A Role for Src in Signal Relay by the Platelet-derived Growth Factor α Receptor*

Julie A. Gelderloos‡, Stephan Rosenkrantz‡, Chantal Bazenet†, and Andrius Kazlauskas§

From The Schepens Eye Research Institute, Harvard Medical School, Boston, Massachusetts 02114

Previous studies have shown that Src is required for platelet-derived growth factor (PDGF)-dependent cell cycle progression in fibroblasts. Since fibroblasts usually express both PDGF receptors (PDGFRs), these findings suggested that Src was mandatory for signal relay by both the α and βPDGFRs. In this study, we have focused on the role of Src in signal relay by the αPDGFR. In response to stimulation with PDGF-AA, which selectively engages the αPDGFR, Src family members (Src) associated with the αPDGFR and Src kinase were activated. A mutant receptor, in which tyrosines 572 and 574 were replaced with phenylalanine (F72/74), failed to efficiently associate with Src or activate Src. The wild type (WT) and F72/74 receptors induced the expression of c-myc and c-fos to comparable levels. Furthermore, an equivalent extent of PDGF-dependent soft agar growth was observed in cells expressing the WT or the F72/74 αPDGFR. Comparing the ability of these two receptors to initiate tyrosine phosphorylation of signaling molecules indicated that both receptors mediated phosphorylation of the receptor itself, phospholipase Cγ1, and SHP-2 to similar levels. In contrast, the F72/74 receptor triggered phosphorylation of Shc to 1 and 20% of the WT levels for the 55- and 46-kDa Shc isoforms, respectively. These findings indicate that after exposure of cells to PDGF-AA, Src stably associates with the αPDGFR, and Src activity is increased. Furthermore, Src is required for the PDGF-dependent phosphorylation of signaling molecules such as Shc. Finally, activation of Src during the G0/G1 transition does not appear to be required for latter cell cycle events such as induction of c-myc or cell proliferation.

Platelet-derived growth factor (PDGF)1 is a homo- and heterodimer consisting of two homologous chains, A and B. All possible isoforms exist and are biologically active. Like the ligand, the receptor is also a dimer that can exist as a homo or hetero combination of the α and β subunits of the PDGF receptor (PDGFR). Exposure of cells to PDGF assembles the monomeric receptor subunits into dimers, in which each of the subunits binds one of the chains of the dimeric ligand. The PDGFR subunits differ in their ability to bind to the two PDGF chains. The β subunit binds only the B chain with high affinity, whereas the α subunit binds both PDGF chains. Consequently, the PDGF isoform will determine the subunit composition of the PDGFR. PDGF-AA assembles only αα homodimers, PDGF-AB induces αα and αβ dimers, and PDGF-BB, the universal ligand, leads to formation of all possible PDGFR subunit dimers (1). In this work we will focus on the αPDGFR, which is activated by all forms of PDGF and can be selectively activated with PDGF-AA.

Once the PDGFR has been activated, it triggers a number of signaling cascades. Comparison of the signal relay pathways engaged by the α and βPDGFRs demonstrates that although there are many similarities, there are also a number of differences. For instance, the SH2 domain-containing phosphotyrosine phosphatase, SHP-2, binds to the kinase insert of the αPDGFR (2), but to the tail of the βPDGFR (3). Furthermore, although both of the receptors recruit and associate with phosphatidylinositol 3-kinase, phospholipase Cγ (PLCγ), and SHP-2, only the βPDGFR stably associates with GTPase-activating protein of Ras (RasGAP) (4, 5). In regard to which of these signaling molecules are required for sending a biological signal, reports to date have shown that the βPDGFR initiates multiple signal relay cascades and that at least some of the receptor-associated proteins are required for PDGF-dependent DNA synthesis (6, 7). In contrast, preventing the αPDGFR from individually associating with phosphatidylinositol 3-kinase, PLCγ, or SHP-2 does not severely impair the mitogenic signal of the receptor (2, 8–10). Thus, which if any of these recruited signaling molecules is required for mitogenic signal relay from the αPDGFR remains an open question.

A number of groups have investigated the role of Src in PDGF-dependent signal relay. The initial observations were that Src activity increased in a PDGF-stimulated cell (11, 12). Subsequent studies demonstrated that all three Src family members that are expressed in a fibroblast are activated in response to PDGF and associate with the βPDGFR (13). Binding of Src family members to the βPDGFR requires the presence of two tyrosine phosphorylation sites (579 and 581) in the βPDGFR juxtamembrane domain (14). The binding of Src to the receptor is dependent on the Src SH2 domains, and these events are thought to contribute to activation of Src in a PDGF-stimulated cell (14–16).

