-Tyr fraction contains more PI 3-kinase activity (Fig. 11A).

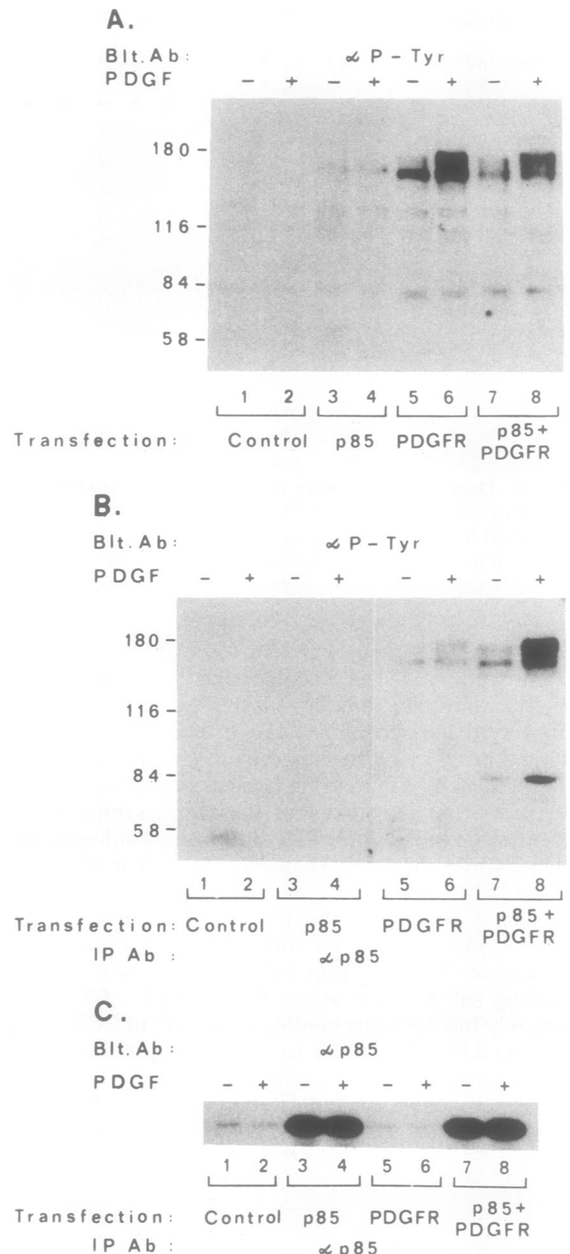

FIG. 9. PDGF-dependent tyrosine phosphorylation of p85 upon overexpression in 293 cells. Cells were transiently transfected with human p85 and PDGF receptor cDNAs and lysed, and lysates were immunoprecipitated as described in Materials and Methods. (A) Anti-P-Tyr immunoblotting of cell lysates. Thirty microliters of lysate was analyzed per lane. (B) Anti-P-Tyr immunoblotting of anti-p85 immunoprecipitates. (C) Anti-p85 immunoblotting of anti-p85 immunoprecipitates. Lysate from $\sim 5 \times 10^6$ cells was immunoprecipitated with Ab51; 80% of the immunoprecipitate was analyzed in panel B, and the remainder was analyzed in panel C. Immunoblotting was performed as described for Fig. 2. Immunoblots were exposed for 12 h at $-70°C$. Positions of molecular weight markers are indicated in thousands.

DISCUSSION

In this study, we have demonstrated that PI 3-kinase-associated p85 associates with EGF and PDGF receptors in a ligand-dependent manner. Either SH2 domain of p85 is sufficient for the formation of these complexes. The affinity of the N-terminal SH2 domain for EGF and PDGF receptors, as measured by inhibition of binding by P-Tyr, corre-

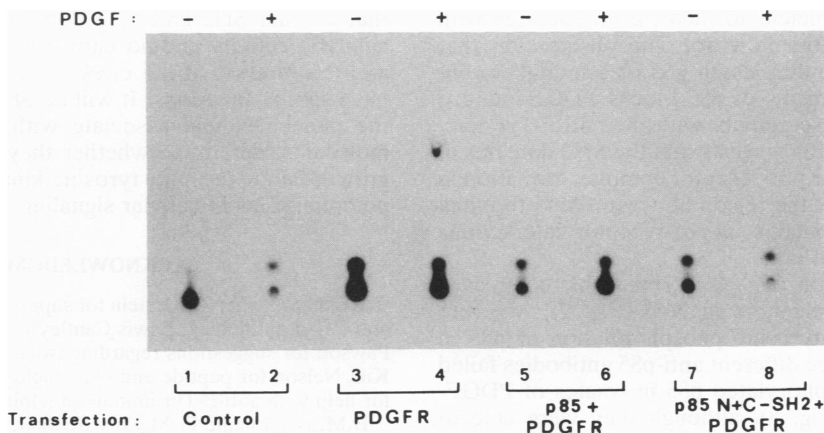

FIG. 10. PI 3-kinase activity in anti-P-Tyr immunoprecipitates from 293 cells overexpressing PDGF receptor and p85. Cells were transiently transfected and processed as described for Fig. 9. Anti-P-Tyr immunoprecipitates were assayed for PI 3-kinase activity as described for Fig. 1.

