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SHP-1 Associates with Both Platelet-derived Growth Factor Receptor and the p85 Subunit of Phosphatidylinositol 3-Kinase*

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The Src homology 2 (SH2)-containing protein tyrosine phosphatase 1, SHP-1, is highly expressed in all hematopoietic cells as well as in many non-hematopoietic cells, particularly in some malignant epithelial cell lines. In hematopoietic cells, SHP-1 negatively regulates multiple cytokine receptor pathways. The precise function and the targets of SHP-1 in non-hematopoietic cells, however, are largely unknown. Here we demonstrate that SHP-1 associates with both the tyrosine-phosphorylated platelet-derived growth factor (PDGF) receptor and the p85 subunit of phosphatidylinositol 3-kinase in MCF-7 and TRMP cells. Through the use of mutant PDGF receptors and performing peptide competition for immunoprecipitation, it was determined that SHP-1 independently associates with the PDGF receptor and p85 and that its N-terminal SH2 domain is directly responsible for the interactions. Overexpression of SHP-1 in TRMP cells transfected with the PDGF receptor markedly inhibited PDGF-induced c-*fos* promoter activation, whereas the expression of three catalytically inactive SHP-1 mutants increased the c-*fos* promoter activation in response to PDGF stimulation. These results indicate that SHP-1 might negatively regulate PDGF receptor-mediated signaling in these cells. Identification of the association of SHP-1 with the PDGF receptor and p85 in MCF-7 and TRMP cells furthers our understanding of the function of SHP-1 in non-hematopoietic cells.

Protein tyrosine phosphorylation is critical in many cellular processes including signal transductions, neoplastic transformation, and the control of the mitotic cycle. These cellular processes are regulated by the activities of both protein tyrosine kinases and protein tyrosine phosphatases (PTPs).[1] One subfamily of cytoplasmic PTPs, referred to as SHP (1), contains two SH2 domains at their N terminus. Three members of SHP have been identified. They are SHP-1, SHP-2, and *Drosophila* Csw. Csw (2), the likely homology of mammalian SHP-2, is essential in signaling from the Torso and Sevenless receptor tyrosine kinases (2, 3). SHP-2 (previously also known as PTP2C, SH-PTP2, PTP1D, Syp, and SHPTP3) (1) is widely expressed and involved in many signaling pathways mediated by multiple non-hematopoietic receptor protein tyrosine kinase and by hematopoietic receptors as well (reviewed in Ref. 4). Generally, SHP-2 plays a positive role in growth factor-stimulated signaling. However, SHP-2 also negatively regulates some cellular processes, such as PDGF receptor-mediated signaling and cell membrane ruffling (5–7) and EGF-dependent cell growth (8). SHP-1 (previously known as PTP1C, SH-PTP1, HCP, PTPN6, or SHP) (1) is predominantly expressed in hematopoietic cells, where it generally functions as a negative regulator (reviewed in Ref. 4), although with some exceptions (9). Recent studies, however, revealed that SHP-1 is also substantially expressed under the control of an alternative tissue-specific promoter in a variety of non-hematopoietic cells, especially in some malignant epithelial cells (10–14). In non-hematopoietic cells, the phosphatase activity of SHP-1 has been shown to positively regulate EGF- or serum-activated mitogenic signaling in 293 cells (15). The negative effect of SHP-1 on cytokine receptor-mediated signaling is exerted by dephosphorylation of the tyrosine-phosphorylated cytokine receptor itself or receptor-associated tyrosine-phosphorylated mediators (Refs. 16 and 17 and reviewed in Ref. 4). In other pathways, however, the mechanism(s) by which SHP-1 and SHP-2 positively or negatively regulate signaling is not fully understood. Recently, various methods for detecting protein interaction have identified potential targets for SHP-1 and SHP-2, including SHPS-1 (SIRPs), CD22, CTLA4, ZAP70, Killer inhibitory receptors, SHIP, STATs, the 97-kDa (100-kDa) and 135-kDa proteins (Ref. 18–26 and reviewed in Ref. 4). Identification of interacting proteins with these two SHPs can greatly facilitate the elucidation of the roles of these phosphatases in the signaling pathways. In this report, we demonstrate that SHP-1 directly associated with both the PDGF receptor and the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) in non-hematopoietic MCF-7 and TRMP cells stimulated with PDGF and that its phosphatase activity negatively regulated PDGF receptor-mediated activation of the c-*fos* promoter.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—MCF-7 (human breast carcinoma) and TRMP (canine kidney epithelial) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Antibodies used were rabbit anti-SHP-1 polyclonal antibody generated as described previously (27), mouse anti-SHP-1 monoclonal antibody (anti-PTP 1C) (Transduction Laboratories), rabbit anti-human PDGF type β receptor polyclonal antibody (Upstate Biotechnology), mouse anti-PDGF type β receptor monoclonal antibody (Genzyme), rabbit anti-p85α polyclonal antibody (Santa Cruz Biotechnology),

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[1] The abbreviations used are: PTP, protein tyrosine phosphatase; SH2, *src* homology 2; SHP, SH2 domain-containing PTP; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; EGF, epidermal growth factor; EpoR, erythropoietin receptor; GST, glutathione *S*-transferase; PI, phosphatidylinositol; Tyr(P), phosphotyrosine; SRE, serum-responsive element; PAGE, polyacrylamide gel electrophoresis; Luc, luciferase; PCR, polymerase chain reaction.

mouse anti-p85α monoclonal antibody (Transduction Laboratories), goat anti-GST polyclonal antibody (Pharmacia Biotech Inc.), and mouse anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology). Protein A-Sepharose CL-4B was obtained from Pharmacia Biotech Inc. Nitrocellulose membrane Hybond-C, anti-mouse IgG-horseradish peroxidase, and the enhanced chemiluminescence (ECL) kit were purchased from Amersham Corp. Human recombinant platelet-derived growth factor BB (PDGF) was purchased from Upstate Biotechnology.