Recent studies have defined two Src-dependent stages in the cell cycle, mid/late G1, as well as G2/M. PDGF-BB-dependent entry into the S phase of the cell cycle was largely eliminated by microinjection of NIH 3T3 cells with either dominant negative forms of Src and Fyn or a neutralizing antibody that
inhibits the activity of Src, Fyn, and Yes (17). Progression to the S phase of the cell cycle could be blocked by microinjection of the reagents at the time of PDGF stimulation or even up to 6 h after stimulating with PDGF (17). Constitutive expression of c-myc, but not fos or jun, rescued the block induced by dominant negative Src (18), leading to the idea that Src contributes to PDGF mitogenic signaling by increasing c-myc expression. The observations that Src is activated during mitosis (19–21) suggested that Src is performing an important function during this stage of the cell cycle as well. Indeed, Roche et al. (22) have demonstrated that the activity of Src family kinases is essential for the G2 to M transition. Thus Src kinase activity is required at mid G1 and at the G2/M transition in order for PDGF-stimulated cells to traverse the cell cycle.

PDGF-dependent signaling is not unique in its requirement for the activity of Src kinases. A number of other signaling pathways, including those initiated by B and T cell receptors, cytokine receptors, integrins, as well as the receptors for epitope-specific antibodies, are involved in a variety of signaling systems, and it is important to precisely define what events Src is regulating.

Although Src is activated at both the G1/G0 transition and at the G2/M transition, the relative importance of Src activation at the early point in the cell cycle has not been thoroughly investigated. In addition, the role of Src in signaling by the αPDGFRI has not been addressed directly. In this study, our goal was to assess the importance of the initial increase in Src activity for signal relay by the αPDGFRI. Our approach was to generate a receptor mutant that was unable to activate Src and then to compare the ability of the WT and mutant receptors to mediate signal transduction events and drive cell proliferation.

In our system, the Src family members were not manipulated directly, and we have demonstrated that the activity of Src family kinases is essential for the G2 to M transition. Thus Src kinase activity is required at mid G1 and at the G2/M transition in order for PDGF-stimulated cells to traverse the cell cycle.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The mouse embryo 3T3 Patch B (PhB) cell line was derived from Ph/Ph mouse embryos and was kindly provided by Dr. Dan Bowen-Pope (24). PhB cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% calf serum. The human WT and F72/74 αPDGFRI constructs were subcloned into the pLNCX2 retroviral vector, which is a 3.5-kilobase pair vector. The αPDGFRI has a 3.5-kilobase pair EcoRI-HindIII fragment from resting or PDGF-stimulated cells, as described above, using the appropriate antibodies, except the first set of washes were with EB instead of radioligand precipitation buffer. Immunoprecipitates representing 1.5 × 10^6 cells were then subjected to an anti-phosphotyrosine Western blot analysis. In vitro Kinase Assay.—Src immunoprecipitates from approximately 1–2 × 10^6 cells were incubated with 1.6 μg of acid-denatured rabbit muscle enolase (Sigma), 20 μg PIPES (pH 7.0), 10 μg MnCl2, 20 μg/ml aprotinin, and 0.25 μg/ml SHP-2, at 30 °C in a total reaction volume of 20 μl. The reaction was stopped by adding 20 μl of 2.5% TCA buffer (10 mM EDTA, 4% SDS, 5.6 M urea, and 0.2% mercaptoethanol, 20% glycerol, 200 μM Thr-HCl (pH 6.8), 1% bromphenol blue). Kinase assays with the PDGFR were performed under identical conditions, except that 0.5 μg of glutathione S-transferase-PLCβ was used as the substrate SKII– and includes the entire αPDGFRI cDNA (2). Finally, the NotI-BamHI fragment from 18F was subcloned into the NotI-BamHI digested pLNCX2 retroviral vector.

**Antibodies**—The αPDGFRI antibodies were crude rabbit polyclonal antibodies raised against a glutathione S-transferase-fusion protein, including either the carboxyl terminus (amino acids 951–1089; 27P) or a portion of the first immunoglobulin domain (amino acids 52–94; 80.8) of the human αPDGFRI. The αPDGFRI antisera recognizes the mouse and human αPDGFRI and do not cross-react with the βPDGFRI of either species. The Src-2 antibody used for immunoprecipitation of Src family members was purchased from Santa Cruz and is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 509–533 of c-Src. This antibody recognizes all three of the Src family members that are expressed in fibroblasts, Src, Yes, and Fyn. The Src antibody used for Western blotting, 327, is a mouse monoclonal that is thought to recognize the SH3 domain of c-Src and v-Src (26) (Oncogene Science Inc.) and was used at a 1:1,000 dilution. The immunoprecipitating anti-phosphotyrosine antibody used in these studies was PY20, a monoclonal antibody purchased from Transduction Labs. For anti-phosphotyrosine Western blot analysis, a combination of PY20 (Transduction Labs) and 4G10 (Upstate Biotechnology, Inc.), each at a 1:1,000 dilution, was used. To immunoprecipitate PLCγ, we used a polyclonal antibody, 36.3, which was raised against a glutathione S-transferase-PLCγ1 fusion protein that included amino acids 550–850 of rat PLCγ (27). In Western blot analysis was performed using a mixture of monoclonal anti-PLCγ1 antibodies (Upstate) at a concentration of 0.25 μg/ml. SHP-2 was immunoprecipitated subjected to Western blot analysis as described previously (27). SHP-2 was immunoprecipitated and immunoblotted (using a dilution of 1:100) with an anti-Shc polyclonal antibody purchased from Upstate.

