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SH2 Domains of the p85α Subunit of Phosphatidylinositol 3-Kinase Regulate Binding to Growth Factor Receptors

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The binding of cytoplasmic signaling proteins such as phospholipase C-γ1 and Ras GTPase-activating protein to autophosphorylated growth factor receptors is directed by their noncatalytic Src homology region 2 (SH2) domains. The p85α regulatory subunit of phosphatidylinositol (PI) 3-kinase, which associates with several receptor protein-tyrosine kinases, also contains two SH2 domains. Both p85α SH2 domains, when expressed individually as fusion proteins in bacteria, bound stably to the activated β receptor for platelet-derived growth factor (PDGF). Complex formation required PDGF stimulation and was dependent on receptor tyrosine kinase activity. The bacterial p85α SH2 domains recognized activated βPDGF receptor which had been immobilized on a filter, indicating that SH2 domains contact autophosphorylated receptors directly. Several receptor tyrosine kinases within the PDGF receptor subfamily, including the colony-stimulating factor 1 receptor and the Steel factor receptor (Kit), also associate with PI 3-kinase in vivo. Bacterially expressed SH2 domains derived from the p85α subunit of PI 3-kinase bound in vitro to the activated colony-stimulating factor 1 receptor and to Kit. We infer that the SH2 domains of p85α bind to high-affinity sites on these receptors, whose creation is dependent on receptor autophosphorylation. The SH2 domains of p85 are therefore primarily responsible for the binding of PI 3-kinase to activated growth factor receptors.

The activation of signal transduction pathways by receptor tyrosine kinases involves a series of protein-protein interactions between receptors and signaling proteins (5, 23). The binding of a growth factor, such as platelet-derived growth factor (PDGF) or epidermal growth factor (EGF), to its receptor induces the receptor to dimerize and to autophosphorylate at specific tyrosine residues within its intracellular region (50). Autophosphorylation induces the βPDGF receptor (PDGFR) and EGF receptor (EGFR) to bind and phosphorylate cytoplasmic proteins that control distinct signal transduction pathways. Signaling proteins that physically associate with activated growth factor receptors include phospholipase C-γ1 (PLC-γ1) (29, 34, 52), Ras GTPase-activating protein (GAP) (16, 20), Src family tyrosine kinases (24), and phosphatidylinositol (PI) 3-kinase (4, 7, 18).

These signaling proteins are likely biological targets of receptor kinase activity. The γ1 and γ2 isoforms of PLC can couple PDGF stimulation to PI turnover, unlike other PLC isoforms that are not tyrosine kinase substrates (30, 49). Furthermore, phosphorylation of PLC-γ1 at Tyr-783 is required for in vivo hydrolysis of PI-4,5-P_2 in response to PDGF (21). Similarly, in responsive cells stimulated with PDGF or colony-stimulating factor 1 (CSF-1), the levels of PI-3,4-P_2 and PI-3,4,5-P_3 are increased (3, 51), suggesting that the interactions of PI 3-kinase with the PDGFR and CSF-1 receptor (CSF-1R) (15, 41, 46) have functional consequences. GAP is a strong candidate for the molecule that links tyrosine kinases to the activation of $p21^{ras}$ (10, 27, 35, 37), although the mechanisms by which this might occur are not fully established.

Although PLC-γ1 and GAP have quite distinct functions, they each possess two copies of the noncatalytic Src homology region 2 (SH2) domain (23, 45), which form stable complexes with activated βPDGFR or EGFR in vitro (1, 28, 36). Purified PI 3-kinase is a heterodimer of an 85-kDa and a 110-kDa protein (p85 and p110) (6, 38). cDNAs for two isoforms of p85 have recently been cloned and shown to encode proteins with two SH2 domains, one at the C terminus and another in the middle of the protein (12, 39, 47). Purified p85 does not possess PI 3-kinase activity, although it binds to autophosphorylated EGFR and PDGFR (39), suggesting that p85 is an adaptor that couples the PI 3-kinase catalytic subunit, possibly p110, to tyrosine kinases by virtue of its SH2 domains.