The peptides were synthesized and purified at the Biotechnology Research Institute (Quebec, Canada). Mass spectral analysis gave the expected molecular mass. The peptide sequences are shown in Table I, and the phosphotyrosine sites are indicated.

Construction and Expression of the PDGF Receptor and SHP-1 in MCF-7 Cells—Human PDGF receptor β, wild type SHP-1, and catalytically inactive SHP-1 with a Cys^{455} to Ser mutation (SHP-1 C455S) were constructed into pRc/cytomegalovirus vector as described previously (28). The other two catalytically inactive mutants, SHP-1 D421A (Asp^{421} to Ala) and SHP-1Δ-(455–461) (deletion of Cys^{455}-Ser-Ala-Gly-Ile-Gly-Arg^{461}), were constructed by two-step PCR. For SHP-1 D421A, the primers used were GGAATTCATGCTGTCCCGTGGGTGG (primer 1, forward) with GGGGACCCCATGGGCGGGCCAGCTCAGGT (reverse), and GAGCTGGCCCGCCCATGGGGTCCCCAGTG (forward) with GGGGTCGACCTGAGGACAGCACCGCT (primer 2, reverse). For constructing SHP-1Δ-(455–461) (deletion mutation), primers used were primer 1 (forward) with TGGTGCCTGTGCCGTGCACGATGATGGGC-CCTGC (reverse) and CATCGTGCACGGCACAGGCACCATTGTCATC (forward) with primer 2 (reverse). The two PCR fragments encompassing the mutation were annealed with each other and extended, and the extended DNA fragments were used as new templates for the secondary PCR using primer 1 and primer 2 (in the first PCR) as primers. The secondary PCR product was digested with *Eco*RI and *Sal*I, and the digested fragments were inserted into the mammalian expression vector pcDNA3 at the *Hin*dIII site by blunt-end ligation. The sequence and direction were confirmed by DNA sequencing.

The constructs mentioned above were transfected into MCF-7 cells by standard calcium phosphate co-precipitation technique, and clonal cell lines overexpressing the PDGF receptor, SHP-1 or mutant SHP-1 C455S, were isolated by selection in 400 µg/ml G418 and used throughout the study.

TRMP cells that express stably transfected wild type PDGF receptor or its mutants PDGFR Y740F, Y751F, Y771F, Y1009F, and Y1021F were kindly provided by Dr. J. A. Cooper, Fred Hutchinson Cancer Research Center (29).

Immunoprecipitation and Immunoblot—70–80% confluent cells growing in a 100-mm tissue culture dish were lysed in 1 ml of buffer A (50 mM β-glycerophosphate, pH 7.3, 2 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 100 mM NaCl, 1% Triton X-100, 0.2 mM $NaVO_4$, 0.1 µM microcystin, 1.0 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 1 µM pepstatin A, and 1 µM/ml aprotinin). Cell lysates were clarified by centrifuging at 14,000 rpm at 4 °C for 10 min. Fifty µl of a 50% slurry of protein A-Sepharose CL-4B was added to 1 ml of cell lysates and incubated for 30 min with gentle shaking. The precleared lysates were subjected to immunoprecipitation and immunoblotting as described previously (15). For Western blot analyses, one-fifth of the precipitates were subjected to SDS-PAGE gels and electrotransferred to nitrocellulose membranes. The membranes were probed with specified antibodies using the concentrations and conditions recommended by the manufacturers. Immunoreactive protein bands were revealed by chemiluminescence with ECL detection according to the manufacturer's instruction (Amersham Corp.).

In Vivo Peptide Competition—MCF-7 cells were permeabilized in 40 mM Hepes buffer, pH 7.4, with 10 mM $MnCl_2$, 2 mM EGTA, 300 µM $CaCl_2$, 1 mM 2-mercaptoethanol, and 285 µg/ml α-lysophosphatidylcholine, palmitoyl (Mire's buffer), either alone or containing 1.4 mM peptides, on ice for 1 min. Cells were warmed for 2 min at 37 °C before stimulation with 50 ng/ml PDGF. Cells were then lysed with buffer A as described above.

GST Fusion Proteins and in Vitro Binding Assays—The DNA fragment encoding amino acids 1–213 of SHP-1 was amplified by PCR and inserted into pGEX-2T (Pharmacia). The fusion protein and GST alone were expressed in *Escherichia coli* strain DH5α and freshly prepared for the *in vitro* binding experiments. For binding assays, glutathione-Sepharose beads with approximately 1 µg of bound GST or GST fusion protein were incubated at 4 °C for 2 h with 1 ml of cell lysates that were prepared from the cells grown in a 100-mm tissue culture dish to 70–80% confluence as described above. The beads were washed four times with ice-cold lysis buffer (buffer A), and one-fifth of the bound proteins were analyzed by SDS-PAGE and Western blotting.

Far Western Blotting—p85 was immunoprecipitated from PDGF-stimulated or unstimulated MCF-7 cells under the conditions as described above (see also Ref. 15). The complexes were dissociated by boiling the immunoprecipitates in SDS sample buffer, and the eluted proteins were separated by SDS-PAGE gel and transferred to nitrocellulose membrane. After blocking with 5% milk overnight at 4 °C, the membrane was incubated with 1 µg/ml GST fusion protein containing the SH2 domains of SHP-1 for 2 h and then subjected to Western blotting using the polyclonal anti-GST antibody and appropriate secondary antibody (15).

Luciferase Activity Assay—Transfection of the luciferase reporter gene (SRE-Luc) and assay of luciferase activity were described previously (15).