**Immunoprecipitation and Western Blot Analysis of Src**—PhB cells expressing the WT or F72/74 αPDGFRI were grown to confluence, incubated overnight in DME containing 0.1% CS, and were left resting or stimulated with 50 ng/ml PDGF-AA for 5 min. The cells were washed and lysed in EB (10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% bovine serum albumin, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, and 20 μg/ml aprotinin), and Src was immunoprecipitated using the Src-2 antibody. Immune complexes were bound to formalin-fixed Staphylococcus aureus membranes, spun through EB + 10% sucrose, washed twice with 1 μl of radioligand precipitation buffer (150 mM NaCl, 10 mM NaPO4 (pH 7.0), 2 mM EDTA, 1% sodium deoxycholate, 1% Nonidet-P40, 0.1% SDS, 20 μg/ml aprotinin, 50 mM NaF, 2 mM Na3VO4, 0.1% 2-mercaptoethanol, twice with 1.0 ml of PAN (10 mM PIPES (pH 7.0), 100 mM NaCl, 20 μg/ml aprotinin) + 0.5% Nonidet-P40) twice with 1.0 ml of PAN, and finally resuspended in PAN and stored at –70 °C. Immune complexes from 3 × 10^6 cells were resolved on a 10% SDS-polyacrylamide gel electrophoresis gel, and the proteins were transferred to Immobilon. The membranes were incubated for 1 h at room temperature in Blotto (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mg/ml nonfat dry milk, 0.05% Tween 20, 0.005% Na3PO4). The membranes were further incubated for 2 h at room temperature with primary antibody dilution in Blotto (the anti-αPDGFRI antibodies were a combination of 80.8 + 27P each at 1:1,000; the anti-Src antibody was the 327 monoclonal diluted 1:1,000). The membranes were then incubated with secondary antibody: a horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit diluted 1/2,000 in Blotto. Finally, ECL (Amersham Corp.) was used to develop the Western blot.

**Anti-phosphotyrosine Western Blot Analysis of Immunoprecipitated Proteins**—The αPDGFRI, PLCγ, and Shc were immunoprecipitated from resting or PDGF-stimulated cells, as described above, using the appropriate antibodies, except the first set of washes were with EB instead of radioligand precipitation buffer. Immunoprecipitates representing 1.5 × 10^6 cells were then subjected to an anti-phosphotyrosine Western blot. In vitro Kinase Assay.—Src immunoprecipitates from approximately 1–2 × 10^6 cells were incubated with 1.6 μg of acid-denatured rabbit muscle enolase (Sigma), 20 μg PIPES (pH 7.0), 10 μg MnCl2, 20 μg/ml aprotinin, and 0.25 μg of [γ-32P]ATP for 10 min at 30 °C in a total reaction volume of 20 μl. The reaction was stopped by adding 20 μl of 2.5% TCA buffer (10 mM EDTA, 4% SDS, 5.6 M urea, and 0.2% mercaptoethanol, 20% glycerol, 200 μM Thr-HCl (pH 6.8), 1% bromphenol blue). Kinase assays with the PDGFR were performed under identical conditions, except that 0.5 μg of glutathione S-transferase-PLCβ was used as the substrate SKII— and includes the entire αPDGFRI cDNA (2). Finally, the NotI-BamHI fragment from 18F was subcloned into the NotI-BamHI digested pLNCX2 retroviral vector.

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exogenous substrate instead of enolase. After the kinase assay, the samples were incubated for 4 min at 98 °C and spun at 8,000 rpm for 5 min, and the supernatant was resolved on a 10% SDS-polyacrylamide electrophoresis gel.