A variety of experiments employing receptor mutants and phosphorylated receptor fragments suggest that the SH2 domains of signaling proteins bind to tyrosine-phosphorylated sites on the PDGFR, CSF-1R, and EGFR. In particular, the C-terminal tail of the EGFR and the noncatalytic kinase insert regions of the α- and βPDGFRs and the CSF-1R provide binding sites for specific SH2-containing proteins (7, 11, 18, 19, 28, 44, 47, 55). Receptor autophosphorylation may therefore act as a switch to elicit high-affinity binding of SH2-containing signaling proteins.

The βPDGFR, the CSF-1R, and Kit belong to a group of closely related receptor tyrosine kinases that contain an insert within their kinase domains of approximately 70 to 100

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amino acids (8, 43, 53, 54). The βPDGFR, the CSF-1R, and Kit (which serves as a receptor for a factor variously known as Steel factor, mast cell growth factor, stem cell factor, and Kit ligand [2, 13, 56]) all associate with PI 3-kinase following growth factor stimulation (7, 9, 26, 41, 42, 44, 46). Efficient PI 3-kinase association is dependent on tyrosine phosphorylation within the kinase insert regions (18, 19, 55). Phosphorylation of Tyr-740 and Tyr-751 of the human βPDGFR, corresponding to Tyr-708 and Tyr-719 of the mouse βPDGFR, and Tyr-721 of the mouse CSF-1R creates PI 3-kinase-binding sites (11, 18, 19, 41a). These tyrosine residues lie within a consensus sequence, (E/D)(E/D)Y(V/M)(P/D/E)M, which has been proposed as a phosphorylation-dependent PI 3-kinase-binding motif (5). These results suggest that the context of a receptor tyrosine autophosphorylation site may determine its ability to bind different SH2 signaling proteins.

To investigate the molecular basis for the interactions between PI 3-kinase and growth factor receptors, we have examined the binding of the PI 3-kinase SH2 domains to the βPDGFR, the CSF-1R, and Kit.

MATERIALS AND METHODS

Bacterial fusion proteins. Regions of bovine p85α PI 3-kinase corresponding to amino acids 312 to 444 (SH2-N), 612 to 722 (SH2-C), or 312 to 722 (SH2-N+C) were isolated by the polymerase chain reaction and subcloned into pATH or pGEX bacterial expression vectors. pATH vectors containing fragments of human GAP and bovine PLC-γ1 were constructed as previously described (1) and contain the following residues: GAP SH2-N, human GAP 178 to 277; GAP SH2-N+C, GAP 171 to 448; and PLC-γ1 SH2-N+C, bovine PLC-γ1 547 to 752.

Bacterial cultures expressing pATH expression vectors were grown, induced and lysed, and TrpE fusion proteins were recovered by using rabbit anti-TrpE antiserum as previously described (1, 36). The amount of TrpE fusion protein in the immune complexes was determined by immunoblotting with monoclonal anti-TrpE antibody (Oncogene Science). Bacterial cultures expressing pGEX expression plasmids were grown in LB containing ampicillin (100 μg/ml) and induced with 1 mM isopropylthiogalactopyranoside (IPTG) for 3 to 6 h. The induced bacteria were lysed by sonication in PLC-lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM $MgCl_2$ 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml). Glutathione *S*-transferase (GST) fusion proteins were recovered from clarified lysates by using glutathione-agarose (Pharmacia) (48). The amount of each GST fusion protein was determined by Coomassie blue staining. Both TrpE and GST fusion proteins were aliquoted, flash frozen, and stored at −80°C.

Cell culture. Rat-2, Rat-2 c-*fms*, R1hER (Rat-1 cells expressing the human EGFR), and Cos-1 cells were routinely maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The dog epithelial cell lines (TRMP) expressing wild-type or mutant PDGFR (18) were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum and 40 μg of G418 per ml. Growth factor stimulation of Rat-2 or TRMP cells was with 75 ng of BB-PDGF (UBI) per ml, or 80 nM EGF for R1hER cells, for 5 min following 48 h of serum starvation (0.5% fetal calf serum). Rat-2 c-*fms* cells (41) were similarly stimulated with 10 nM CSF-1. Cos-1 cells were transfected with $pKitA^+$ and stimulated with 1 μg of recombinant Steel factor (a gift of S. Lyman and D. Williams, Immunex) per ml as previously described (42).

