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The C-Terminal SH2 Domain of p85 Accounts for the High Affinity and Specificity of the Binding of Phosphatidylinositol 3-Kinase to Phosphorylated Platelet-Derived Growth Factor β Receptor

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Upon stimulation by its ligand, the platelet-derived growth factor (PDGF) receptor associates with the 85-kDa subunit of phosphatidylinositol (PI) 3-kinase. The 85-kDa protein (p85) contains two Src homology 2 (SH2) domains and one SH3 domain. To define the part of p85 that interacts with the PDGF receptor, a series of truncated p85 mutants was analyzed for association with immobilized PDGF receptor in vitro. We found that a fragment of p85 that contains a single Src homology domain, the C-terminal SH2 domain (SH2-C), was sufficient for directing the high-affinity interaction with the receptor. Half-maximal binding of SH2-C to the receptor was observed at an SH2-C concentration of 0.06 nM. SH2-C, like full-length p85, was able to distinguish between wild-type PDGF receptor and a mutant receptor lacking the PI 3-kinase binding site. An excess of SH2-C blocked binding of full-length p85 and PI 3-kinase to the receptor but did not interfere with the binding of two other SH2-containing proteins, phospholipase C-γ and GTPase-activating protein. These results demonstrate that a region of p85 containing a single SH2 domain accounts both for the high affinity and specificity of binding of PI 3-kinase to the PDGF receptor.

Proteins containing Src homology 2 (SH2) domain sequences, which are homologous to noncatalytic regions of tyrosine kinases, bind to phosphorylated proteins through direct interaction of the SH2 domains with phosphotyrosine residues (1, 13, 22–24, 27). SH2 domains appear to mediate the binding of enzymes such as phospholipase C-γ (PLC-γ), GTPase-activating protein (GAP), and phosphatidylinositol (PI) 3-kinase to activated growth factor receptors (for a review, see reference 19). These interactions are thought to be important in the mechanism by which growth factor receptors transduce mitogenic signals. An important issue is how SH2-containing proteins selectively recognize specific tyrosine-phosphorylated sequences.

Several studies have suggested that there is specificity in the interaction between SH2-containing proteins and tyrosine-phosphorylated receptors (19). For example, PI 3-kinase, an enzyme thought to be important in growth regulation (2, 4, 5, 15, 16), binds to activated wild-type platelet-derived growth factor (PDGF) receptor through a recognition sequence in the kinase insert region of the receptor (4, 6, 8). PI 3-kinase does not bind to receptor mutants that lack this recognition sequence despite the presence of other phosphotyrosine residues (10, 17, 38). In contrast, PLC-γ binds equally well to the wild-type PDGF receptor and to a mutant receptor, ΔKI, that lacks the kinase insert region including the PI 3-kinase binding sequence (4, 30). Therefore, PI 3-kinase and PLC-γ seem to recognize different phosphotyrosine residues on the receptor. However, it is not known for these proteins, or for any other SH2-containing protein, whether SH2 domains are responsible for determining the selectivity of the interaction with a specific phosphotyrosine.

PI 3-kinase is an enzyme that consists of two subunits of 85 and 110 kDa (3, 28, 32). The 85-kDa subunit binds directly to the PDGF receptor and has two SH2 domains and one SH3 domain (8, 31, 33). In this study, we have shown that a fragment of p85 containing the C-terminal SH2 domain accounts for both the high affinity and specificity of the interaction of PI 3-kinase with the PDGF receptor. Full-length p85 and the fragment containing only a single SH2 domain (the C-terminal SH2 domain [SH2-C]) bound with the same apparent affinity to phosphorylated PDGF receptors. The SH2-C fragment could discriminate between wild-type receptor and the ΔKI mutant of the receptor, which lacked the PI 3-kinase binding site, whereas the SH2 domain fragment of PLC-γ bound to wild-type and mutant receptors equally well. It seems likely that the remainder of p85 is involved in interactions with other proteins. These results show that a region of p85 containing the single C-terminal SH2 domain determines the specificity and affinity of the interaction of an intracellular molecule with tyrosine-phosphorylated PDGF receptor.

MATERIALS AND METHODS

Cell culture. BALB/c 3T3 cells (clone A31 from C. D. Scher, Children's Hospital, Philadelphia, Pa.) and COS-7 cells were cultured at 37°C in Dulbecco's modified Eagle medium containing 10% bovine calf serum plus penicillin and streptomycin (50 μg/ml each). *Spodoptera frugiperda* (Sf9) cells (from M. Summers, Texas A&M University, College Station) were grown in Grace's medium supplemented with 10% fetal bovine serum, 3.3 g of yeastolate per liter, 3.3 g of lactalbumin hydrolysate per liter, 50 μg of penicillin per ml, and 50 μg of streptomycin per ml. Recombinant baculovirus expressing wild-type PDGF β receptor or a receptor mutant lacking the kinase insert region, ΔKI, was harvested from the supernatant of Sf9 cells 4 days after infection at a multiplicity of 1 (29, 35). *Escherichia coli* K-12 strain DH5α (Bethesda Research Laboratories) was used for plasmid propagation. *E. coli* B strain BL21 (DE3)plysS (34) served as

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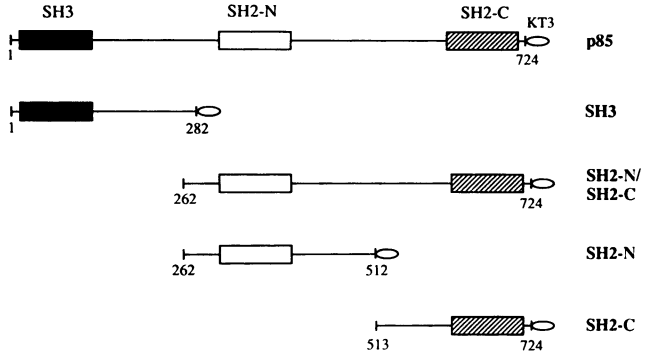

FIG. 1. Schematic structures of full-length p85 and truncated p85 fragments expressed in bacterial and mammalian systems. The presumed functional domains SH3, SH2-N, and SH2-C are represented by black, shaded, and hatched boxes, as indicated above the full-length p85 molecule at the top; the KT3 epitope, which was fused to each C-terminal end, is shown as an oval. The first and last amino acids of each fragment are numbered according to their relative positions within the wild-type p85 sequence (the initiator methionine is number 1).

the host to achieve high expression levels of p85-derived proteins, which are under control of the T7 promoter. *E. coli* RR1 strains expressing the two SH2 regions of PLC-γ (PLC-SH2[N+C]) or the SH2/SH3 region of GAP as TrpE fusion proteins (1) were kindly provided by D. Anderson and T. Pawson, Mount Sinai Hospital, Toronto, Ontario, Canada. After induction of protein expression, bacterial lysates were prepared as described previously (1, 27).