**Northern Blotting**—RNA was isolated from the PhB cells after lysis in guanidine isothiocyanate and centrifugation through a CsCl gradient (28). RNA (10 μg) was separated and electrophoresed to an agarose gel and electro-transferred to Nytran (Schleicher and Schuell). RNA was cross-linked to the filter with UV light using a Stratalinker (Stratagene). Hybridizations were performed at 45 °C in 50% formamide, 5× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate), 100 mM sodium phosphate, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin, 0.1% SDS, 250 μg/ml salmon sperm DNA. After hybridization, blots were washed twice at room temperature with 2× SSC, 0.5% SDS and three times at 45 °C with 0.1× SSC, 0.5% SDS. 32P-labeled probes were prepared with random primers using the Prime-it II kit (Stratagene) and (α-32P)dCTP. The following cDNAs were used as probes: a 548-base pair HindIII-XbaI fragment of pKS321 containing the human glyceraldehyde-3-phosphate dehydrogenase gene (29), a 1.5-kilobase pair EcorI-PstI fragment of murine c-fos/pGEM3 (30), a 400-base pair PstI fragment of murine c-myc that includes exon 2.

**RESULTS**

**Characterization of the F72/74 αPDGFR**—To determine whether Src family members (hereafter collectively referred to as Src) are involved in αPDGFR signaling relay, we tested if Src is activated after engagement of the αPDGFR. Our preliminary experiments (data not shown; see Figs. 2 and 3 below) indicated that αPDGFR is indeed able to activate Src; we next wanted to generate an αPDGFR mutant that failed to stimulate the kinase activity of Src. For the βPDGFR, phosphorylation of tyrosines 579 and 581 in the juxtamembrane domain enables Src to associate with the receptor and also appears to activate the kinase activity of Src (14). Since these two tyrosines, as well as the flanking sequences are identical in the α and βPDGFRs, we reasoned that mutating them in the αPDGFR would generate a mutant αPDGFR that would be unable to activate or bind Src. To test this hypothesis, we mutated tyrosine 572 and 574 to phenylalanine residues, and the resulting receptor was expressed in Ph cells, a fibroblast cell line that expresses the βPDGFR, but not the αPDGFR.

To analyze the expression level of the introduced αPDGFR, lysates were prepared from resting or PDG-stimulated cells, and the samples were subjected to an anti-αPDGFR Western blot. As shown in Fig. 1A, the WT and F72/74 cells expressed comparable levels of the introduced receptor, which we have previously estimated to be similar to the level of the endogenous βPDGFR (4). Next we compared the kinase activity of the WT and F72/74 receptors. Ph cells expressing the WT αPDGFR or the F72/74 mutant were arrested by serum deprivation then left resting or exposed to PDGF-AA; finally, the cells were lysed, and the αPDGFR was immunoprecipitated. The kinase activity of the resulting immunoprecipitates was compared by testing their ability to phosphorylate an exogenous substrate. When the WT αPDGFR was isolated from PDG-stimulated cells, it phosphorylated the exogenous substrate 5.9 times more than the WT receptor isolated from resting cells (Fig. 1B). The kinase activity of the F72/74 αPDGFR was also activated by PDGF, and the extent of activation was comparable to the WT receptor. Western blot analysis of the immunoprecipitates demonstrated that there was a similar amount of receptor present in all of the samples (Fig. 1C). No differences were observed in the kinase activity of the WT and F72/74 receptors when they were compared using additional exogenous substrates (data not shown). These data indicate that mutation of tyrosines 572 and 574 did not impair the kinase activity of the αPDGFR.

We next tested if Src associates with the αPDGFR and whether mutating tyrosines 572 and 574 had any effect on this association. Cells were exposed to buffer or PDG-AA for 5 min, Src was immunoprecipitated, and the samples were sub-
The αPDGFR Needs Src to Efficiently Phosphorylate Shc

![Image](https://via.placeholder.com/150)

**FIG. 2.** Mutation of tyrosines 572 and 574 disrupts association of Src with the αPDGFR. PhB cells expressing either the WT or mutated (F72/74) αPDGFR were grown to 80% confluence, starved for 18 h in serum-free DME, and then left resting (−) or exposed for 5 min to 35 ng/ml PDGF-AA. The cells were lysed, and the lysates were immunoprecipitated with an antisemur that recognizes Src family members (Src-2; Santa Cruz). Immunoprecipitates (IP) were then immunoblotted with anti-αPDGFR antiserum (80.8 kDa) to detect coimmunoprecipitation of the αPDGFR (A), or with anti-Src (327) (B) to determine the amount of Src in each of the samples.

jected to anti-αPDGFR or anti-Src Western blot analysis. Blotting of the Src immunoprecipitates with an αPDGFR antibody demonstrated that the WT αPDGFR coimmunoprecipitated with Src, provided that the cells had been stimulated with PDGF (Fig. 2A). In contrast, only trace amounts (approximately 1% of that seen with the WT receptor) of the F72/74 receptor was observed coprecipitating with Src from activated cells. An anti-Src Western blot of these Src immunoprecipitates indicated that equal amounts of Src were present in all of the samples (Fig. 2B). These data indicated that the αPDGFR does indeed associate with Src, and furthermore, that tyrosine 572 and/or 574 is required for this event.