Protein complex formation. Serum-starved or growth factor-stimulated cells were lysed in PLC-LB (approximately 5×10^6 to 1×10^7 cells per ml). One milliliter of clarified lysate was added to immobilized TrpE or GST fusion proteins, and the lysate was incubated for 90 min at 4°C. TrpE fusion proteins were present at approximately 0.1 to 0.5 μg/ml, whereas GST fusion proteins were added to approximately 5 μg/ml. The complexes were washed three times with HNTG buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM sodium orthovanadate) and boiled for 3 min in sodium dodecyl sulfate (SDS) sample buffer. Whole cell lysates of TRMP cells were prepared by adding SDS sample buffer directly to cell monolayers; total protein concentrations were determined as previously described (22).

Immunoblotting and antibodies. All samples were resolved by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose in a semidry blotting apparatus at 0.8 mA/cm^2 for 60 min. Blots were blocked and then probed with 1 μg of affinity-purified rabbit antiphosphotyrosine antibody per ml, 1:500 dilutions of rabbit anti-PDGFR or anti-EGFR antiserum, or 1 μg of monoclonal anti-TrpE antibody (Oncogene Science) per ml followed by 1 μg of rabbit anti-mouse immunoglobulin G (Organon Teknika) per ml. Blots were then probed with ^{125}I-labeled protein A (35 μCi/μg; Amersham) and exposed to Kodak XAR film with an intensifying screen at −80°C. Rabbit anti-Kit antiserum and its use in immunoprecipitation have been previously described (44).

SH2 blotting. Serum-starved or growth factor-stimulated R1hER and TRMP cells expressing wild-type PDGFR were lysed in PLC-LB and immunoprecipitated with either 10 μl of anti-EGFR antiserum or 20 μl of anti-PDGFR antiserum, respectively. Four equal aliquots of each immunoprecipitate were resolved and transferred to nitrocellulose as described above. Blots were blocked in TBS-T (20 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20) containing 3% milk proteins. Lysates of *Escherichia coli* expressing TrpE or TrpE fusions with the p85α SH2-N or SH2-C domain were made by sonication of induced bacteria in phosphate-buffered saline containing 1% Triton X-100, 1% Tween 20, 10 mM dithiothreitol, and 10 μg each of aprotinin and leupeptin per ml. Omission of the reducing agent from the bacterial lysis buffer led to an artificially low signal for the C-terminal p85α SH2 domain. The amount of fusion protein in the lysates was determined by Coomassie blue staining. Equivalent blots were probed either with antiphosphotyrosine antibodies or with approximately 5 μg of TrpE, or TrpE fusion proteins, per ml in TBS-T containing 3% milk proteins for 1 to 2 h at room temperature. Blots probed with fusion proteins were then incubated with anti-TrpE antibodies followed by rabbit anti-mouse immunoglobulin G antibodies and finally probed with ^{125}I-labeled protein A as described above. Blots were exposed to Kodak XAR film with an intensifying screen at −80°C.

RESULTS

SH2 domains of the p85α PI 3-kinase subunit bind to the PDGFR. The binding activities of the p85 SH2 domains were investigated by constructing bacterial expression vectors containing portions of the bovine p85α cDNA. The more

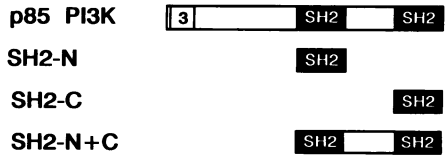

FIG. 1. Structures of p85α PI-3 kinase and bacterial fusion proteins. Locations of the SH2 and SH3 domains in p85α are indicated. The regions of p85α contained in bacterial TrpE or GST fusion proteins used in binding experiments are also illustrated.