Antibodies. The rabbit anti-PDGF β-receptor antibody (antibody 77) used was directed against a synthetic peptide derived from amino acids 425 to 446 of the extracellular receptor domain (7). The murine anti-PLC-γ monoclonal antibody was kindly provided by S. G. Rhee, National Institutes of Health, Bethesda, Md.; anti-GAP rabbit polyclonal antibodies were obtained from F. McCormick, Cetus Corp., Emeryville, Calif. The murine monoclonal antiphosphotyrosine antibody was used as described previously (29). The murine monoclonal anti-TrpE antibody was obtained from Oncogene Science. The murine monoclonal anti-KT3 antibody was kindly provided by G. Walter (University of California, San Diego).

Plasmid constructions. Schematic structures of p85 fragments cloned into expression vectors are shown in Fig. 1. DNA fragments suitable for subcloning of single p85 domains were obtained by polymerase chain reaction, using mouse p85 cDNA (8) as the template. The N-terminal half was generated with primer p85-NdeI (5′-ATG CAT ATG AGT GCA GAG GGC TAC CAG TAC-3′) containing an *Nde*I site at the start codon and primer p85-ScaI (5′-GAG GTG TTC AGT ACT ATC AGA GC-3′), which introduces a *Sca*I site at nucleotide 845 of the p85 coding sequence (A of the start codon is number 1; nucleotides that are changed with respect to the wild-type sequence are underlined). The DNA ends were repaired with T4 DNA polymerase and phosphorylated by using T4 polynucleotide kinase. The DNA fragment was ligated into the *Sma*I site of pTZ19U (25). The resulting plasmid, pTZ-85-N, carries the N-terminal part of the p85 sequence (nucleotides 1 to 843 of the coding region). The sequence of p85-derived inserts was confirmed by DNA sequence analysis. The C-terminal half of p85 was generated by using primer p85-B5′ (5′-G CTT CAG TAT TTG CTC AAG CA-3′) spanning nucleotides 717 to 737 of the coding strand and primer p85-KT3 (5′-AA GGA TCC TCA AGT CTC AGG CTC AGG AGG AGG AGT TCC TCG CCT CTG TTG TGC ATA-3′) to modify the 3′ end of the p85 sequence. This generated a fragment in which the wild-type p85 coding region was extended by a sequence encoding the eight-amino-acid KT3 epitope (TPPPEPET [21]) and in which a *Bam*HI site and a stop codon were introduced downstream of that sequence.

For expression of p85 and various truncations tagged with the KT3 epitope in *E. coli*, the respective DNA fragments were cloned into the T7 expression plasmid pHB40P (generous gift of Andrew Vershon, University of California, San Francisco); pHB40P is a derivative of pET3a (34) with a modified polylinker. The following expression vectors were prepared from restriction fragments of the above-described polymerase chain reaction products: pHB-85 · KT3 encoding the full-length p85 on an *Nde*I-*Bam*HI DNA fragment (nucleotides 1 to 2172 of the p85 coding region), pHB-SH2#5 · KT3 containing the two SH2 domains of p85 in their wild-type configuration on a *Bsm*I-*Bam*HI DNA fragment (nucleotides 784 to 2172) (SH2-N/SH2-C; Fig. 1), and pHB-SH2#C · KT3 encoding the C-terminal SH2 domain on a *Dra*I-*Bam*HI fragment (nucleotides 1537 to 2172) (SH2-C; Fig. 1). For construction of the following expression plasmids, a double-stranded 30-bp oligonucleotide (coding strand: 5′-AGT ACT CCT CCT CCT GAG CCT GAG ACT TGA-3′; 5′-TCA AGT CTC AGG CTC AGG AGG AGG AGT ACT-3′), which encodes the KT3 tag flanked by a *Sca*I site at its 5′ end and a TGA stop codon at its 3′ end, was phosphorylated and cloned into the *Sma*I site of pTZ19U. pTZ-KT3 plasmids, in which the stop codon of the KT3 insert was placed next to the *Bam*HI site in the vector polylinker, were selected. pHB-SH2#N · KT3 encoding a region that overlaps the N-terminal SH2 domain on an *Nde*I-*Bam*HI fragment (nucleotides 784 to 1536 of the p85 coding region) (SH2-N; Fig. 1) and pHB-SH3 · KT3, which expresses the N-terminal portion of p85 that includes the SH3 domain on an *Nde*I-*Sca*I DNA fragment (nucleotides 1 to 846) (SH3; Fig. 1), were obtained by fusing the KT3 insert of pTZ · KT3 to the respective restriction fragments of the p85 coding region. The correct fusion of the p85-derived fragments to the ATG provided by the vector was confirmed by DNA sequencing.

To express p85 and its isolated domains in mammalian systems, the respective DNA fragments were cloned into vector pBJ-I. In pBJ-I, transcription is under control of the SRα promoter, which represents a simian virus 40-human immunodeficiency virus type 1 promoter hybrid (36).

Expression of recombinant p85 and its isolated domains in *E. coli*. The T7 expression vectors described above were introduced into the bacterial strain BL21(DE3)plysS (34) for production of the respective p85-derived proteins. Bacterial cells were propagated in dYT medium (26) containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) unless they were inoculated for protein production, in which case only ampicillin was added. To achieve high expression levels of p85 fragments, the cultures were grown at 37°C to an optical density at 600 nm of 0.6 to 0.7 and induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a concentration of 0.04 mM for cells harboring pHB-SH2#N · KT3 and at 0.5 mM for all other constructs. Cultures containing pHB-85 · KT3 and pHB-SH3 · KT3 were further grown at 37°C; bacteria containing pHB-SH2#5 · KT3, pHB-SH2#N · KT3, and pHB-SH2#C · KT3 were placed at 20°C after induction and then incubated for an additional 2 h. Cells were harvested and frozen at −80°C. After thawing on ice,

bacterial lysis buffer (50 mM Tris-HCl [pH 7.5], 50% sucrose, 5 mM EDTA, 5 mM EGTA, 0.25% Nonidet P-40, 10 μg of aprotinin per ml, 10 μg of pepstatin per ml, 10 μg of Leupeptin per ml, 2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol) was added to the cells at 1/25 of the initial culture volume. Lysis was completed by sonication five times for 5 s each time; the extracts were cleared by centrifugation for 15 min at 16,000 × *g* at 4°C. The final yield of soluble protein was in a range of 1.2 to 60 mg/liter of culture.