RESULTS

SHP-1 Associates with p85 and PDGF Receptor—As MCF-7 cells, a breast carcinoma cell line, express both SHP-1 and the PDGF receptor, we initially employed this cell line for experiments. To address the potential role of SHP-1 in PDGF receptor-mediated signaling, we first performed immunoprecipitation with anti-SHP-1 antibody in cells stimulated with PDGF. Proteins that were co-precipitated with SHP-1 were examined by Western blot analysis with anti-phosphotyrosine antibody 4G10. As shown in Fig. 1*A* (*lane 2*), one intensified band and two faint bands with molecular masses of approximately 160–180, 80–90, and 65 kDa were detected. To clarify the identity of the associated proteins, we overexpressed the wild type and the catalytically inactive mutant SHP-1 C455S (28) in these cells. From various stable cell clones, two wild type SHP-1 and two mutants (SHP-1 C455S) were chosen from independent transfections for further characterization. These clones typically expressed a high and comparable level of SHP-1 or SHP-1 C455S (Fig. 1*B*). Interestingly, in the SHP-1-overexpressed cells (*lane 4* in Fig. 1*A*), the intensity of the 80–90- and 65-kDa bands was increased slightly, whereas the tyrosine-phosphorylated signal of 160–180-kDa band was substantially reduced in comparison with the parental cells (*lane 2* in Fig. 1*A*). In cells transfected with the mutant SHP-1 C455S, all three bands became much stronger than those observed in the SHP-1-overexpressed cells, although the expression level of SHP-1 and mutant SHP-1 C455S in the cells were comparable (Fig. 1*B*). The 65- and 160–180-kDa proteins were easily proved to be SHP-1 and the PDGF receptor, respectively, by immunoblotting the same samples with anti-SHP-1 and anti-PDGF receptor antibodies (Fig. 1*C*). To identify the nature of the 80–90-kDa protein, we probed the precipitates with antibodies to various candidate proteins. Eventually, it was revealed that the 80–90-kDa protein was the α type 85-kDa subunit (p85) of phosphatidylinositol 3-kinase (PI 3-kinase) (Fig. 1*C*). As with previous results (28), the overexpressed SHP-1, when tyrosine-phosphorylated in response to PDGF stimulation (*lane 4* in Fig. 1*A*), was rapidly dephosphorylated by its own activity. Retention of the high level of tyrosine phosphorylation on p85 and the PDGF receptor in the SHP-1 C455S transfected cells (*lane 6* in Fig. 1*A*) implies that these two proteins, particularly the PDGF receptor, might be substrates of SHP-1 in PDGF-activated signaling pathway.

To demonstrate further the interaction of these proteins, reciprocal experiments were performed, *i.e.* the cell lysates were subjected to immunoprecipitation with anti-PDGF receptor and anti-p85 antibodies, respectively, and the immunoprecipitates were immunoblotted with anti-SHP-1 antibody. As expected, SHP-1 was detected in both the PDGF receptor and p85 immunoprecipitates produced from PDGF-stimulated cells (Fig. 1, *D* and *E*).

SHP-1 Independently Associates with Both the PDGF Receptor and p85—We have shown that SHP-1 was co-immunoprecipitated with both the tyrosine-phosphorylated PDGF receptor

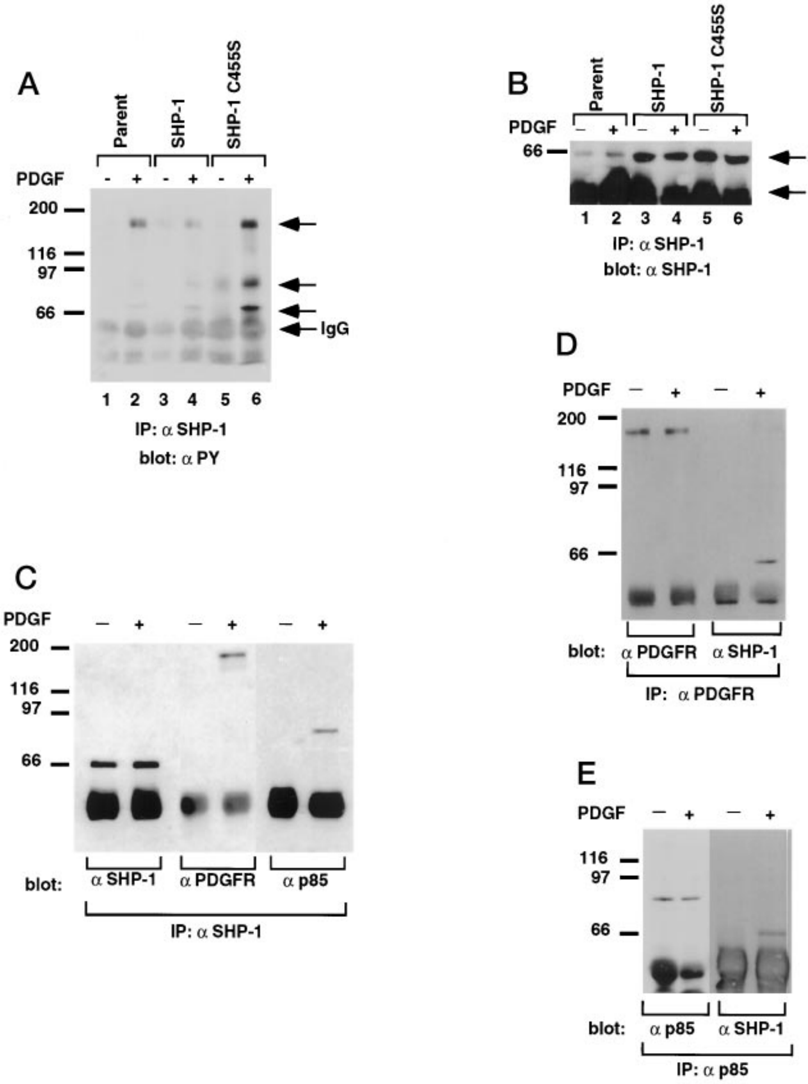

FIG. 1. **Association of SHP-1 with the activated PDGF receptor and the p85 subunit of PI 3-kinase.** *A* and *B*, parental MCF-7 cells or MCF-7 cells overexpressing SHP-1 or SHP-1 C455S were left resting or stimulated for 5 min at 37 °C with 50 ng/ml PDGF following starvation for 24 h. Cells were lysed, and lysates were used for immunoprecipitation (*IP*) with anti-SHP-1 and immunoblotted by anti-phosphotyrosine antibody (*A*) and anti-SHP-1 antibody (*B*) respectively. *C–E,* the cell lysates prepared from MCF-7 cells overexpressing SHP-1 which were treated as in *A* were used for immunoprecipitation with anti-SHP-1 (*C*), anti-PDGF receptor (*D*), and anti-p85 (*E*) polyclonal antibodies, respectively, and immunoblotted by anti-SHP-1, anti-PDGF receptor, or anti-p85 monoclonal antibody as indicated. The positions of the protein molecular mass markers are indicated in kilodaltons (kDa).