**Activation of Src—Stimulation of quiescent fibroblasts with PDGF-BB has been shown to increase the kinase activity of Src, Fyn, and Yes by 2–5-fold (13). Since PDGF-BB activates both α and βPDGFRs, which are expressed in most fibroblasts, these studies did not distinguish the contribution of the two receptors to the Src activation event. Mori et al. (14) clearly demonstrated that the βPDGFR can activate Src; thus we were interested in learning whether the αPDGFR also activates Src and if so, whether association of Src with the receptor was required for this event. Src was immunoprecipitated from WT and F72/74 αPDGFR expressing PhB cells that were either left resting or stimulated with 35 ng/ml PDGF-AA for 5, 15, 30, 60, or 120 min. The kinase activity of the immunoprecipitated Src was measured in an in vitro kinase assay, using enolase as an exogenous substrate (Fig. 3A). PDGF-AA stimulation of WT cells for 5 min resulted in a 1.8-fold increase in the in vitro phosphorylation of enolase, which was 1.9-fold after 15 min and peaked at 30 min with a 2.2-fold increase. After 60 min, enolase phosphorylation began to decrease with only a 1.6-fold and 1.2-fold increase observed at 60 and 120 min, respectively. In Src immunoprecipitates from F72/74 cells, enolase phosphorylation did not increase after 5 min and was only slightly increased (1.2-fold) after 15 min of stimulation. After 30 min, enolase phosphorylation decreased below the level seen before PDGF stimulation. Western blotting the Src immunoprecipitates with an anti-Src antibody indicated that comparable amounts of Src were present in all of the samples (Fig. 3B). These experiments demonstrate that engagement of the αPDGFR leads to activation of Src. Furthermore, the F72/74 receptor, which associates with Src poorly, is unable to efficiently activate Src, suggesting that binding of Src to the αPDGFR is required for maximal PDGF-dependent activation of Src kinase activity.

**Induction of Immediate Early Genes—**Previous studies have linked PDGF-BB-dependent Src activation in NIH 3T3 cells with induction of c-myc but not fos or jun (18). We were curious whether the αPDGFR was able to trigger an increase in the c-myc mRNA level and if this event required activation of Src. To this end, RNA was harvested from resting cells and cells treated with 35 ng/ml PDGF-AA for 20–120 min. In both WT and F72/74 cells, PDGF-AA increased transcription of c-myc (Fig. 4). The fos mRNA level also rose in both WT and F72/74 cells after PDGF-AA treatment (Fig. 4). In both cell types, the amount of fos mRNA decreased slightly after 20 min and then increased at the 60 and 120 min time points. Equal loading of RNA was confirmed by probing with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Cell Proliferation—**We also evaluated the importance of the increase in Src activity in PDGF-stimulated cells for PDGF-dependent cell proliferation. Ph cells expressing an empty vector...
or the WT or F72/74 αPDGFR were plated in soft agar supplemented with PDGF or buffer. After 10 days, the cultures were photographed, and the number of colonies was counted (Fig. 5). Although this assay is a commonly used indication of cell transformation, since colony formation requires cell proliferation, we have used it here to evaluate PDGF-dependent cell proliferation. All three cell types were able to form a limited number of small colonies in the absence of PDGF, and this background level of colony formation was comparable in all of the cell lines. Adding PDGF-AA, which selectively activates the αPDGFR, had only a marginal effect on the empty vector expressing cells, whereas PDGF-BB, which activates the endogenous βPDGFR, triggered a 9.5-fold increase in colony formation. Activation of the αPDGFR with PDGF-AA stimulated a 27.5-fold increase in colony number for the WT receptor-expressing cells and a 25.5-fold increase in the F72/74 receptor-expressing cells. In addition, the colonies that grew in the presence of PDGF-AA were of similar size and morphology for the WT and F72/74 cells. Finally, PDGF-BB was also able to drive colony formation in the cells expressing the WT and F72/74 receptors, and the colony number and morphology was comparable. A likely reason that PDGF-BB (the universal ligand) stimulated more colonies in the WT and F72/74 cells as compared with the vector-expressing cells is that the WT and F72/74 cells have two forms of PDGFRs, whereas the vector-expressing cells have only a single receptor, the endogenous βPDGFR. These data demonstrate that the F72/74 receptor is able to drive growth of cells in soft agar to a comparable extent as the WT αPDGFR and thus suggest that Src activation during the G0/G1 transition is not required for PDGF-AA-dependent cell growth.