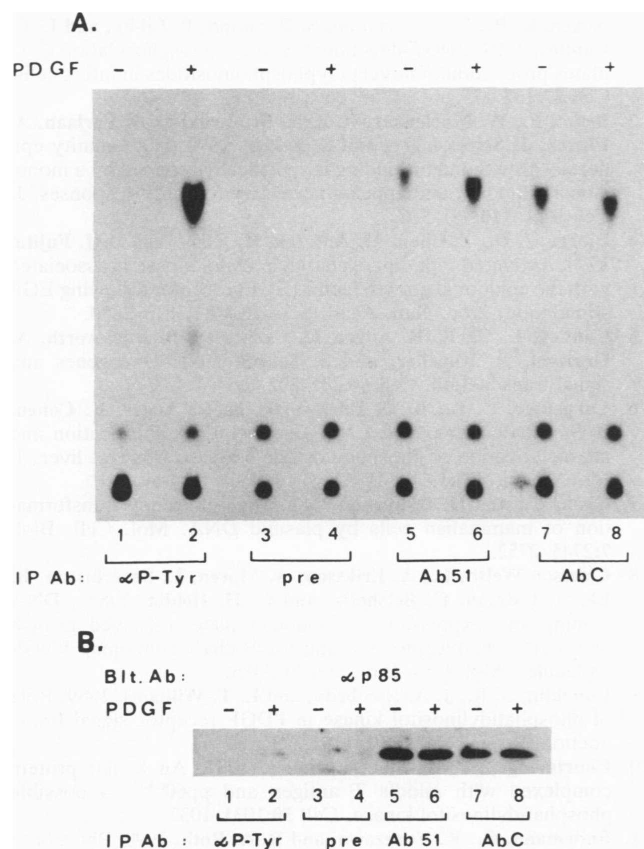

FIG. 11. PI 3-kinase activity and p85 levels in anti-P-Tyr and anti-p85 immunoprecipitates of HER14 cell lysates. HER14 cells were treated with PDGF (50 ng/ml) for 5 min at 37°C. Lysates were immunoprecipitated with anti-P-Tyr or anti-p85 antibodies. The immunoprecipitates were split into two equal portions and assayed concomitantly for PI 3-kinase activity and p85 levels. (A) Immunoprecipitates were assayed as described for Fig. 1; (B) immunoprecipitates were subjected to 8% SDS-PAGE and immunoblotted with anti-p85 antibodies as described for Fig. 2. Immunoblots were exposed for 18 h at −70°C.

lates with the steady-state level of p85-receptor complex and the amount of PI 3-kinase activity recruited to the anti-P-Tyr fraction upon growth factor treatment of HER14 cells. As expected, growth factor treatment increased the amount of p85 associated with the anti-P-Tyr fraction of HER14 lysates as well. We investigated the tyrosine phosphorylation of p85 in response to PDGF treatment by using three different anti-p85 antibodies and three distinct approaches: anti-P-Tyr immunoblotting of anti-p85 immunoprecipitates, immunoprecipitation of $^{32}P_i$-labeled cell lysates, and anti-P-Tyr immunoblotting of anti-p85 immunoprecipitates from cells transiently overexpressing PDGF receptors. None of these approaches allowed us to detect tyrosine phosphorylation of endogenous p85 in response to PDGF. Only when p85 was overexpressed transiently with the PDGF receptor were we able to demonstrate ligand-dependent tyrosine phosphorylation of p85. Overexpression of p85 also abolished PI 3-kinase activity in anti-P-Tyr immunoprecipitates from cells transiently expressing PDGF receptors.

The relatively low levels of anti-P-Tyr-associated PI 3-kinase activity and receptor-p85 complex formation observed upon EGF treatment of a cell line that overexpresses EGF receptors suggest that PI 3-kinase plays a minor role in EGF receptor-mediated signal transduction. Surprisingly, binding of p85 SH2 domains to the EGF receptor in vitro (Fig. 4) did not correlate well with in vivo p85-receptor interactions (Fig. 2). This finding may reflect the fact that p85 exists as a heterodimer with p110 in vivo. Indeed, it has been shown that free p85 binds more avidly to the EGF receptor than the p85/p110 complex does (34); this may explain the differential effects of EGF and PDGF on PI 3-kinase activity. Our data on the differential inhibition of EGF and PDGF receptor–N-SH2 complex formation by P-Tyr (Fig. 5 and 6) suggest that p85 itself may have a much lower affinity for EGF receptors than for PDGF receptors. This is consistent with the finding that the EGF receptor does not contain a consensus PI 3-kinase binding motif found in the PDGF receptor kinase insert and other growth factor receptor tyrosine kinases (5) and supports a role for amino acid residues flanking P-Tyr residues in determining the stability of interactions between SH2 domains and activated growth factor receptors.