and p85. However, it is not clear whether SHP-1 binds independently to the PDGF receptor and p85 or the three proteins associate together through a high order complex. This is possibly due to the presence of the SH2 domains in both SHP-1 and p85, and the induction of tyrosine-phosphorylation following PDGF stimulation on these proteins, resulting in their utilization as adaptors to interact with each other. To investigate the nature of the association between SHP-1, the PDGF receptor, and p85, we used TRMP cells that stably express transfected wild type or mutant PDGF receptors lacking various tyrosine phosphorylation sites. As shown in Fig. 2*A*, p85 could not be co-immunoprecipitated with the receptor mutants utilized, either PDGFR-Y740F or PDGFR-Y751F. This confirms the finding that the association of p85 with the PDGF receptor is through both the phosphorylated Tyr^{740} and Tyr^{751} residues on the receptor (29, 30). However, mutation of these two binding sites on the receptor did not abolish the co-precipitation of SHP-1 with the mutant PDGF receptor (Y740F) (Fig. 2*B*). These results indicate that SHP-1 can directly associate with the PDGF receptor without the mediation of p85. The interaction of SHP-1 with p85 was also examined in the TRMP cells. Although p85 was not co-precipitated with the mutant PDGF receptors as shown in Fig. 2*A*, this protein could be co-precipitated with SHP-1 in TRMP cells expressing the mutant PDGF receptor (Fig. 2*C*). The amount of SHP-1 precipitated by p85 in cells expressing the mutant receptor was as high as that detected in cells expressing the wild type receptor (Fig. 2*C*). This result, taken together with those in Fig. 2, *A* and *B*, indicates that SHP-1 can associate with p85 without the binding of p85 to the PDGF receptor. Thus, SHP-1 likely associates directly with the PDGF receptor and p85, respectively. Further evidence for the direct association of SHP-1 with the PDGF receptor and p85 will be presented below.

Whether p85 can be tyrosine-phosphorylated as a result of activation of the PDGF receptor has not been clearly established. To demonstrate directly tyrosine phosphorylation of p85 in response to PDGF stimulation, p85 was immunoprecipitated with anti-p85 antibody. The immunoprecipitates were probed with anti-phosphotyrosine antibody. Fig. 2*D* shows that p85, as with the co-precipitated PDGF receptor, was tyrosine-phosphorylated in response to PDGF stimulation. A reciprocal experiment was also performed in which cell lysates were immunoprecipitated by anti-phosphotyrosine antibody and then examined with an anti-p85 antibody. Again, p85 was proved to be precipitated by anti-phosphotyrosine antibody (data not shown). Interestingly, in response to PDGF stimulation, p85 even can be tyrosine-phosphorylated in TRMP cells expressing only the mutant PDGFR-Y740F (Fig. 2*D*), suggesting that tyrosine phosphorylation of p85 does not require its binding to the receptor, although its association with the PDGF receptor increased the intensity of its tyrosine phosphorylation (Fig. 2*D*).