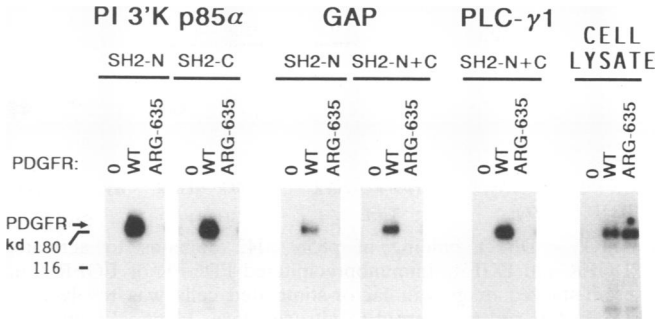

FIG. 2. Evidence that binding of p85α PI 3-kinase SH2 domains to PDGFR requires receptor kinase activity. TrpE fusion proteins synthesized in bacteria were immobilized and incubated with lysates from PDGF-stimulated TRMP cells expressing either wild-type (WT) PDGFR or a mutant receptor containing a lysine-to-arginine substitution at residue 635 (Arg-635). TRMP cells expressing no receptor (0) were treated in a similar manner. The association of PDGFR with bacterial fusion proteins was monitored by anti-PDGFR immunoblotting. From left to right: TRMP cell lysate mixed with p85α SH2-N or SH2-C, GAP SH2-N or SH2-N+C, and PLCγ1 SH2-N+C. Total cell lysates were also analyzed by anti-PDGFR immunoblotting to confirm the presence of equivalent amounts of receptor in cells lines expressing wild-type or mutant receptors. Blots were incubated with ^{125}I-protein A, and autoradiography was for 16 h.

N-terminal SH2 domain (SH2-N) and the C-terminal SH2 domain (SH2-C) were isolated by polymerase chain reaction and subcloned into TrpE and GST expression vectors (Fig. 1). Similar vectors containing both SH2 domains and the intervening region (designated SH2-N+C) were also constructed. The TrpE-SH2 bacterial fusion proteins were induced and immunoprecipitated from bacterial lysates with anti-TrpE antibody; the amounts of TrpE proteins in the immune complexes were assessed by immunoblotting with anti-TrpE antibodies. GST fusion proteins were purified from bacterial lysates by using glutathione-agarose beads.

To test the binding of p85α SH2 domains to the PDGFR, we used derivatives of a dog epithelial cell line (TRMP) which normally contain no endogenous PDGFR. This epithelial line has been engineered to express wild-type human βPDGFR or a mutant receptor which lacks kinase activity as a consequence of the substitution of Lys-635 in the ATP-binding site with arginine (18, 20). Cells were starved of serum and then incubated with 75 ng of PDGF per ml for 5 min. Stimulated and unstimulated cells were lysed, and the clarified cell lysates were incubated with immobilized bacterial TrpE fusion proteins. The association of PDGFR with the bacterial polypeptides was monitored by immunoblotting the washed complexes with anti-PDGFR antibodies (Fig. 2). None of the bacterial proteins associated with the PDGFR prior to PDGF stimulation, nor did the parental TrpE bacterial protein bind to the PDGFR in lysates of PDGF-stimulated cells (data not shown). Following PDGF stimulation, however, the TrpE fusion proteins containing N- or C-terminal p85α SH2 domains bound the PDGFR in lysates of dog epithelial TRMP cells expressing the wild-type human βPDGF-R. These results suggest that both the SH2-N and SH2-C domains of bovine p85α can complex with the activated PDGFR with similar efficiencies. However, no p85α SH2 binding was detected following stimulation of epithelial cells expressing the kinase-inactive Lys-635 mutant PDGFR, even though the mutant receptor was highly expressed (Fig. 2). Hence, p85α SH2 binding requires not only PDGF stimulation but also receptor kinase activity. As previously reported, bacterial TrpE fusion proteins containing the GAP and PLC-γ1 SH2 domains bound the activated human PDGFR; as with the p85α SH2 domains, association with the PDGFR required receptor kinase activity, since no binding was detected with the kinase-inactive mutant receptor (Fig. 2).