Transient expression of recombinant p85 and its truncated fragments in COS-7 cells. COS-7 cells (60 to 70% confluent on a 10-cm plate) were transfected with the pBJ-I-derived p85 constructs described above, using the DEAE-dextran method (12). At 72 h after transfection, cells were lysed and the recombinant p85 and its fragments were analyzed by a receptor association assay (see below).

Receptor association assays. In vitro associations using immobilized receptor were performed as previously described (6, 29). Briefly, lysates of approximately 10^6 Sf9 cells expressing wild-type PDGF β receptor or ΔKI mutant PDGF receptor were incubated with PDGF β-receptor antibody overnight at 4°C at a 1:500 dilution. Protein A-Sepharose beads (Sigma) were used to precipitate the receptor-antibody complexes. The beads were washed with Triton-lysis buffer containing 0.05% sodium dodecyl sulfate (SDS), twice with wash buffer 1 (50 mM Tris-HCl [pH 7.5], 0.5 M LiCl, 0.5% Triton X-100), and once with distilled water. For experiments involving an in vitro protein kinase assay, the immobilized receptor was subjected to autophosphorylation as previously described (29). This did not affect the association of p85 and its derivatives with the receptor (data not shown). The immunoprecipitates were washed twice with wash buffer 1 and once with Triton-lysis buffer before the incubation with recombinant p85 proteins. Associations were performed in 0.5 to 1 ml of lysis buffer containing 5 mM EDTA at 4°C for 2 h. In competition experiments using peptides or BALB/c 3T3 lysate, the p85-derived molecules were preincubated for 30 min at 4°C with the respective peptides (150 μM) or the 3T3 lysate (from 6 × 10^6 cells) prior to the incubation with PDGF receptor. Lysates from quiescent, unstimulated BALB/c 3T3 cells were prepared as previously described (29). The complexes were then washed twice with wash buffer 1, twice with wash buffer 2 (50 mM Tris-HCl [pH 7.5], 0.5 M LiCl), and once with distilled water. Proteins associated with the receptor were analyzed by immunoblotting with the appropriate antibodies and by in vitro kinase and PI 3-kinase assays as described below.

Immunoblotting. Receptor immunoprecipitates were boiled in Laemmli sample (20) buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose filters (Schleicher & Schuell). Filters were blocked in TBST buffer (10 mM Tris-HCl [pH 7.5], 0.9% NaCl, 0.05% Tween 20, 0.2% sodium azide) containing 3% dried milk; for immunoblotting with phosphotyrosine antibody, TBST plus 3% bovine serum albumin was used. The respective antibodies were added in TBST at appropriate dilutions. Bound antibody was detected with anti-mouse or anti-rabbit antibody conjugated with alkaline phosphatase (Promega) in TBST, washed, and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Promega).

In vitro protein kinase assay and PI 3-kinase assay. Associated molecules were phosphorylated by PDGF receptor in an in vitro protein kinase reaction as described previously (6, 8, 29). After incubation with [γ-^{32}P]ATP in buffer containing $MnCl_2$, the reaction was stopped in Laemmli sample buffer and analyzed by denaturing SDS-PAGE (20) and autoradiography. Alternatively, the relative amount of radiolabeled phosphoproteins was quantitated by using a PhosphoImager (Molecular Dynamics). Receptor immunoprecipitates containing associated proteins were assayed for PI 3-kinase activity in PI 3-kinase buffer (30 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4], 30 mM $MgCl_2$, 50 μM ATP, 200 μM adenosine, 0.2 mg of sonicated PI, 10 μCi of [γ-^{32}P]ATP [5,000 Ci/mmol]) for 20 min at 25°C. Adenosine was added to inhibit any contaminating PI 4-kinase activity (37). Reactions were stopped by adding 100 μl of 1 M HCl, and the phospholipids were extracted with 200 μl of a 1:1 mixture of chloroform-methanol. The reaction products were separated by thin-layer chromatography as previously described (16). The conversion of PI to PI 3-phosphate was determined by autoradiography or quantitated by using a PhosphoImager.

RESULTS

Fragments of p85 containing the C-terminal SH2 region bind to the PDGF receptor with high affinity. Fragments containing the presumed functional domains of p85 (Fig. 1) were expressed in bacterial or COS-7 cells. Each of the p85-derived fragments was tagged at the C terminus with an eight-amino-acid KT3 epitope of simian virus 40 large T antigen (21). The KT3 tag did not affect the association of the p85 molecule with the PDGF receptor, as determined by comparing p85 and KT3-tagged p85 expressed in COS cells (data not shown).

To identify the domain(s) in p85 which interacts with the PDGF receptor, bacterial lysates containing full-length p85 protein, the SH3 domain fragment (SH3), the N-terminal SH2 domain (SH2-N), or the SH2-C fragment (Fig. 1) were incubated with baculovirus-expressed PDGF receptor immobilized on protein A-Sepharose beads (6). After extensive washing of the complexes, the association of the p85 fragments with the receptor was detected by immunoblotting with an anti-KT3 antibody. The concentrations of the p85 fragments used were adjusted in comparison with protein standards visualized by Coomassie stain and by immunoblotting with an anti-KT3 antibody (Fig. 2B). As shown in Fig. 2A, only full-length p85 and SH2-C were able to bind to the PDGF receptor at a concentration of 0.12 nM, indicating a high-affinity interaction. No binding of SH3 was observed at 18 nM. The isolated SH2-N fragment bound to the receptor at 12 nM, which is 100-fold higher than the concentration necessary for SH2-C fragment association (0.12 nM). A fragment that contained both the N-terminal and the C-terminal SH2 domains of p85 (SH2-N/SH2-C; Fig. 1) bound with the same apparent affinity as did the fragment that included only SH2-C (see Fig. 4A).