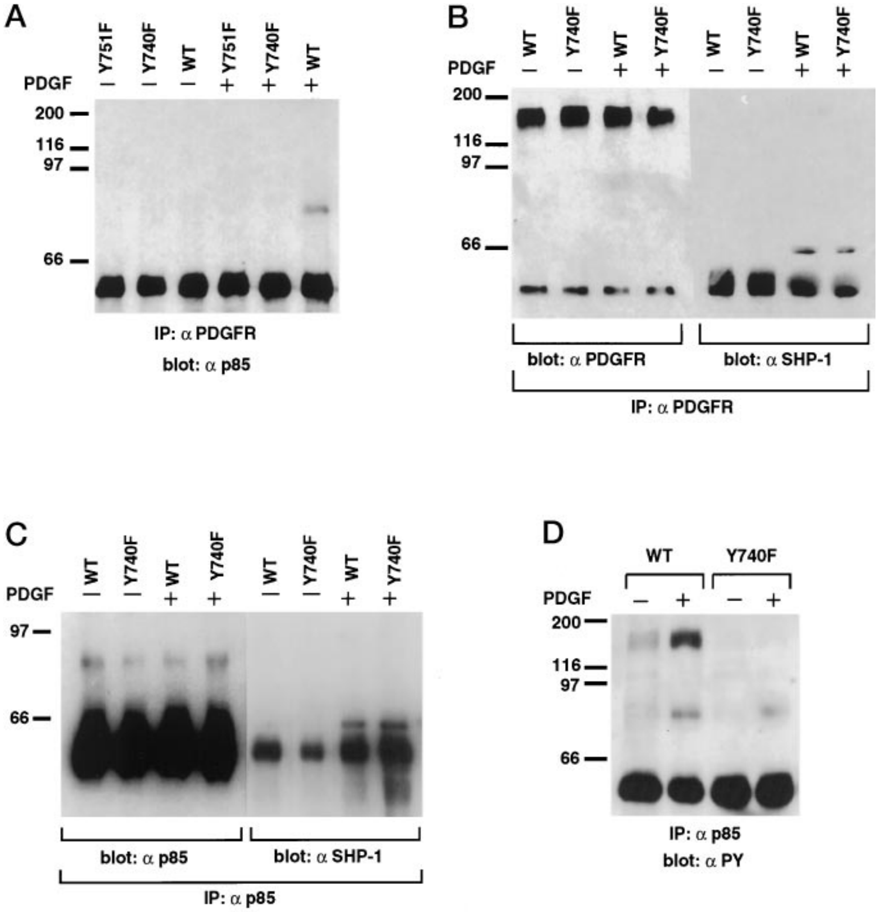

FIG. 2. **Effect of mutant PDGFR Y740F or Y751F on the association of SHP-1 with p85 and with the PDGF receptor.** TRMP cells expressing the wild type (*WT*) or the mutant PDGF receptor (*Y740F* or *Y751F*) were transiently transfected with SHP-1 by using standard calcium phosphate co-precipitation technique. Twenty four hours after transfection, the cells were starved by maintaining in serum-free medium for another 24 h and then left resting or stimulated for 5 min at 37 °C with 50 ng/ml PDGF. The cells were lysed, and the lysates were used for immunoprecipitation (*IP*) with anti-PDGF receptor (*A* and *B*) or anti-p85 (*C* and *D*) polyclonal antibody. The immunoprecipitates were subjected to electrophoresis on SDS-polyacrylamide gel and immunoblotted with anti-p85, anti-SHP-1, anti-PDGF receptor, or anti-phosphotyrosine monoclonal antibody as indicated. The positions of the protein molecular mass markers are indicated in kDa.

The SH2 Domain of SHP-1 Directly Binds to p85 and the PDGF Receptor—To investigate the mechanism of interaction between SHP-1 and the PDGF receptor and between SHP-1 and p85, *in vitro* binding experiments were performed. GST fusion protein containing the two SH2 domains of SHP-1 (GST-SHP-1 SH2) and GST alone were immobilized on glutathione-Sepharose beads, respectively. The beads were incubated with the lysates of unstimulated or PDGF-stimulated MCF-7 cells. The proteins precipitated by the immobilized proteins were analyzed by immunoblotting with anti-phosphotyrosine, anti-PDGF receptor, and anti-p85 antibodies, respectively. As shown in Fig. 3*A*, only two major bands were detected in the anti-phosphotyrosine immunoblots of the GST-SHP-1 SH2 precipitates from PDGF-stimulated cells but not from unstimulated cells. The much lower intensity band with a molecular mass of approximately 85 kDa was identified to be p85 by immunoblotting with anti-p85 antibody (Fig. 3*A*). The high intensity tyrosine-phosphorylated band with molecular mass of approximately 170 kDa was confirmed to be the PDGF receptor in immunoblotting with anti-PDGF receptor antibody (Fig. 3*A*). In the control experiments, no tyrosine-phosphorylated PDGF receptor and p85 were precipitated by the GST protein alone from either unstimulated or PDGF-stimulated cells (Fig. 3*A*). These results suggest that the association of SHP-1 with both the PDGF receptor and p85 is through the SH2 domains of SHP-1, and the association is dependent on the tyrosine phosphorylation of the receptor and the p85 protein subunit.

To confirm the direct association of p85 with SHP-1 as described above, we used far Western blot analysis. p85 immunoprecipitates were transferred to nitrocellulose, and the membranes were incubated with a fusion protein containing the SH2 domains of SHP-1 (GST-SHP-1 SH2) and then probed with anti-GST antibody. As shown in Fig. 3*B*, GST-SHP-1 SH2 directly bound to the p85 band immunoprecipitated from the PDGF-stimulated MCF-7 cells but not to that from the unstimulated cells. This experiment also revealed that a protein in the p85 immunoprecipitates with a molecular mass of approximately 180 kDa directly interacts with GST-SHP-1 SH2. This protein is likely the PDGF receptor co-precipitated by p85. In addition, we found that p85 was still co-precipitated with the mutant SHP-1 Y538F and SHP-1 Y543F (data not shown). Since Tyr^{538} and Tyr^{543} are the major tyrosine phosphorylation sites in SHP-1 (28, 35, 36), mutation of these sites should disturb potential binding of the SH2 domain of p85 to SHP-1.

Taken together, these results strongly suggest that p85 directly associates with SHP-1 through the binding of the p85 subunit to the SH2 domain of SHP-1, and not conversely through the SH2 domain of p85 with tyrosine-phosphorylated SHP-1. Since SHP-1 was detected independently to be associated solely with the PDGF receptor and p85, *i.e.* no other apparent tyrosine-phosphorylated proteins were detected in the *in vitro* binding experiment using GST fusion protein of the SH2 domain of SHP-1 (Fig. 3*A*), it is likely that SHP-1 also directly interacts with the PDGF receptor.

SHP-1 Associates with the PDGF Receptor and p85 via Its N-terminal SH2 Domain—Since SHP-1 contains two SH2 domains and each SH2 domain can bind specific tyrosine-phosphorylated proteins (17, 31), we were interested in assessing which SH2 domain of the enzyme is involved in the interaction with these two proteins. We therefore performed *in vivo* peptide competition experiments based on two tyrosine-phosphorylated peptides specifically binding to the individual SH2 domains of SHP-1. One phosphopeptide, EpoR Tyr(P)^{429}, contains the sequence surrounding Tyr^{429} in the erythropoietin receptor, which has been shown previously to bind to the N-terminal