Since the p85α SH2 domains were expressed in bacteria as fusion proteins with N-terminal TrpE sequences, the linked TrpE residues might have had an effect on ligand binding. We therefore expressed identical p85α SH2 sequences as GST fusion proteins. These polypeptides, containing either N- or C-terminal p85α SH2 domains, bound the activated wild-type human PDGFR in a similar fashion to the corresponding TrpE fusion proteins (Fig. 3). The ability of bacterially expressed p85α SH2 domains to bind activated PDGFR is therefore independent of the linked bacterial tag.

Direct binding of p85α SH2 domains to the autophosphorylated PDGFR. The association of immobilized p85α SH2 domains with the PDGFR in a cell lysate could result from a direct interaction of the bacterial fusion proteins with the receptor or could occur through an intermediate polypeptide. The ability of bacterial TrpE fusion proteins to bind directly to the PDGFR was addressed by using an SH2 blotting procedure. Serum-starved TRMP cells expressing the wild-type βPDGFR were either stimulated with PDGF or left unstimulated. Cells were lysed and immunoprecipitated with anti-PDGFR antibodies; the immune complexes were then boiled in SDS, subjected to SDS-PAGE, and transferred to a nitrocellulose filter. When the filter was immuno-

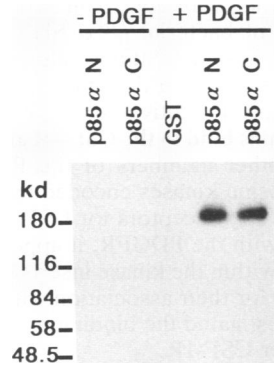

FIG. 3. Binding of activated PDGFR by bacterial GST-SH2 fusion proteins. Bacterially produced GST or GST fusion proteins containing the p85α SH2-N or SH2-C domain were immobilized on glutathione-agarose beads and mixed with lysates of serum-starved (−PDGF) or PDGF-stimulated (+PDGF) TRMP cells expressing wild-type PDGFR. The protein complexes formed were resolved by SDS-PAGE and immunoblotted with anti-PDGFR antibodies.

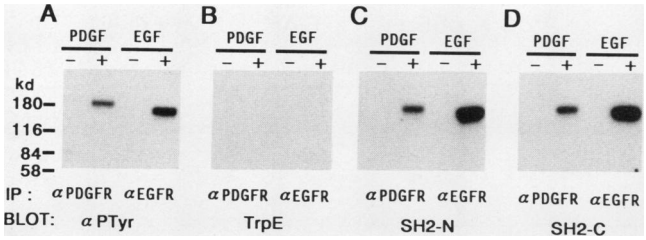

FIG. 4. Direct binding of p85α SH2 domains to activated PDGFR and EGFR. Immunoprecipitated PDGFR or EGFR from serum-starved or growth factor-stimulated cells was resolved by SDS-PAGE and transferred to nitrocellulose. Identical blots were probed with antiphosphotyrosine antibody (A), TrpE-containing bacterial lysate (B), bacterial lysate containing approximately 5 μg of the TrpE–SH2-N domain per ml (C), or bacterial lysate containing approximately 5 μg of the TrpE–SH2-C domain per ml (D). Bacterial lysates were prepared as described in Materials and Methods. Bacterial TrpE fusion proteins which bound to the filters in panels B to D were detected by using monoclonal anti-TrpE antibodies. All blots were incubated with ^{125}I-protein A, and autoradiography was for 16 h.