Relative binding affinities of p85 and p85 fragments for tyrosine-phosphorylated PDGF receptor. To examine the binding affinities of full-length p85 and its isolated SH2 domain fragments, the association of p85, SH2-N, and SH2-C with the PDGF receptor was analyzed over a wider range of protein concentration. Tyrosine-phosphorylated PDGF receptor was present in limiting amounts (approximately 10 pM) to ensure that the binding reactions were dependent on the input concentration of p85 and p85 fragments. To facilitate measurement of small amounts of p85 or the SH2 fragments that bound to the receptor under these conditions, the bound fragments were detected by an in vitro phosphorylation reaction. The phosphorylation of associ-

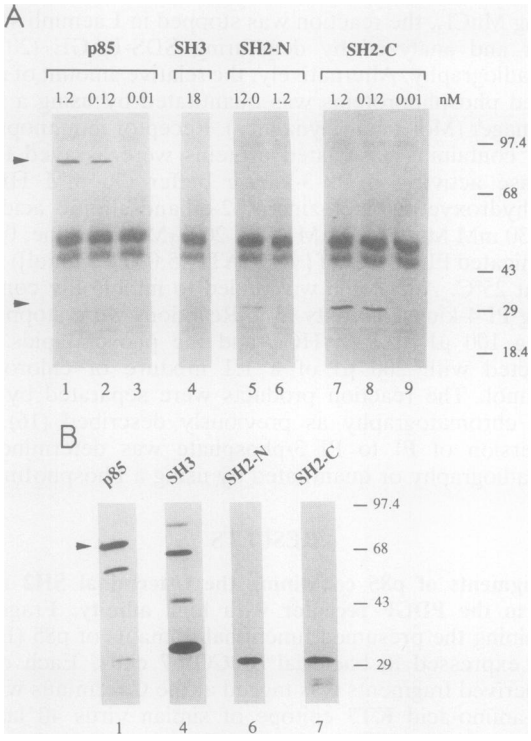

FIG. 2. (A) Association of bacterially expressed p85 and truncated fragments with tyrosine-phosphorylated PDGF receptor. The indicated concentrations of the full-length p85 or its truncated fragments were tested for association in vitro with immobilized baculovirus-expressed receptor. After extensive washes, the immunocomplexes were separated by 8 to 12% step gradient SDS-PAGE and transferred to a nitrocellulose membrane. p85 molecules associated with the receptor were visualized via their KT3 tag, using an anti-KT3 antibody. The positions of p85 and SH2 fragments are indicated by arrowheads at the left. The appearance of the immunoglobulin G band of antibody 77, which was used for immunoprecipitating the PDGF receptor, is due to some cross-reactivity of the alkaline phosphatase-conjugated anti-mouse antibody. Molecular size markers on the right are in kilodaltons. (B) Quantitation of the bacterially expressed p85 derivatives. The amount of p85 or its fragments used for lanes 1, 4, 6, and 7 shown in panel A was assessed by anti-KT3 immunoblot analysis of the respective bacterial lysates. The concentration of p85, SH2-N, and SH2-C (lanes 1, 6, and 7) was 1.2 nM and the concentration of SH3 (lane 4) was 18 nM in comparison with standards visualized by Coomassie stain. The position of recombinant p85 molecules is indicated by the arrowhead. Minor products detected by the anti-KT3 antibody might be due to proteolytic degradation of the p85-derived species or to secondary translational initiation sites present on runaway transcripts.

ated p85 or p85 fragments by the tyrosine kinase activity of PDGF receptor was quantitated by using a PhosphoImager after separation of the respective reaction mixtures by SDS-PAGE. Full-length p85 and the SH2 domain fragments were phosphorylated to the same extent when they bound maximally to the receptor (data not shown). The association curves obtained by the phosphorylation assay (Fig. 3) were in agreement with the data obtained by immunoblotting p85 or its fragments (Fig. 2A). Full-length p85 and SH2-C bound half-maximally to the receptor at a concentration of approximately 0.06 nM; SH2-N bound half-maximally at a concentration of approximately 40 nM. This result suggests that SH2-C mediates the high-affinity interaction between p85

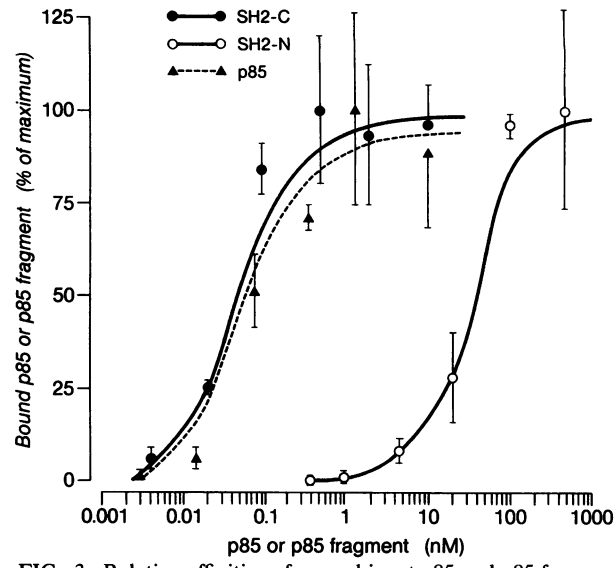

FIG. 3. Relative affinities of recombinant p85 and p85 fragments for tyrosine-phosphorylated PDGF receptor. Various concentrations of p85, SH2-N, or SH2-C were incubated with immobilized PDGF receptor. After stringent washes, associated molecules were phosphorylated in the presence of radiolabeled ATP by the receptor tyrosine kinase activity in an in vitro protein kinase assay (see Materials and Methods). The reaction mixtures were separated by SDS-PAGE, and the relative amount of radiolabeled p85 or p85 fragment in each lane was quantitated by using a PhosphoImager. Each data point represents the mean of triplicate samples ± standard deviation. The apparent dissociation constant was estimated to be 0.06 nM for full-length p85, 0.06 nM for SH2-C, and 40 nM for SH2-N from the concentration that gave half-maximal binding.

and the PDGF receptor. Binding of p85 or PI 3-kinase activity to the receptor could be blocked by fragments containing the C-terminal SH2 domain (SH2-C or SH2-N/SH2-C) but not by fragments containing only the N-terminal SH2 domain (see below).