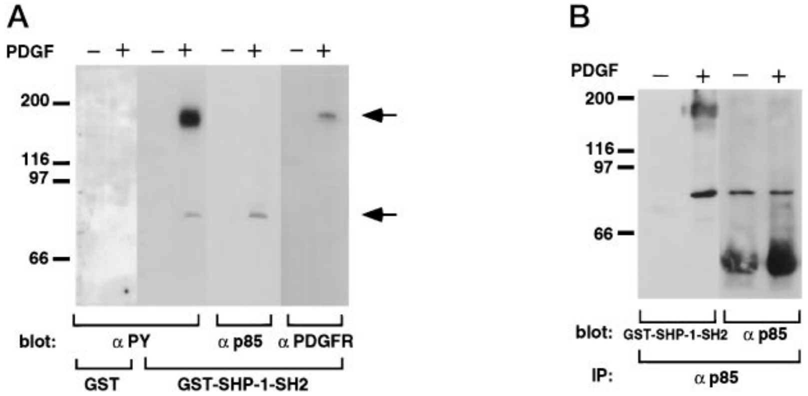

FIG. 3. **SHP-1 directly binds to p85 via the SH2 domains of SHP-1.** MCF-7 cells were left resting or stimulated for 5 min at 37 °C with 50 ng/ml PDGF following cell starvation for 24 h and then lysed. *A*, the lysates were incubated with GST or GST fusion protein containing the SH2 domains of SHP-1 (GST-SHP-1 SH2) for 2 h and washed with lysis buffer 4 times. The proteins in the complexes were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-phosphotyrosine, anti-p85, and anti-PDGF receptor monoclonal antibodies, respectively. *B*, p85 was immunoprecipitated (*IP*) from the cell lysates. The immunoprecipitates were used for far Western blot analysis with GST-SHP-1 SH2 fusion protein or blot with p85 monoclonal antibody as described under "Experimental Procedures." The positions of the protein molecular mass markers are indicated in kDa.

SH2 domain of SHP-1 (17). The other peptide, FcγRIIB1 Tyr(P)^{309}, contains the sequence surrounding Tyr^{309} in FcγRIIB1 ITIM motif. This tyrosine-phosphorylated peptide specifically binds to the C-terminal SH2 domain of SHP-1 (31). The other tyrosine-phosphorylated peptides used are listed in Table I, including PDGFR Tyr(P)^{1009} surrounding the SHP-2-binding site (32, 33) and PDGFR Tyr(P)^{771} surrounding the GTPase activating protein-binding site (29, 30). Three other peptides, Tyr(P)^{368}, Tyr(P)^{580}, and Tyr(P)^{607} designed from the three reported tyrosine-phosphorylated sites on p85 (34), were also included. MCF-7 cells stably overexpressing SHP-1 were permeabilized to allow entrance of phosphopeptides prior to stimulation with PDGF. As shown in Fig. 4*A*, the tyrosine-phosphorylated peptide EpoR Tyr(P)^{420} completely blocked the co-precipitation of SHP-1 with the PDGF receptor. The other three tyrosine-phosphorylated peptides, FcγRIIB1 Tyr(P)^{309}, PDGFR Tyr(P)^{1009}, and PDGFR Tyr(P)^{771}, did not significantly affect the co-precipitation. Similarly, the tyrosine-phosphorylated peptide EpoR Tyr(P)^{429} also effectively abolished the co-precipitation of SHP-1 with tyrosine-phosphorylated p85 (Fig. 4*B*), whereas the other four phosphorylated peptides, FcγRIIB1 Tyr(P)^{309}, Tyr(P)^{368}, Tyr(P)^{580}, and Tyr(P)^{607}, did not display any detectable effect on the co-immunoprecipitation. This experiment also shows that the association of SHP-1 with p85 is not very likely to occur through the binding of the SH2 domain of SHP-1 to any of the three known tyrosine phosphorylation sites previously identified on p85 following insulin stimulation (34). The effect of peptide EpoR Tyr(P)^{429} on blocking the co-precipitation of SHP1 with both the PDGF receptor and p85 is phosphorylation-dependent. The non-tyrosine-phosphorylated EpoR Tyr^{429} did not display its competition in the co-immunoprecipitations (Fig. 4*C*). These results clearly demonstrate that SHP-1 associates with both the PDGF receptor and p85 through its N-terminal SH2 domain.

TABLE I

The peptides used in in vivo peptide competition experiments

The peptides are named based on the parent proteins, and the phosphotyrosine position in these proteins is numbered accordingly in the peptides.

Peptide name (Tyr(P) position)	Sequence	Parent protein
PDGFR Tyr(P)^{771}	ADIESSNpYMAPYDNYVDS	PDGF receptor
PDGFR Tyr(P)^{1009}	LDTSSVLpYTAVQPNEG	PDGF receptor
EpoR Tyr(P)^{429}	PHIKpYLYLVVSDS	Epo receptor
EpoR Tyr^{429}	PHIKYLYLVVSDS	Epo receptor
FcγRIIB1 Tyr(P)^{309}	EAENTITpYSLLKH	FcγRIIB1
p85 Tyr(P)^{368}	MHGDpYTLTLRKGGNNK	p85
p85 Tyr(P)^{580}	TRDQpYLMWLTQKGVRQ	p85
p85 Tyr(P)^{607}	TEDQpYSLVEDDEDLPH	p85

Effect of Overexpressed SHP-1 on the PDGF-stimulated c-fos Promoter Activation—To investigate the role of SHP-1 in PDGF-activated signaling, we examined the effect of wild type and catalytically inactive mutants of SHP-1 on PDGF-induced activation of the c-*fos* promoter. The c-*fos* promoter contains a well characterized serum-responsive element (SRE) whose activity can be stimulated upon mitogenic activation of appropriate receptors. The plasmid construct containing the c-*fos* promoter-driven luciferase gene (SRE-Luc) was co-transfected with SHP-1 or its mutants, SHP-1 C455S, SHP-1 D421A, and SHP-1Δ-(455–461), into TRMP cells. It has been shown that all three mutants (SHP-1 C455S, SHP-1 D421A, and SHP-1Δ-(455–461)) have very little or no catalytic activity (37). The mutations in these phosphatases, however, do not affect the affinity of the SH2 domain binding to tyrosine-phosphorylated proteins, and additionally, mutant SHP-1 C455S and SHP-1 D421A retain their ability to interact with substrates (37). Accordingly, these mutants would be predicted to act as biochemically dominant negative modulators of SHP-1 *in vivo*. As shown in Fig. 5, the luciferase activity driven by SRE promoter was induced 2.9-fold following PDGF stimulation in the control TRMP cells (which were transfected by SRE-Luc and vector alone), whereas overexpression of wild type SHP-1 caused an induction of only 2.1-fold, resulting in a 30% reduction in the PDGF-stimulated luciferase activity. In contrast, expression of all three catalytically inactive mutants enhanced PDGF induction of the c-*fos* promoter-driven luciferase expression by approximately 20–45%. Moreover, the inhibitory effect of the overexpressed wild type SHP-1 on the PDGF-stimulated c-*fos* promoter-driven luciferase activity could be blocked by overexpressing the catalytically inactive SHP-1 (SHP-1 C455S) (data not shown). Taken together, these results suggest that SHP-1 negatively regulates PDGF receptor-mediated signaling in the SRE-controlled transcription.

