Identification of the Receptor-associated Signaling Enzymes That Are Required for Platelet-derived Growth Factor-AA-dependent Chemotaxis and DNA Synthesis*

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Activation of the platelet-derived growth factor (PDGF) α receptor (αPDGFR) leads to cell migration and DNA synthesis. These events are preceded by the ligand-induced tyrosine phosphorylation of the receptor and its association with SH2-containing signaling enzymes including Src family members (Src), the phosphotyrosine phosphatase SHP-2, phosphatidylinositol 3-kinase (PI3K), and phospholipase C-γ1 (PLCγ). In this study, we sought to systematically evaluate the relative roles of the signaling enzymes that are recruited to the αPDGFR for DNA synthesis and cell migration. Our approach was to generate and characterize tyrosine to phenylalanine αPDGFR mutants that failed to associate with one or more of the above listed signaling enzymes. In a 3T3-like cell line (Ph cells), PDGF-dependent DNA synthesis was strictly dependent on only one of the receptor-associated proteins, PI3K. In contrast, multiple signaling enzymes were required for maximal chemotaxis, as receptors unable to associate with either Src, PI3K, or PLCγ initiated chemotaxis to 4, 47, or 56% of the wild-type level, respectively. Furthermore, coexpression of mutant receptors revealed that these signaling enzymes do not need to be on the same receptor for a cell to respond chemotactically to PDGF. We conclude that for the αPDGFR, PI3K plays a major role in initiating DNA synthesis, whereas PI3K, PLCγ, and especially Src are required for chemotaxis.

Receptor tyrosine kinases elicit responses such as cell proliferation and migration via binding and activation of Src homology 2 (SH2) domain-containing signaling molecules. Upon ligand binding, receptor tyrosine kinases dimerize and autophosphorylate, and the phosphorylated tyrosines serve as a key component of the docking sites for SH2 domain-containing signal relay enzymes. There are at least several different ways in which signaling enzymes associate with a tyrosine-phosphorylated receptor tyrosine kinase. For the epidermal growth factor receptor, each of the signaling enzymes appears to bind to any one of the phosphorylation sites. The hepatocyte growth factor receptor contains a pair of tyrosine phosphorylation sites, which are required for stable recruitment of at least four signaling enzymes. In contrast, at least some of the signaling enzymes that associate with the βPDGFR and the fibroblast growth factor receptor have specific binding sites (1).

Activation of the αPDGFR results in its phosphorylation at numerous tyrosine residues, and eight such phosphorylation sites have been identified (reviewed in Ref. 2). One of the consequences of phosphorylation of the αPDGFR is the selective recruitment of SH2-containing signaling enzymes such as Src family members (Src), the phosphotyrosine phosphatase SHP-2, phosphatidylinositol 3-kinase (PI3K), and phospholipase C-γ1 (PLCγ).

Recent studies have shown that some of the αPDGFR-associated proteins are not essential for biological responses such as cell cycle progression. Src family members and SHP-2 are not required for cell proliferation or DNA synthesis (3–5). Interestingly, although PI3K and PLCγ are required for PDGF-dependent DNA synthesis initiated by the βPDGFR (6, 7), preventing the αPDGFR from individually associating with these signaling enzymes does not severely impair the mitogenic signal of the receptor (8–10). Thus, which, if any, of the recruited signaling molecules is required for mitogenic signal relay from the αPDGFR remains an open question.

A number of groups (9, 11–12) have investigated PDGF-dependent cell migration and have found that PDGF-mediated chemotaxis appears to be largely cell type-specific. Engagement of the αPDGFR promotes chemotaxis in some cell types such as lung fibroblasts, Swiss 3T3 cells, and hematopoietic 32D cells. In other cell types (foreskin fibroblasts, aortic endothelial cells, and vascular smooth muscle cells), PDGF-AA does not promote chemotaxis, but it inhibits the chemotactic response induced by other agents (13–15). In regard to which of the signaling molecules are involved with PDGF-dependent chemotaxis, PI3K was absolutely required for PDGF-AA-induced migration of NIH3T3 cells; however, it was not required for αPDGFR-mediated chemotaxis of hematopoietic 32D cells (9, 16). Finally, in transfected porcine aortic endothelial cells, Src family members did not contribute to αPDGFR-mediated chemotaxis (4).