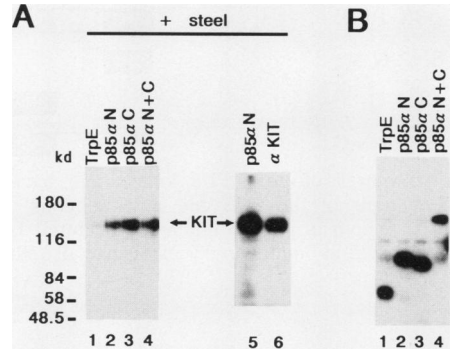

FIG. 5. Binding of p85α SH2 domains in vitro to activated Kit. (A) Cos-1 cells transfected with the pKitA$^+$ expression plasmid were stimulated with Steel factor, and cell lysates were incubated with immobilized TrpE (lane 1) or p85α SH2-N (lanes 2 and 5), p85α SH2-C (lane 3), and p85α SH2-N+C (lane 4) fusion proteins or immunoprecipitated with 10 μl of anti-Kit antiserum (lane 6). Protein complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine antibodies. Aliquots of samples in panel A were also analyzed by anti-TrpE immunoblotting to determine the amount of TrpE fusion protein present in each complex (B). Blots were incubated with ^{125}I-protein A, and autoradiography was for 16 h (A) or 3 h (B).

blotted with antiphosphotyrosine antibodies, a 185-kDa protein corresponding to the autophosphorylated PDGFR was detected in the sample from PDGF-stimulated cells (Fig. 4A). Replica filters were then incubated with bacterial extracts containing parental TrpE or the p85α SH2-N– or SH2-C–TrpE fusion protein. Any TrpE fusion proteins that bound to polypeptides immobilized on the filter were detected by incubation with anti-TrpE antibodies followed by ^{125}I-protein A. The parental TrpE bacterial protein did not show any binding activity (Fig. 4B). However, both the SH2-N– and SH2-C–TrpE fusions recognized a 185-kDa protein from the PDGF-stimulated cells that comigrated with the PDGFR (Fig. 4C and D). Binding of the bacterial p85α SH2 domains to immobilized PDGFR was contingent on prior PDGF stimulation. The p85α SH2-N and SH2-C domains bound the immobilized βPDGFR with equivalent efficiency. These results indicate that both SH2 domains of p85α can interact directly with the activated PDGFR. Recognition of the denatured PDGFR by the p85α SH2 domains is consistent with the suggestion that SH2 domains recognize short peptide sequences containing phosphotyrosine. The concentrations of TrpE-SH2 fusion proteins used in these blotting experiments were approximately 100 nM. The efficient association of bacterial p85α SH2 domains with the βPDGFR under these conditions suggests a high-affinity interaction. Experiments to determine the true affinities of the p85 SH2 domains for activated receptors are in progress.

p85α SH2 domains bind to the CSF-1R and Kit. PI 3-kinase associates with other members of the PDGFR subfamily, including the tyrosine kinases encoded by the c-*fms* and *kit* genes, which serve as receptors for CSF-1 and Steel factor, respectively. As with the PDGFR, it appears that autophosphorylation sites within the kinase inserts of the CSF-1R and Kit are important for their association with PI 3-kinase (44). We therefore investigated the binding of p85α SH2 domains to activated Kit or CSF-1R.

To test the binding of SH2 domains to the Kit receptor tyrosine kinase, we used an isoform of Kit, KitA+, which contains an insert of four amino acids in the extracellular domain (42). We have previously shown that the activity of the KitA+ tyrosine kinase expressed in Cos monkey cells is fully inducible with Steel factor and that the KitA+ receptor is the most highly tyrosine phosphorylated protein identified following stimulation with Steel factor (42). The TrpE fusion proteins containing the p85α SH2-N and SH2-C domains complexed in vitro with a 150-kDa phosphotyrosine-containing protein in lysates of Kit-expressing Cos cells which had been stimulated with Steel factor (Fig. 5A, lanes 2 to 4). This 150-kDa p85α SH2-binding protein comigrated with autophosphorylated Kit, identified by immunoprecipitation with anti-Kit antibodies (Fig. 5A, lanes 5 and 6). Similar results have been obtained by using a mouse NIH 3T3 fibroblast cell line engineered to stably overexpress the KitA+ receptor tyrosine kinase (data not shown).