The SH2-C domain fragment of p85 exhibits the same specificity in binding to the PDGF receptor as does full length p85. A mutant PDGF receptor, ΔKI, which lacks a large portion of the kinase insert region, does not associate with p85 or PI 3-kinase activity (4, 6, 9). However, the ΔKI mutant receptor is able to bind PLC-γ and to activate PLC-dependent pathways (4, 30). To analyze the specificity of the interaction of isolated p85 domains with the receptor in more detail, the association of the various p85 fragments with the wild-type PDGF receptor or the ΔKI mutant receptor was determined. A fragment containing both SH2 regions of PLC-γ (PLC-SH2[N+C]) served as a control. The fragments which contain the SH2-C domain of p85 (p85, SH2-N/SH2-C, and SH2-C) all bound to the wild-type PDGF receptor at a concentration of 0.5 nM (Fig. 4A); no binding of SH3 or SH2-N was observed at that concentration. The fragments containing SH2-C did not bind to the ΔKI mutant receptor. However, the SH2 fragment of PLC-γ bound equally well to the PDGF receptor and ΔKI mutant (Fig. 4B). This result shows that the isolated SH2-C fragment retains the same specificity as does full-length p85 and is able to distinguish the wild-type from the mutant PDGF receptor.

An alternative way to assess the specificity of the interaction between p85/PI 3-kinase and the PDGF receptor is to block this association by the use of a tyrosine-phosphorylated peptide (Y719P). This 20-amino-acid peptide repre-

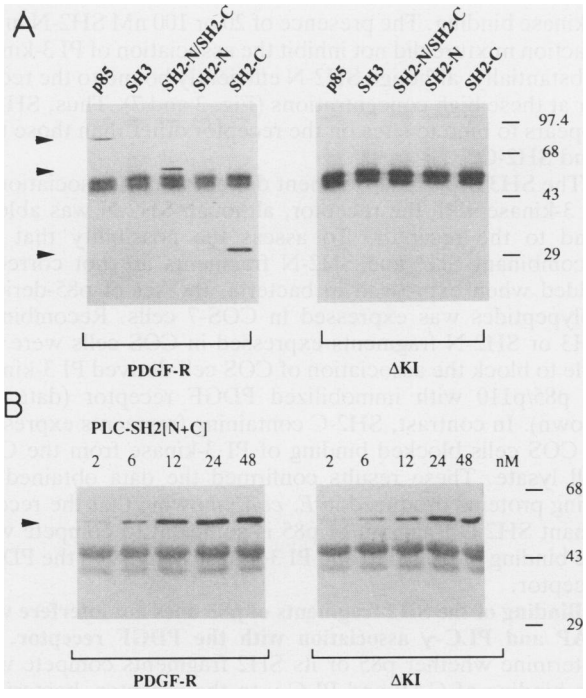

FIG. 4. (A) Association of recombinant p85 and its fragments with the wild-type or ΔKI mutant PDGF receptor in vitro. Each p85-derived polypeptide (0.5 nM) was assayed for binding to the immobilized receptor molecules as indicated. Equal amounts of tyrosine-phosphorylated wild-type and ΔKI mutant receptor were used, as estimated from antiphosphotyrosine immunoblotting. The immunoprecipitates were analyzed by SDS-PAGE and anti-KT3 immunoblotting as described in the legend to Fig. 2. Molecular size markers are given in kilodaltons. (B) Association of a fragment containing both SH2 regions of PLC-γ (PLC-SH2[N+C]) with wild-type or ΔKI mutant PDGF receptor. Various concentrations of PLC-SH2[N+C] were incubated with immobilized receptor proteins as indicated above each lane. Molecules which were bound to the receptors were detected by immunoblotting with an anti-TrpE antibody. The positions of the associated molecules are marked by arrowheads on the left.

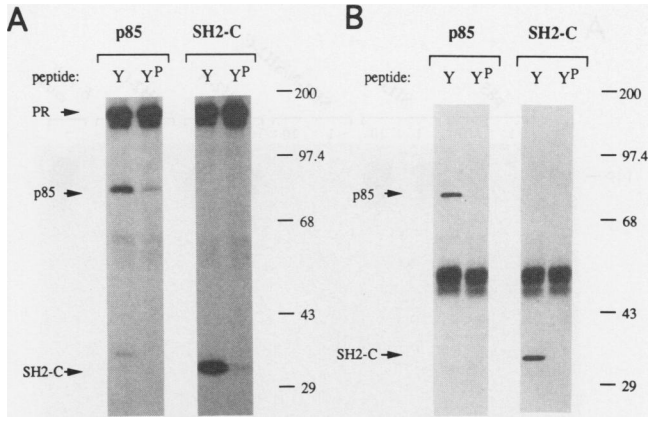

FIG. 5. Specificity of binding of the SH2-C fragment of p85 to the PDGF receptor. Recombinant p85 or the SH2-C fragment was mixed at a concentration of 6 nM with 150 μM tyrosine-phosphorylated peptide Y719P (Y^P) or unphosphorylated peptide Y719 (Y) derived from the kinase insert region of the receptor. After incubation of the mixture with immobilized PDGF receptor, the complexes were washed extensively and subjected to an in vitro protein kinase reaction. The samples were separated by 8 to 12% step gradient SDS-PAGE and transferred to a nitrocellulose filter. Molecules associated with the receptor were visualized by autoradiography (A), revealing proteins which were phosphorylated by the receptor, and by anti-KT3 immunoblot analysis (B) of the same filter. The positions of PDGF receptor (PR), p85, and SH2-C are indicated at the left, and molecular size markers are shown in kilodaltons on the right.

sents the presumed site of interaction for p85 in the receptor molecule and was shown to inhibit the binding of p85/PI 3-kinase to the PDGF receptor (6). p85 or the SH2-C fragment was incubated with a tyrosine-phosphorylated five-amino-acid peptide version (Y^PVPML) of Y719P, which was recently shown to specifically block p85 binding to the receptor (10). The unphosphorylated peptide (Y719) was used as a control. After addition of these mixtures to immobilized receptor, association of p85 or the SH2-C fragment was determined by an in vitro protein kinase assay and by anti-KT3 immunoblot analysis (Fig. 5). The phosphorylated peptide blocked association of p85, as well as the SH2-C polypeptide, with the receptor. The unphosphorylated peptide in the control reactions did not inhibit receptor binding. The association of other SH2-containing molecules with the PDGF receptor, such as PLC-γ and GAP, was not affected by the Y719P peptide (6). These experiments show that the region of p85 that interacts with this peptide is located within the SH2-C fragment.