Tyrosine Phosphorylation of Proteins—The observation that activation of Src during the first 2 h of PDGF-AA stimulation is not mandatory for driving the mitogenic signal of the αPDGFR raises the question of the nature of the role of Src in αPDGFR signaling. Hence, we tested the hypothesis that Src is required for PDGF-dependent tyrosine phosphorylation of proteins. To this end, Ph cells expressing the empty vector, WT, or F72/74 constructs were grown to 85–90% confluence, arrested by serum starvation, and then left resting or stimulated with 50 ng/ml PDGF AA for 5 min. The cells were washed and lysed, and several different proteins were immunoprecipitated and subjected to anti-phosphotyrosine Western blot analysis (Fig. 6). To check the levels of immunoprecipitated protein in the samples, the anti-phosphotyrosine blots were stripped and then reprobed with the immunoprecipitating antibody (Fig. 6, lower panel of each pair). Stimulation of the empty vector-expressing cells did not increase the level of phosphotyrosine in any of the proteins. Upon stimulation of the WT receptor-expressing cells with PDGF AA, PLCγ, Shc, and SHP-2 were robustly tyrosine-phosphorylated. Stimulation of cells expressing the F72/74 receptor increased tyrosine phosphorylation of PLCγ and SHP-2, and the extent of phosphorylation was comparable to that seen in the WT receptor-expressing cells (Fig. 6, lower panel of each pair). Stimulation of the empty vector-expressing cells did not increase the level of phosphotyrosine in any of the proteins. Upon stimulation of the WT receptor-expressing cells with PDGF AA, PLCγ, Shc, and SHP-2 were robustly tyrosine-phosphorylated. Stimulation of cells expressing the F72/74 receptor increased tyrosine phosphorylation of PLCγ and SHP-2, and the extent of phosphorylation was comparable to that seen in the WT receptor-expressing cells (Fig. 6, lower panel of each pair). Stimulation of the empty vector-expressing cells did not increase the level of phosphotyrosine in any of the proteins. Upon stimulation of the WT receptor-expressing cells with PDGF AA, PLCγ, Shc, and SHP-2 were robustly tyrosine-phosphorylated. Stimulation of cells expressing the F72/74 receptor increased tyrosine phosphorylation of PLCγ and SHP-2, and the extent of phosphorylation was comparable to that seen in the WT receptor-expressing cells (Fig. 6, lower panel of each pair). Stimulation of the empty vector-expressing cells did not increase the level of phosphotyrosine in any of the proteins. Upon stimulation of the WT receptor-expressing cells with PDGF AA, PLCγ, Shc, and SHP-2 were robustly tyrosine-phosphorylated. Stimulation of cells expressing the F72/74 receptor increased tyrosine phosphorylation of PLCγ and SHP-2, and the extent of phosphorylation was comparable to that seen in the WT receptor-expressing cells (Fig. 6, lower panel of each pair).
We found that when the receptor mutants were left resting (−) or stimulated (+) with 50 ng/ml PDGF AA. The cells were lysed and immunoprecipitated with antibodies against PLCγ (A), SHP-2 (B), Shc (C), or PDGFR (D). The immunoprecipitates representing approximately 1.5 x 10⁶ cells were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon, and anti-phosphotyrosine Western blot analyses were performed (top panel). The Western blots were then stripped and re-probed with antibodies against the protein immunoprecipitated (bottom panel) to determine the levels of protein present in the immunoprecipitates. The signal in the top left corner of the upper panel of B is the molecular weight marker that nonspecifically absorbs the primary and/or secondary antibody during the Western blot analysis. The molecular weights of the three Shc isoforms are indicated in the left-hand margin of panel C. CX², pLNCC."}

initiated comparable phosphorylation of both receptors (Fig. 6D). These data demonstrate that tyrosine phosphorylation of most proteins in a PDGF-AA-stimulated cell do not require Src, and also, that some of the proteins, such as Shc, are not phosphorylated efficiently when Src is not activated. These findings suggest that the αPDGFR is not able to phosphorylate all of the proteins that are often thought of as substrates of receptor tyrosine kinases and that other kinases such as Src contribute to the overall level of substrate phosphorylation in an acutely stimulated cell.