DISCUSSION

As is the case with other receptor protein tyrosine kinases, upon binding of its ligand, the PDGF receptor is activated via dimerization, resulting in autophosphorylation of multiple tyrosine residues within its intracellular domain. The autophosphorylated PDGF receptor provides docking sites to recruit

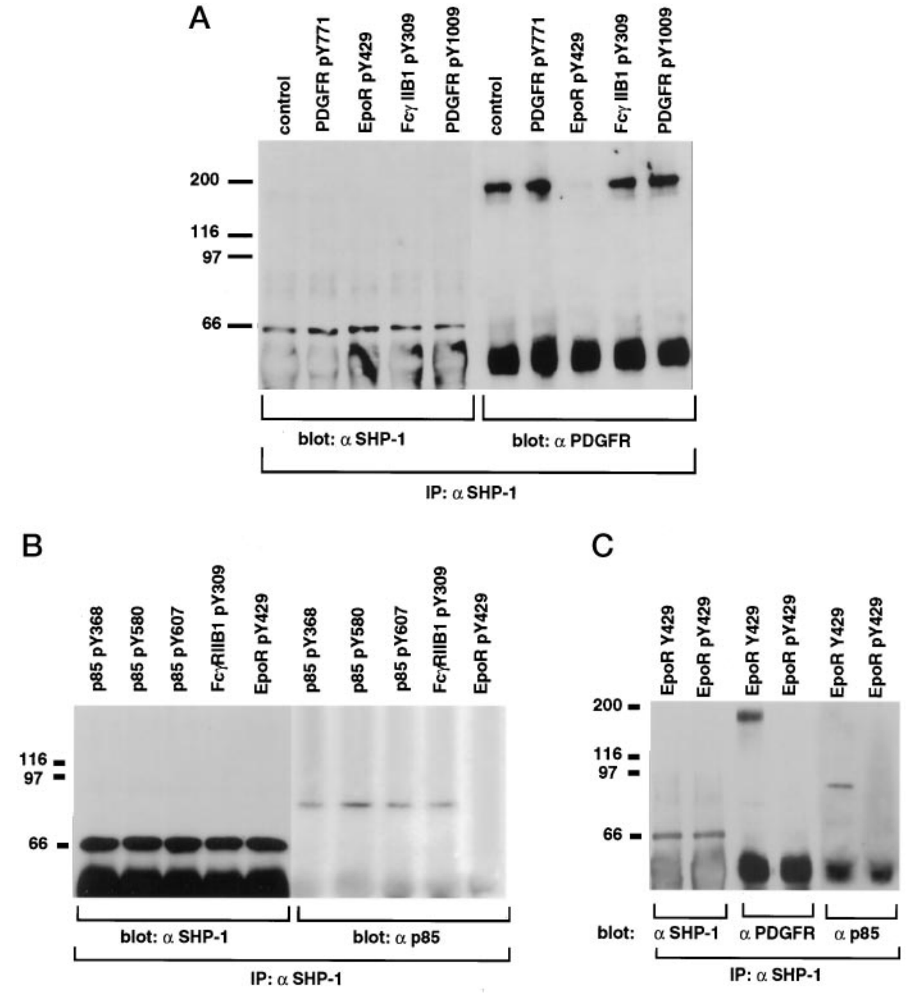

FIG. 4. **Association of SHP-1 with the PDGF receptor and p85 by its N-terminal SH2 domain.** MCF-7 cells expressing stably transfected SHP-1 were permeabilized with 1.4 mM tyrosine-phosphorylated or non-phosphorylated peptides or without peptide (*control*) as described under "Experimental Procedures" and then stimulated for 5 min at 37 °C with 50 ng/ml PDGF. The cells were lysed, and the lysates were used for immunoprecipitation (*IP*) with anti-SHP-1 polyclonal antibody. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-SHP-1 (*A–C*), anti-PDGF receptor (*A* and *C*), or anti-p85 (*B* and *C*) monoclonal antibody as indicated. The positions of the protein molecular mass standards are indicated in kDa.

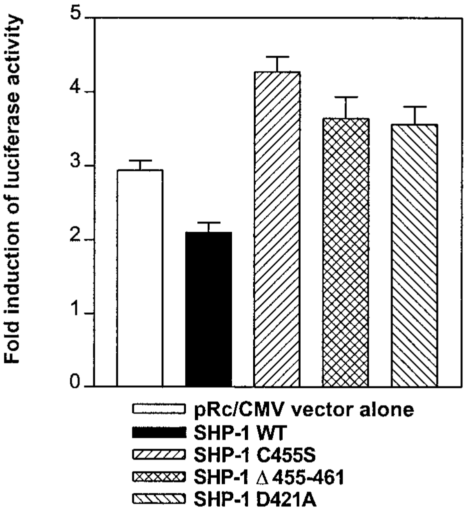

FIG. 5. **Effects of overexpression of wild type and catalytically inactive SHP-1 on PDGF-stimulated c-*fos* promoter activation.** TRMP cells with the stably expressed PDGF receptor were transiently transfected with 1 μg of SRE-Luc reporter plasmids plus 20 μg of individual expression vector of SHP-1 and its mutants. Cells were left resting or stimulated with 50 ng/ml PDGF, and luciferase activity was assayed as described under "Experimental Procedures." Luciferase activity was shown as the ratio of PDGF-stimulated to unstimulated samples. The data are presented as means + S.E. from three independent experiments each performed in triplicate. The constructs of SHP-1 and its mutants are described under "Experimental Procedures."