The SH2-C fragment of p85 is sufficient to block association of PI 3-kinase activity with the PDGF receptor. If PI 3-kinase directly interacts with the PDGF receptor via the SH2-C part of the p85 subunit, it should be possible to block binding of PI 3-kinase activity to the receptor by adding an excess of the C-terminal SH2 fragment of p85. Full-length p85 or the p85 fragments were mixed with BALB/c 3T3 lysates, and the mixtures were added to immobilized receptor. After stringent washes, binding of PI 3-kinase from the 3T3 lysate to the receptor was monitored by measuring PI 3-phosphate production in a PI 3-kinase activity assay (6, 15) (Fig. 6A). In addition, the association of the p85 and p110 subunits of PI 3-kinase with the receptor was analyzed by allowing the receptor to phosphorylate bound molecules in vitro in the presence of radiolabeled ATP (6, 30) (Fig. 6B). The presence of 10 nM SH3 or SH2-N fragment did not inhibit the binding of PI 3-kinase or p85/p110 to the PDGF receptor. In contrast, recombinant full-length p85 or p85 fragments containing the SH2-C domain (1 nM) blocked the association of PI 3-kinase activity (Fig. 6A) and the p85/p110 proteins from the 3T3 cell lysate (Fig. 6B) with the receptor. Recombinant full-length p85 inhibited the binding of cell lysate p110. It was not possible to assess the inhibition of p85 from the 3T3 cell lysate by recombinant p85, since the recombinant and 3T3 cell-derived p85 are indistinguishable by SDS-PAGE.

If the SH2-N fragment of p85 does not play a major role in the association of PI 3-kinase with the PDGF receptor, full-length p85 and SH2-C should compete equally well with the binding of PI 3-kinase over a wide range of protein concentrations. Binding of PI 3-kinase to the receptor in the presence of recombinant p85, SH2-N, or SH2-C was analyzed as described above. PI 3-phosphate production was quantitated by using a PhosphoImager. The competition curves obtained were very similar for full-length p85 and SH2-C (Fig. 6C). The binding of PI 3-kinase to the receptor was inhibited half-maximally in the presence of approximately 0.1 nM p85 or 0.2 nM SH2-C; either protein at a concentration of 1 or 10 nM blocked essentially all PI

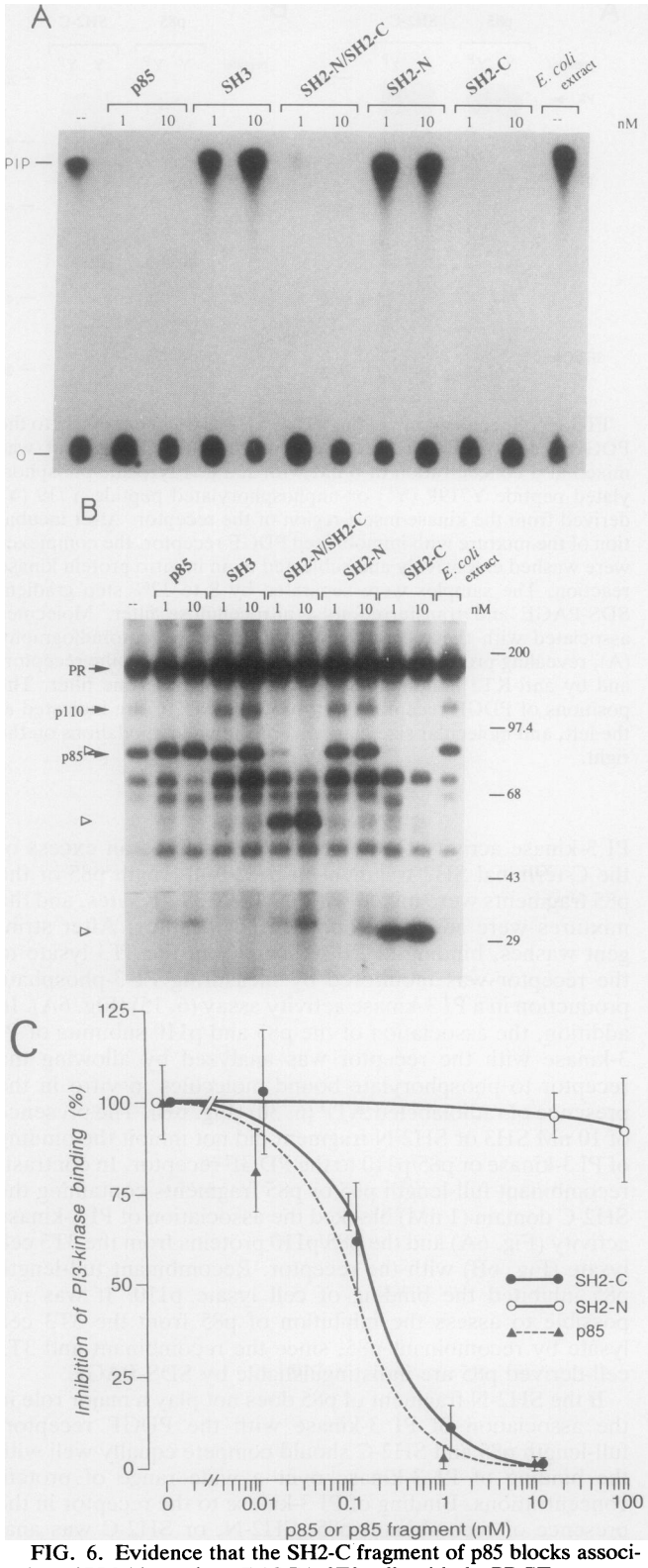

FIG. 6. Evidence that the SH2-C fragment of p85 blocks association of PI 3-kinase from BALB/c 3T3 cells with the PDGF receptor. The indicated concentrations of recombinant p85 or its truncated fragments were mixed with unstimulated 3T3 cell lysate prior to incubation with immobilized receptor (see Materials and Methods). The immunocomplexes were analyzed in parallel for the presence of PI 3-kinase activity by thin-layer chromatography (A) and for the presence of receptor-associated phosphoproteins detected by an in vitro kinase assay (B) as described in Materials and Methods. In the first lane of each panel, only 3T3 lysate was incubated with immobilized receptor; in the last lane, the 3T3 lysate was premixed with extract from *E. coli* cells harboring the pHB40P expression vector without insert, before addition to the receptor. In panel A, the origin (O) of the chromatogram and the position of PI 3-phosphate (PIP) are marked. In panel B, the in vitro kinase reactions were separated by 8 to 12% step gradient SDS-PAGE. The positions of PDGF receptor (PR) and 3T3 lysate-derived p110 and p85 are shown. The positions of associated recombinant p85 and p85 fragments are marked by open arrowheads. Molecular size markers are in kilodaltons. (C) Full-length p85, SH2-C, and SH2-N were compared for their abilities to block binding of PI 3-kinase to the receptor, using the same assay as in panel A. PI 3-phosphate production was quantitated by using a PhosphoImager. Each data point represents the mean of triplicate samples ± standard deviation for p85 and SH2-C or duplicate samples ± standard deviation for SH2-N.