DISCUSSION

The present study reveals that in response to stimulation with PDGF-AA, the αPDGFR associates with and activates Src and that these events are dependent on tyrosines 572 and 574. Furthermore, it sheds light on two aspects of the signal relay pathway downstream of the αPDGFR: 1) the mechanism by which proteins are tyrosine-phosphorylated in a PDGF-stimulated cell, and 2) the importance of the initial increase of Src which proteins are tyrosine-phosphorylated in a PDGF-stimulated cell. Shc is somewhat unique in its dependence on Src activity to be efficiently tyrosine-phosphorylated, as the receptor itself, PLCγ, and SHP-2 were all comparably phosphorylated in PDGF-stimulated cells expressing the WT or F72/74 receptors (Fig. 6). To see if there were additional proteins that were poorly phosphorylated in the F72/74 receptor-expressing cells, we performed antiphosphotyrosine Western blots on antiphosphotyrosine immunoprecipitates from resting and PDGF-AA-stimulated cells. There were numerous proteins detected in this assay, and we did not see differences in the extent of phosphorylation in any of them from the WT versus F72/74 receptor-expressing cells. Although this assay does not detect all tyrosine-phosphorylated proteins in a cell (for instance, we did not see Shc by this approach), it does suggest that Src is required for phosphorylation of only a small subset of the proteins that are tyrosine-phosphorylated in a PDGF-AA-activated cell. Thus, although the αPDGFR, or perhaps some other kinase, phosphorylates many of the proteins that associate with the αPDGFR, a subset of the signaling molecules requires Src for efficient tyrosine phosphorylation.

It is not obvious to us why Shc differs from the other proteins that become tyrosine-phosphorylated in a PDGF-AA-stimulated cell. One possibility is that Shc has a special role in signaling by the αPDGFR and is subject to additional layers of regulation. For instance, Shc may be involved with Ras activation, as appears to be the case in other signaling systems. As compared with the βPDGFR, the αPDGFR associates poorly with putative regulators of the Ras pathway such as Grb2 and the GTPase activating protein of Ras (2, 4, 5). We are currently investigating the role of Shc and Src in regulating Ras activation in PDGF-AA-stimulated cells.

We found that Src plays a similar role in PDGF-dependent phosphorylation of proteins in a PDGF-AA-stimulated cell. She was somewhat unique in its dependence on Src activity to be efficiently tyrosine-phosphorylated, as the receptor itself, PLCγ, and SHP-2 were all comparably phosphorylated in PDGF-stimulated cells expressing the WT or F72/74 receptors (Fig. 6). To see if there were additional proteins that were poorly phosphorylated in the F72/74 receptor-expressing cells, we performed antiphosphotyrosine Western blots on antiphosphotyrosine immunoprecipitates from resting and PDGF-AA-stimulated cells. There were numerous proteins detected in this assay, and we did not see differences in the extent of phosphorylation in any of them from the WT versus F72/74 receptor-expressing cells. Although this assay does not detect all tyrosine-phosphorylated proteins in a cell (for instance, we did not see Shc by this approach), it does suggest that Src is required for phosphorylation of only a small subset of the proteins that are tyrosine-phosphorylated in a PDGF-AA-activated cell. Thus, although the αPDGFR, or perhaps some other kinase, phosphorylates many of the proteins that associate with the αPDGFR, a subset of the signaling molecules requires Src for efficient tyrosine phosphorylation.

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phosphorylation of proteins in Ph cells expressing a chimeric \( \beta \)PDGFR. This chimera consists of the extracellular, transmembrane, and juxtamembrane domains of the \( \alpha \)PDGFR, and the kinase, kinase insert, and tail are all \( \beta \)PDGFR. Using this chimera as a model for signaling by the \( \beta \)PDGFR, we found that Src appears to be required for efficient phosphorylation of not only Shc, but also for PLC\( \gamma \), the GTPase activating protein of Ras, and SHP-2.\(^4,5\) At the present time we do not know if Src is directly phosphorylating these proteins in PDGF-stimulated cells or if Src is activating additional kinases to perform this function. Support for the former possibility comes from the observation that Shc is phosphorylated soon after cells expressing a temperature-sensitive v-Src are shifted to the permissive temperature (33).

What is the physiological relevance of Src contributing to phosphorylation of cellular substrates in a PDGF-stimulated cell? It does not appear to be an absolute requirement for cell growth, since the F72/74 receptor-expressing cells grow in soft agar as well as the WT receptor-expressing cells. An intriguing possibility is that Src changes the nature of the signal and thereby alters the type of biological response initiated by the growth factor. Alternatively, Src may amplify the signal of the receptor and thereby enhances a given biological response such as proliferation. The observation that expression of \( \gamma \)-Src or overexpression of c-Src potentiates epidermal growth factor-dependent transformation of cells suggests that such a situation exists for Src and the epidermal growth factor receptor (34–37).