Our in vitro binding data on p85 SH2 domains and the PDGF receptor (Fig. 4) are in agreement with previous studies (1, 26, 28, 30, 32, 40) and demonstrate that the SH2

domains of p85 are sufficient to mediate interactions with activated PDGF receptors in vitro. The observation that overexpression of either full-length p85 or a fragment consisting of both SH2 domains of p85 blocks PDGF-induced association of PI 3-kinase activity with the anti-P-Tyr fraction in living cells (Fig. 10) suggests that the SH2 domains of p85 are also sufficient for p85-receptor complex formation in vivo, although a role for the region between the N-terminal and C-terminal SH2 domains in p85-receptor interactions cannot formally be ruled out.

Despite the observation that p85 forms stable complexes with activated PDGF receptors in vivo (Fig. 2), we were unable to demonstrate tyrosine phosphorylation of p85 in response to PDGF. Three different anti-p85 antibodies failed to detect tyrosine-phosphorylated p85 in lysates of PDGF-treated HER14 cells (Fig. 8), although they were able to immunoprecipitate tyrosine-phosphorylated p85 (Fig. 9B) and PI 3-kinase activity (Fig. 11A). In vitro kinase assays demonstrate that an 85-kDa protein in the anti-P-Tyr fraction of PDGF-treated HER14 cells can be phosphorylated; this protein is specifically immunoprecipitated by anti-p85 antibodies (data not shown). This finding together with the fact that tyrosine-phosphorylated p85 is detectable only upon p85 overexpression (Fig. 9B) suggests either that PDGF induces tyrosine phosphorylation of endogenous p85 at very low stoichiometry or that p85 is an efficient substrate for protein tyrosine phosphatases.

The absence of a tyrosine-phosphorylated 110-kDa protein in anti-p85 immunoprecipitates of PDGF-treated HER14 cells (Fig. 8) suggests that p110 may also not be substantially tyrosine phosphorylated in the steady state. Anti-p85 immunoprecipitates of [^{35}S]methionine-labeled HER14 cell lysates revealed the presence of a 110-kDa protein that could be p110, but numerous other proteins were also present in the immunoprecipitate. It is noteworthy that the various components which are essential for the activity and regulation of PI 3-kinase are not yet determined.

The possibility that endogenous p85 is not tyrosine phosphorylated in response to PDGF raises the question of how PI 3-kinase is activated upon growth factor treatment. The increase in PI 3-kinase products observed in intact cells upon PDGF treatment (2) could represent activation; alternatively, it may simply reflect localization of constitutive PI 3-kinase activity to the cell membrane, where PI 3-kinase substrates are more abundant. It is noteworthy that PI 3-kinase assays of anti-p85 immunoprecipitates do not demonstrate a major increase in activity after PDGF treatment (Fig. 11A). However, attempts to correlate p85 levels with PI 3-kinase activity show that the relatively small amount of p85 associated with the anti-P-Tyr fraction is associated with a disproportionate amount of PI 3-kinase activity (Fig. 11). A definitive determination of whether PI 3-kinase is activated by PDGF awaits the generation of specific antibodies against the presumptive catalytic p110 subunit.

Our data suggest that endogenous p85 associates readily with but is not tyrosine phosphorylated by activated PDGF receptors. Only when overexpressed does p85 become tyrosine phosphorylated in response to PDGF. Thus, p85 may not be a good substrate for the PDGF receptor tyrosine kinase. It is possible that p85 serves as an adaptor molecule or a regulatory subunit of PI 3-kinase whose function is to target the catalytic moiety of PI 3-kinase to activated tyrosine kinases. It will be interesting to determine whether the p110 subunit is tyrosine phosphorylated in response to PDGF and other growth factors and whether the presence of p85 enhances the phosphorylation of p110. Other molecules that contain SH2 and SH3 domains but have no apparent catalytic activity and no known function, such as Nck (23) and the product of the *c-crk* proto-oncogene (28, 29), may have similar functions. It will be of great interest to identify the molecules that associate with these putative adaptor molecules, determine whether they serve as substrates for growth factor receptor tyrosine kinases, and elucidate their potential roles in cellular signaling.

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