3-kinase binding. The presence of 20 or 100 nM SH2-N in the reaction mixture did not inhibit the association of PI 3-kinase substantially, although SH2-N efficiently bound to the receptor at these high concentrations (Fig. 3 and 7). Thus, SH2-N appears to bind to sites on the receptor other than those that bind SH2-C.

The SH3 or SH2-N fragment did not inhibit association of PI 3-kinase with the receptor, although SH2-N was able to bind to the receptor. To assess the possibility that the recombinant SH3 and SH2-N fragments are not correctly folded when expressed in bacteria, the set of p85-derived polypeptides was expressed in COS-7 cells. Recombinant SH3 or SH2-N fragments expressed in COS cells were not able to block the association of COS cell-derived PI 3-kinase or p85/p110 with immobilized PDGF receptor (data not shown). In contrast, SH2-C containing fragments expressed in COS cells blocked binding of PI 3-kinase from the COS cell lysate. These results confirmed the data obtained by using proteins produced in *E. coli*, showing that the recombinant SH2-C fragment of p85 is sufficient to compete with the binding of p85/p110 and PI 3-kinase activity to the PDGF receptor.

Binding of the SH2 fragments of p85 does not interfere with GAP and PLC-γ association with the PDGF receptor. To determine whether p85 or its SH2 fragments compete with the binding of GAP and PLC-γ to the receptor, bacterially expressed p85, SH2-N, or SH2-C was mixed with unstimulated 3T3 cell lysates, and the mixture was incubated with immobilized PDGF receptor as described above. p85 and its fragments were present at concentrations at which they efficiently bound to the receptor. The ability of 3T3 cell-derived GAP or PLC-γ to associate with the PDGF receptor was analyzed by immunoblot analysis using an anti-GAP or anti-PLC-γ antibody (Fig. 7A and B). The binding of neither GAP nor PLC-γ to the PDGF receptor was inhibited by an excess of recombinant p85, SH2-C, or SH2-N. Binding of wild-type PLC-γ from the 3T3 cell lysate to the PDGF receptor was blocked by the SH2 region of PLC-γ, and the binding of wild-type GAP was blocked by the SH2/SH3 region of GAP in the respective control reactions (Fig. 7A and B). An in vitro protein kinase assay was performed in parallel to confirm that the binding of 3T3 cell-derived p85/p110 to the PDGF receptor was inhibited by SH2-C in the same experiment (Fig. 7C). The selective inhibition of wild-type GAP by the SH2/SH3 fragment of GAP was also evident in this phosphorylation assay; binding of p85 or p110 was not affected by the GAP SH3/SH2 fragment. Associa-

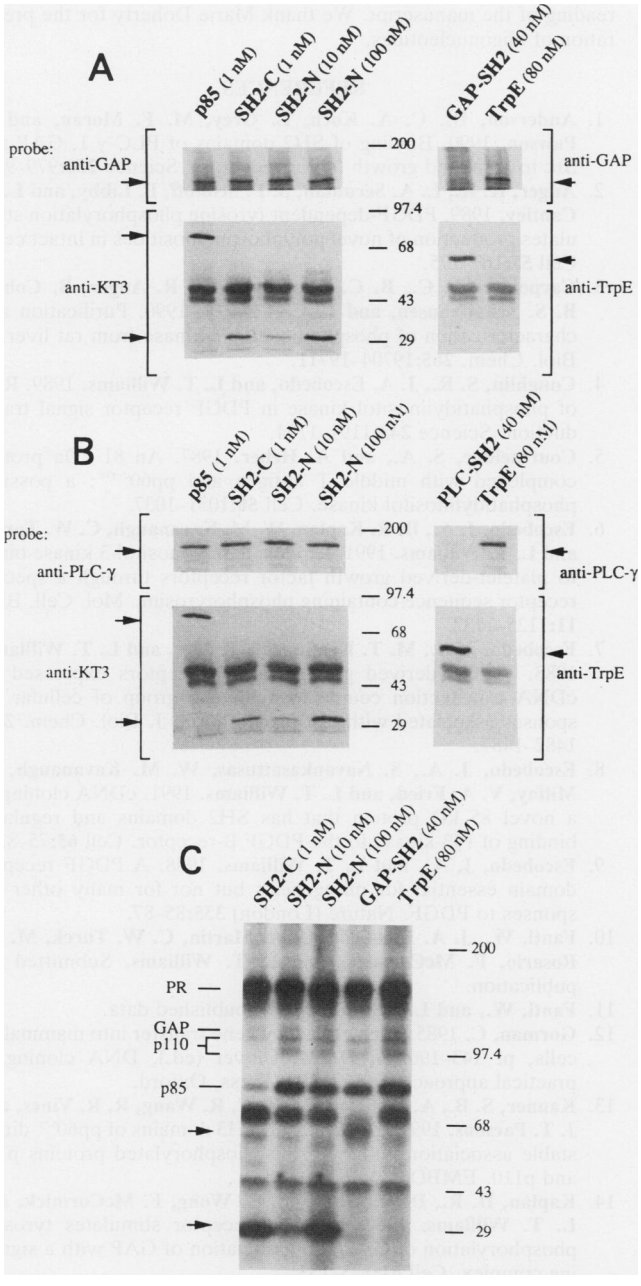

FIG. 7. Evidence that binding of the p85 SH2 fragments does not affect association of the tyrosine-phosphorylated PDGF receptor with GAP or PLC-γ. Unstimulated 3T3 lysates were mixed with recombinant p85, SH2-C, and SH2-N fragments, as indicated, prior to incubation with immobilized baculovirus-expressed receptor. p85 and its fragments were present at concentrations at which they efficiently bound to the receptor. The SH2/SH3 fragment of GAP (GAP-SH2) and the SH2 fragment of PLC-γ (PLC-SH2), which were expressed in *E. coli* as TrpE fusion proteins, and the TrpE fragment were mixed with 3T3 lysates in the respective control reactions. Immunocomplexes were separated by 8 to 12% step gradient SDS-PAGE. After transfer to nitrocellulose, the presence of GAP or PLC-γ in the receptor complex was determined by immunoblot analysis using an anti-GAP (A) or anti-PLC-γ (B) antibody (upper panels). Association of the recombinant p85-derived molecules was detected with an anti-KT3 antibody; association of TrpE-GAP or TrpE–PLC-γ SH2 fragments was monitored with an anti-TrpE antibody (lower panels). Positions of the associated molecules are shown by arrows. (C) One-fourth of each reaction mixture shown in panel A was analyzed for the association of phosphoproteins from 3T3 lysate by an in vitro protein kinase assay. The samples were separated by 7 to 15% SDS-PAGE. The positions of PDGF receptor (PR) and 3T3 lysate-derived p85, p110, and GAP are shown; the positions of recombinant p85 fragments and the SH2/SH3 fragment of GAP are indicated by arrows. Molecular size markers are in kilodaltons.

tion of the SH2-N fragment with the receptor did not interfere with the binding of 3T3-derived p85/p110, GAP, or PLC-γ even at a concentration of 100 nM.