These data indicate that PDGF-AA-dependent responses such as cell cycle progression and chemotaxis are highly dependent on the cell type, and the mechanisms by which the αPDGFR relays a biological signal remain poorly understood. Therefore, we sought to evaluate systematically the role of each PDGF family member in PDGF-mediated chemotaxis.

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† The abbreviations used are: SH2, Src homology 2; PDGF, platelet-derived growth factor; αPDGFR, PDGF α receptor; PI3K, phosphatidylinositol 3-kinase; PLCγ, phospholipase C-γ1; WT, wild type; DME, Dulbecco’s modified Eagle’s; PAGE, polyacrylamide gel electrophoresis; Erk, extracellular signal-regulated kinase.
of the signaling enzymes that are recruited to the activated αPDGFR for PDGFR-dependent DNA synthesis and chemotaxis in a single cell type. Our approach was to generate and characterize tyrosine to phenylalanine αPDGFR mutants that failed to associate with one or more of the signaling enzymes and then to compare the ability of the WT and mutant receptors to mediate signal transduction events and biological responses. We found that PDGFAA-induced DNA synthesis required activation of only one signal relay enzyme, PI3K. In contrast, multiple signaling molecules including Src, PI3K, and PLCγ contributed to αPDGFR-mediated chemotaxis. Furthermore, these three signaling enzymes did not necessarily need to be recruited to the same receptor.

MATERIALS AND METHODS

Cell Lines—Ph cells and their maintenance were previously described (3, 17). The human WT and mutant αPDGFRs were stably expressed in Ph cells to approximately 1 x 10⁶ receptors per cell, using the pLNCl² retroviral vector (3, 18). DNA constructs were transfected into 293GPG cells (19), and the viral supernatant was collected for 7 days and concentrated by centrifugation (25,000 g, 20 min, 4 °C). The virus was titrated, and equal amounts of colony-forming units were used to infect Ph cells. The infected cells were selected in the presence of 1 mg/ml G418, and proliferating populations of drug-resistant cells were used in all of the experiments.

Site-directed Mutagenesis—The 1.7-kilobase pair PstI-BamHI fragment of the human αPDGFR was subcloned into the pBluescript SK² plasmid, and the resulting construct was called 19E. Site-directed mutagenesis was carried out by using the Amersham Pharmacia Biotech oligonucleotide-directed mutagenesis kit. The oligonucleotides used to introduce the phenylalanine changes at Tyr-572/74 and Tyr-720 have been described previously (3, 5). To introduce the phenylalanine substitution at Tyr-731, the following oligonucleotide, which included a BamHI site, was used: 5’-CATGTTCACTAGTCATTCATTTTGGCGAAGAT-3’. To mutate Tyr-742 to phenylalanine, the following oligonucleotide, also introducing a XhoI site, was used: 5’-CCTCTTTTATCTCAGGCATGCT-GTGCGAACAATGCTGTATG-3’. The following oligonucleotide was used to mutate Tyr-988 to phenylalanine: 5’-GACACCAATGAGCTATT-GGTTGAGTCGTCCAGGATC-3’. This oligonucleotide also introduced a SalI site. The following oligonucleotide was used to introduce the phenylalanine substitution at Tyr-1018: 5’-GACAGGGTCTCAGTT-CAAG-CAGAGGAATGATGAAGCCCATGT-3’. This oligonucleotide also introduced a EcoRI site. Finally, the following oligonucleotide was used to introduce the arginine substitution at Lys-627: 5’-TTTC- TAGGATCACCTGGACCTTTCATACCTGAGGAGGGA-3’. This oligonucleotide also introduced a BamHI site. In all cases the introduced restriction site did not alter the amino acid sequence of the receptor. The F7 and add-back mutants were created by subcloning. All mutants were initially identified by restriction enzyme digestion using the introduced restriction sites and then verified by sequencing. The PstI-BamHI fragment of 19E was then subcloned into 18F, which is the full-length human αPDGFR (3.5-kilobase pair NotI-BamHI insert) subcloned into pBluescript SKII + (5). Finally, the NotI-BamHI fragment from 18F was subcloned into the NotI-BamHI-digested pLNCl² retroviral vector.