For the purpose of examining CSF-1R–SH2 interactions, Rat-2 cells expressing the mouse CSF-1R (41) were stimulated with CSF-1, lysed, and incubated with immobilized TrpE bacterial fusion proteins. Tyrosine-phosphorylated proteins that associated with the bacterial SH2 domains were detected with antiphosphotyrosine antibodies. Following stimulation with CSF-1, the SH2-N and SH2-C domains of p85, either individually or together, bound to a 150-kDa tyrosine-phosphorylated protein corresponding to the autophosphorylated CSF-1R (Fig. 6A, lanes 2 to 4). The SH2-binding protein was positively identified as the CSF-1R by stripping the filter of antiphosphotyrosine antibodies and reprobing with anti-CSF-1R antibodies (data not shown).

The EGFR binds directly to p85α SH2 domains in vitro. The EGFR belongs to a distinct subfamily of receptor tyrosine kinases, in which the autophosphorylation sites are clustered within a noncatalytic C-terminal tail. The autophosphorylated EGFR binds weakly to PI 3-kinase activity in EGF-stimulated cells (4), although it is evident that purified p85 will bind to activated EGFR in vitro (39, 47); the site of interaction between the EGFR and p85 apparently involves the C-terminal tail of the receptor (47).

Rat-1 fibroblasts overexpressing the human EGFR were stimulated with EGF, lysed, and immunoprecipitated with anti-EGFR antibodies. The immunoprecipitates were analyzed by blotting with TrpE-SH2 fusion proteins. As with the PDGFR, the SH2-N and SH2-C domains both bound to the immobilized EGFR with similar efficiencies (Fig. 4). Since

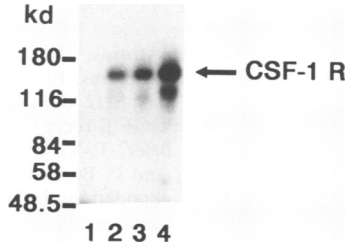

FIG. 6. p85α domains bind in vitro to activated CSF-1R. CSF-1-stimulated lysates from Rat-2 cells expressing the mouse CSF-1R were incubated with immobilized TrpE (lane 1) or p85α SH2-N (lane 2), p85α SH2-C (lane 3), and p85α SH2-N+C (lane 4) bacterial fusion proteins. Protein complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine antibodies.

p85 and its individual SH2 domains bind the EGFR in vitro whereas PI 3-kinase only associates very poorly with the receptor in vivo, there may be constraints imposed on p85 SH2 binding to the EGFR in the cell by another element of the PI 3-kinase complex, likely the p110 subunit (39).

DISCUSSION

Most of the known targets of growth factor receptors are distinguished by a noncatalytic domain, SH2, which binds with high affinity to autophosphorylated receptors and hence is responsible for the formation of physical complexes involving activated receptors and SH2-containing signaling proteins. Such complexes may serve several functions, including the recruitment of signaling proteins to the membrane and their selective phosphorylation and activation by receptor tyrosine kinases. In GAP, PLC-γ and c-Src, the SH2 and catalytic domains are in each case contained within the same polypeptide chain. In contrast, the SH2 domains of PI 3-kinase are located within the regulatory p85 subunit, which is presumed to physically couple the catalytic subunit to activated tyrosine kinases.

The SH2 domains of proteins such as GAP and Src are involved not only in the recognition of growth factor receptors but also in a series of interactions with cytoplasmic phosphoproteins that modulate their own activity and may be involved in their downstream signaling functions (10, 14, 23, 36, 37). A common theme in these interactions is that high-affinity SH2 binding is induced by tyrosine phosphorylation, and in some cases serine phosphorylation (37, 40), of the relevant ligand, be it a growth factor receptor or cytoplasmic protein (11, 18, 28, 31, 33, 36). An alignment of SH2 domain sequences reveals a number of highly conserved residues, including two arginine residues, which might be involved in direct recognition of phosphotyrosine (23). The more variable SH2 residues, by contacting the side chains of residues surrounding the tyrosine-phosphorylated site, could confer high-affinity binding to phosphotyrosine in a specific sequence context (5, 11). We have used members of the PDGFR subfamily to investigate whether SH2 domains of p85 participate in the specific association of PI 3-kinase with receptors.