DISCUSSION

To investigate the interaction of PI 3-kinase with the PDGF receptor in detail, we used an in vitro system (6) that has been used to study the interaction of several intracellular molecules with the receptor (14, 29, 30). We have previously shown that the same set of proteins that associate with the activated PDGF receptor in intact cells bind to the receptor in vitro (6). One of these molecules is p85, the putative subunit of PI 3-kinase (3, 28, 32).

The p85 molecule, like PLC-γ and GAP, has two SH2 domains and one SH3 domain (8, 31, 33). Recently, it was shown that fragments of PLC-γ and GAP bind to tyrosine-phosphorylated receptors and that the SH2 domains of these two proteins competed with each other for binding sites on the PDGF receptor (1). It was concluded that the proteins bind to the same site on the receptor. However, it was not clear whether the SH2 domains of these proteins could bind selectively to specific phosphotyrosine residues. In this study, it was furthermore suggested that the two SH2 domains present in PLC-γ and in GAP act cooperatively, since the individual SH2 domains of these enzymes were not sufficient for maximal binding to tyrosine-phosphorylated receptors (1, 27). In contrast, we have found that a fragment of p85 containing the C-terminal SH2 domain binds to tyrosine-phosphorylated PDGF receptor with the same efficiency as does full-length p85. The apparent equilibrium dissociation constant for both full-length p85 containing two SH2 domains and the SH2-C fragment was estimated to be approximately 0.06 nM (Fig. 2 and 3). SH2-C bound to the wild-type receptor but did not bind to a mutant receptor that lacks the binding site for p85/PI 3-kinase (Fig. 4A). The binding of SH2-C was blocked by a tyrosine-phosphorylated peptide (Fig. 5) which was shown to interact specifically with p85/PI 3-kinase but did not bind to the SH2 regions of PLC-γ or GAP (6, 10). Finally, the SH2-C fragment blocked binding of PI 3-kinase to the receptor but did not block binding of PLC-γ and GAP (Fig. 6 and 7). Proteins containing SH2 domains of PLC-γ and GAP failed to block binding of p85/PI 3-kinase to the receptor (Fig. 7C) (11). These data suggest that the portion of p85 containing the SH2-C fragment is sufficient to account for affinity and specificity of the interaction of PI 3-kinase with the receptor.

Although the SH2-C fragment bound specifically to high-affinity sites when it was present at concentrations near the estimated dissociation constant, it bound nonspecifically when it was present at 100-fold-higher concentrations. The nonspecific interaction was reflected by the ability of high concentrations of SH2-C to bind to the kinase insert deletion mutant of the receptor (18). Since previous studies have shown that PI 3-kinase does not bind to this receptor mutant in PDGF-stimulated cells (4), the binding of SH2-C to the ΔKI receptor probably does not represent a physiological interaction. These findings suggest that under in vitro con-

ditions, high concentrations of SH2 domains may cause binding to low affinity sites which are not major sites of interaction in vivo.

The in vitro binding activities of protein domains isolated from their natural context might not necessarily reflect the in vivo situation, especially when the respective fragments are produced in heterologous systems. Therefore, we expressed the series of p85 fragments in both bacteria and COS-7 cells; in addition, fusion proteins consisting of the SH3 domain linked to either of the two SH2 fragments were analyzed (data not shown). In all experiments, proteins containing SH2-C were able to mimic full-length p85 in competing with PI 3-kinase activity and p85/p110 for binding to the PDGF receptor (Fig. 6). The SH2-N fragments did not compete with binding of PI 3-kinase to the receptor even at concentrations up to 100 nM (Fig. 6 and 7). We also found that SH2-N bound to both wild-type and ΔKI receptors at a 20 nM protein concentration, which is 100-fold higher than the amount of SH2-C required for the specific binding to the wild-type receptor (18). We conclude that at this concentration SH2-N binds to sites other than the main site of p85, which is located in the kinase insert region of the PDGF receptor (4, 10). We cannot rule out the possibility that p85 binds to two sites on the PDGF receptor: a high-affinity site that binds to the SH2-C domain and is the major determinant of the binding of PI 3-kinase, and a low-affinity site that interacts with SH2-N. However, it seems also possible that SH2-N interacts with a separate, yet unidentified protein, since the presence of SH2-N in full-length p85 did not enhance the binding to the receptor compared with SH2-C. At present, we do not know the structural basis for the difference in affinity and specificity between the two SH2 domains of p85. The amino acid sequences of SH2-N and SH2-C are as different as are the sequences of the respective SH2 domains of p85 and GAP (19). Therefore, it is not surprising that the two SH2 domains of p85 exhibit different binding specificities.

We have not detected an interaction between the SH3 domain and the receptor even at concentrations as high as 400 nM. Thus, it is likely that the SH3 domain serves some function other than binding to the PDGF receptor. The function of the SH3 domain, which is a common motif present in a number of intracellular molecules, is not clear. It was suggested that SH3 domains might play a role in subcellular localization (for a review, see reference 19).

The results of our studies indicate that a portion of p85 containing SH2-C accounts for the high affinity and specificity of the interaction between the 85-kDa subunit of PI 3-kinase and the tyrosine-phosphorylated PDGF receptor. This interaction might increase the affinity of PI 3-kinase as a substrate of the receptor tyrosine kinase and may localize PI 3-kinase at the plasma membrane. Since binding of p85 within the kinase insert region of the receptor is required for PI 3-kinase activation and PDGF-induced mitogenesis (10), this specific contact mediated by the SH2-C portion of p85 may be an important step in this PDGF-dependent signaling pathway.

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