specific cellular SH2 domain-containing proteins, many of which have been identified, including the SH2 domain-containing PTP, SHP-2 (32, 33). In our immunoprecipitation experiments, we found that SHP-1, structurally related to SHP-2, also associated with the PDGF receptor in response to ligand stimulation in the non-hematopoietic MCF-7 and TRMP cell lines. Notably, the p85 subunit of PI 3-kinase was also co-precipitated with SHP-1. To address the nature of these interactions, we took the advantage of available TRMP cell lines transfected with mutant PDGFR Y740F and PDGFR Y751F which are unable to bind to p85. We demonstrated that although no p85 was associated with the mutant PDGF receptor, SHP-1 was still co-precipitated with the mutant PDGF receptor, suggesting that SHP-1 associates with the PDGF receptor without p85 mediation. Additionally, in the cells expressing only the mutant PDGF receptor, SHP-1 could associate with p85 without the binding of p85 to the PDGF receptor. These results suggest that SHP-1 independently associates with both the PDGF receptor and p85. The *in vitro* binding experiments further showed that SHP-1 associated with the PDGF receptor and p85 through its SH2 domains, whereas peptide competition experiments subsequently specified that the N-terminal domain of SHP-1 was responsible for both of these interactions. The binding sites for SHP-1 on both the PDGF receptor and p85, however, are presently unknown. We attempted to identify the binding sites by two approaches, phosphopeptide competition and using PDGF receptor mutants for co-immunoprecipitation. Experiments with phosphopeptide competition in co-precipitation (Fig. 4*A*) likely excluded the possibility that SHP-1 bound to Tyr^{1009}, a binding site for the structurally related SHP-2 (32, 33). The experiments also excluded the binding of SHP-1 to Tyr^{771}, a GTPase activating protein-binding site (29, 30). We have used PDGF receptor mutants PDGFR Y740F, Y751F, Y771F, Y1009F, and Y1021F in co-immunoprecipitation with SHP-1. All these mutations were unable to interfere with the co-precipitation of the receptors with SHP-1 (Fig. 2*B*),[2] suggesting that the SH2 domain of SHP-1 does not bind to these phosphorylation sites. Similarly, the SH2 domain of SHP-1 does not appear to bind to the reported phosphorylation sites of Tyr^{368}, Tyr^{580}, and Tyr^{607} in p85 (34) as the corresponding tyrosine-phosphorylated peptides failed to block the association of SHP-1 with p85 in co-immunoprecipitation (Fig. 4*B*).

The function of SHP-1 in PDGF receptor signaling was studied by assessing the effect of wild type SHP-1 and its dominant negative mutants on PDGF-induced c-*fos* promoter activation. In TRMP cells, the overexpression of wild type SHP-1 markedly inhibited the response of the c-*fos* promoter to PDGF stimulation, whereas the expression of biochemically dominant negative mutants increased the response. These results suggest that SHP-1 can negatively regulate PDGF receptor-mediated signaling at least in SRE-regulated transcription in TRMP cells. However, in other cell lines, particularly CCL39 cell, it was reported that overexpression of SHP-1 did not affect PDGF-induced DNA synthesis (38). It appears that, depending on the cell type, its compartmentalization, and its targeting molecules, SHP-1 could play different roles in growth factor-activated pathways, such as a negative role observed here in the PDGF-induced c-*fos* promoter activation, a positive role reported in EGF-stimulated pathway (15), or no apparent effect on PDGF-stimulated DNA synthesis (38) and on EGF-stimulated mitogen-activated protein kinase activation and Elk-1 transaction (39).

At present, the mechanism(s) by which SHP-1 positively or negatively regulates various growth factor-activated pathways is largely unknown. In our co-immunoprecipitation experiments, we observed that overexpression of wild-type SHP-1 substantially reduced the intensity of tyrosine phosphorylation on the PDGF receptor, whereas expression of the catalytically inactive mutant SHP-1 C455S dramatically increased the tyrosine phosphorylation of the receptor and p85. This result may suggest that SHP-1 targets the autophosphorylated PDGF receptor as its substrate and dephosphorylates the receptor on certain tyrosine residues, thus inhibiting the ligand-stimulated signaling, a mechanism similar to that found in cytokine receptor-mediated signalings where SHP1 negatively regulates these signal transduction pathways (16, 17). The intensity of tyrosine-phosphorylated p85 was also greatly increased in cells expressing catalytically inactive mutant SHP1 C455S. It is likely that SHP1 also targets p85 as a substrate. It is unknown whether dephosphorylation of p85 contributes the negative effect of SHP1 on the PDGF receptor relaying signaling.

Interestingly, SHP-2 can also positively or negatively regulate mitogen-stimulated pathways. The positive function of SHP-2 was reported in EGF, insulin, and PDGF-activated signal transductions (38–47). Likewise, it was also reported that SHP-2 had a negative role or was not required in PDGF receptor signaling (7, 39, 48–50). We also found that the dominant negative mutants of SHP-2 dramatically suppressed the EGF-stimulated signal pathway (44),[2] but it had no effect on the PDGF-activated mitogenesis in TRMP cells.[2] The seemingly conflicting reports on the function of SHP-1 and SHP-2 in growth factor receptor protein tyrosine kinase signaling are probably due to redundancy and convergence of multiple signals from the growth factor receptors and the existence of multiple substrates that may be differentially expressed and/or differentially regulated by these PTPs. Thus, both SHP-1 and SHP-2 may have diversified functions in complicated and multiple signaling pathways.

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SHP-1 Associates with Both Platelet-derived Growth Factor Receptor and the p85 Subunit of Phosphatidylinositol 3-Kinase

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