Of all of the SH2-containing signaling proteins, PI 3-kinase appears the most catholic in its ability to complex with growth factor receptors in vivo. The SH2 domains of bovine p85α, expressed as bacterial fusion proteins with either TrpE or GST polypeptides, are sufficient to bind several activated growth factor receptors in vitro, including those for PDGF, CSF-1, and Steel factor. The in vitro association of p85α SH2 domains with these receptors has been assayed in two ways. In one approach, the bacterial SH2 domains were immobilized and then incubated with a soluble cell lysate. In the second technique, the PDGFR was immobilized on a filter and then probed with soluble SH2 domains. These two procedures have given comparable results, indicating that bacterial p85α SH2 domains can bind directly to activated receptors with similar efficiencies. These results are consistent with the suggestion that the p85 SH2 domains provide the primary means by which PI 3-kinase complexes with autophosphorylated receptors. The PDGFR, CSF-1R, and Kit all contain tyrosine residues within their kinase insert domains surrounded by a common consensus sequence, (D/E)(D/E)Y(M/V)(P/D/E)M, whose phosphorylation likely creates PI 3-kinase-binding sites (5, 11, 18, 19). Indeed, the isolated mouse CSF-1R kinase insert binds PI 3-kinase and p85α SH2 domains, provided that it is tyrosine phosphorylated at Tyr-721, which lies within such a sequence (41a).

Conclusions concerning SH2 binding specificities derived from in vitro experiments should be interpreted with caution, since the removal of an SH2 domain from its native context might have unanticipated effects on its binding activity. Several variables, including the choice of bacterial fusion protein and the precise extent of the SH2 sequences selected, could perturb the ability of bacterial SH2 domains to bind their ligands. The concentrations of bacterial SH2 domains used for in vitro binding experiments are also likely to be important, since very high SH2 concentrations might drive the formation of nonphysiological, low-affinity complexes. With these caveats in mind, however, our results imply that the specific binding of PI 3-kinase to members of the PDGFR subfamily is maintained by the isolated SH2 domains. This specificity is not so evident for binding to the EGFR, where the isolated p85 SH2 domains, and p85 itself (39), bind more effectively than does the p85-p110 PI 3-kinase complex. The autophosphorylation sites of the EGFR are characterized by a cluster of acidic, proline, and glutamine residues surrounding the phosphorylated tyrosines; this is also a feature of the C-terminal tail of c-Src, which forms an SH2-binding site when phosphorylated at Tyr-527 (26a). Furthermore, a segment of the serine-rich Bcr B box, whose serine phosphorylation elicits binding to the Abl SH2 domain (40), is rich in proline, glutamine, and acidic amino acids. These may represent a class of SH2-binding sites distinct from those identified in the PDGFR family. Therefore, under some circumstances, the binding activity of SH2 domains may be further regulated by associated polypeptide sequences.

Recent data have indicated that different growth factor receptors interact with distinct, albeit overlapping groups of SH2-containing signaling proteins. Hence, each growth factor receptor may be coupled to a unique set of signal transduction pathways and thereby induce an individual cellular response. The mechanisms by which growth factor receptors discriminate between different signaling proteins is therefore an important issue. Since SH2 domains apparently provide the primary determinant through which signaling proteins bind receptors (1, 23), it is possible that this specificity is inherent in the affinities with which different SH2 domains and receptors interact.

In addition to the SH2-containing proteins of defined biochemical activity, including GAP, PLC-γ1, PI 3-kinase and Src, there are an increasing number of SH2-containing proteins of unknown function. It seems likely that these proteins, such as Crk (32), Vav (17, 23), and Nck (25), couple

tyrosine kinases to unidentified effectors. The number of SH2 signaling proteins with which growth factor receptors might interact is therefore potentially quite large. The specificity of receptor-SH2 interactions is likely to be important in determining the array of targets with which each receptor associates.

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