**Role of Src in Cell Cycle Progression—Hooshmand-Rad et al.**\(^38\) have identified tyrosine 572 and 574 as phosphorylation sites, and in agreement with our results, find that mutating these two tyrosine residues largely eliminates PDGF-AA-mediated Src activation but does not prevent DNA synthesis when the receptors are expressed in pig aortic endothelial cells. Yet published work from several other groups indicates that Src is required for PDGF-dependent cell cycle progression. Microinjection of reagents that neutralize Src function blocked PDGFB-induced entry of NIH 3T3 cells into S phase (17). In addition, kinase-inactive forms of Src were able to inhibit PDGFB-dependent DNA synthesis in cells derived from mice that have the Src gene knocked out (39). One explanation for the lack of consensus regarding the apparent importance of Src in PDGF-mediated signaling is that the \( \alpha \)PDGFR may not require Src for signaling, as is the \( \beta \)PDGFR. However, the above-cited studies used fibroblasts, which usually express both the \( \alpha \) and \( \beta \)PDGFRs. Furthermore, PDGFB was used, and this form of PDGF activates both of the PDGFRs. Consequently, the \( \alpha \)PDGFR should have been able to signal in these cells, provided that it was expressed. We have recently tested the NIH 3T3 (NIH 3T3 c17) cells used in the above-mentioned microinjection studies and found that these NIH 3T3 cells do not express detectable levels of the \( \alpha \)PDGFR but do express high levels of the \( \beta \)PDGFR as compared with the Ph cells used herein. Thus PDGFB-primarily initiates the \( \beta \)PDGFR in the NIH 3T3 c17 cells, and the microinjection experiments do not address the issue of the contribution of Src to the \( \beta \)PDGFR signal relay. Yet this does not provide a satisfying resolution of the discrepancy between the various groups, because the signaling by numerous distinct receptor tyrosine kinases was greatly suppressed when reagents that eliminate Src activity were microinjected into cells (40). As a result, it seems likely that the signaling by the \( \alpha \)PDGFR would be blocked as well.

An explanation for how the \( \alpha \)PDGFR promotes cell proliferation in the absence of robust Src activation is that c-myc is still activated, an event that has been shown to rescue PDGF-dependent cell cycle progression when Src is blocked (18). This leads to the obvious question of how the F72/74 \( \alpha \)PDGFR is activating c-myc? One possibility is that the endogenous \( \beta \)PDGFR assists the F72/74 receptor. This does not seem likely because PDGFA does not activate the PDGFR, and numerous attempts to detect cross-talk between ligand-occupied and unoccupied PDGFRs have not provided any evidence that this occurs (4, 41). A second possibility is that the marginal activation of Src by the F72/74 is sufficient to activate c-myc. Alternatively, there may by multiple pathways initiated by the \( \alpha \)PDGFR that culminate in activation of c-myc. For instance, the F72/74 \( \alpha \)PDGFR receptor is a good kinase (Fig. 1) and is able to recruit and tyrosine-phosphorylate signaling enzymes such as PLC\( \gamma \) (Fig. 6, data not shown, and Ref. 38). PLC\( \gamma \) is involved with activation of protein kinase C family members as well as phospholipase D (42), which produce activators of the protein kinase C family, and activation of protein kinase C has been shown to induce c-myc expression in fibroblasts (31, 32). So it seems plausible that the \( \alpha \)PDGFR is engaging multiple signaling pathways leading to c-myc activation and, as a result the F72/74, is still capable of activating c-myc as well as other signaling events required for triggering cell proliferation.

An additional issue to consider is the fundamental differences in the approaches employed by the different groups studying the role of Src in PDGF-dependent signaling. Microinjection of reagents that suppress Src activity allegedly eliminates Src activity through all stages of the cell cycle because the half-life of the injected reagents is thought to be long. In contrast, using receptor mutants that fail to activate Src abolates only the initial burst of Src activity and leaves Src available at latter stages in the cell cycle. Thus an interpretation that reconciles most of the published observations is that Src makes contributions to cell cycle progression at multiple stages in the cell cycle. Furthermore, although Src activity appears to be absolutely required for progression through G1/S and G2/M, the PDGF-dependent increase in Src activity is largely dispensable for G1/S/G2 transition.

In summary, stimulating a cell via the \( \alpha \)PDGFR results in an increase in kinase activity of Src. This initial burst of Src activity is required for efficient tyrosine phosphorylation of an apparently small subset of the signaling molecules that are phosphorylated in an activated cell. In contrast, later events, such as activation of c-myc and cell proliferation do not require an increase in Src activity during the G1/S/G2 transition. Taken together with the work of other groups, it appears that Src is involved in multiple stages of the cell cycle and that at least one of its roles during the G1/S/G2 transition is to enhance phosphorylation of selected signaling molecules.

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