Antibodies—The rabbit polyclonal αPDGFR antibodies recognize either the carboxyl terminus (27P) or a portion of the first immunoglobulin domain (80-8) of the human αPDGFR (3). The Src-2 antibody used for immunoprecipitation of Src was purchased from Santa Cruz Biotechnology. The rabbit polyclonal anti-PLCγ antibodies (Upstate Biotechnology Inc.) at a concentration of 0.25 µg/ml. For Western blot analysis of PI3K, a polyclonal antibody recognizing the p85 subunit of PI3K (kindly provided by Alex Toker) was used at a 1:1,000 dilution. The monoclonal anti-PLCγ antibody (Transduction Laboratories) was used for Western blot analysis of SHP-2 at a 1:1,000 dilution. For anti-phospho-Erk Western blot analysis, a phospho-specific p44/42 mitogen-activated protein kinase (Thr202/Tyr204) antibody purchased from New England Biolabs (catalog number 9101L) was used at a 1:500 dilution.

Immunoprecipitation and Western Blot Analysis—Ph cells expressing the WT or mutant αPDGFRs were cultured and stimulated (50 ng/ml PDGF-AA) as described previously (3). The αPDGFR was immunoprecipitated exactly as described previously (3). αPDGFR immunoprecipitates representing approximately 3 x 10⁶ cells were resolved on a 7.5% SDS-polyacrylamide electrophoresis gel, and the proteins were transferred to Immobilon and subjected to Western blot analysis as described (3). To monitor the association between Src and the αPDGFR, Src was immunoprecipitated from resting or PDGF-stimulated cells using the Src-2 antibody, and immunoprecipitates representing approximately 3 x 10⁶ cells were subjected to anti-αPDGFR (80.8 + 27P) or anti-Src (327) Western blot analysis.

Erk Activation—Activation of Erk was monitored using 30 µg of clarified total cell lysate as described (21).

In Vivo PI3K Assays—PI3K assays were performed with immunoprecipitates of αPDGFRs exactly as described (20), except the silica gel plates were pretreated with 60 µl EDTA, 2% sodium taurocholate, and 50% EtOH and dried in a 100 °C oven overnight.

[3H]Thymidine Uptake—[3H]thymidine uptake was assayed as follows. Cells were rested 8 x 10⁴ cells/ml in DME containing 5% calf serum in 24-well dishes and incubated at 37 °C for 1 h. They were then washed 2 x in phosphate-buffered saline and arrested in 0.5 ml of DME containing 2 mg/ml bovine serum albumin for 48 h at 37 °C. PDGF buffer or various doses of PDGF-AA were added and incubated for 18-20 h at 37 °C. The cells were pulsed for 4 h with [3H]thymidine and harvested as described previously (21). TriPLICATE cultures were performed for each data point, and three independent experiments gave similar results. The data are expressed as a fold increase over the buffer control.

Chemotaxis Assay—PDGFR-dependent chemotaxis was assayed utilizing a 48-well modified Boyden chemotaxis chamber (NeuroProbe Inc., Baltimore, MD) and polyvinyl pyridone-free polycarbonate filters (8-µm pore size) (Poretics Corp., Livermore, CA) as described previously (22). Briefly, the lower wells of the chamber were filled with DME containing 0.1% calf serum supplemented with 10 ng/ml PDGF-AA or vehicle. The filters were coated with 50 µg/ml rat type I collagen (Collaborative Biomedical Products, Bedford, MA) and fixed atop the bottom wells. Ph cells expressing WT or mutated αPDGFRs were trypsinized, washed, and diluted in DME containing 0.1% calf serum to a final concentration of 4 x 10⁶ cells/ml and 50 µl of this cell suspension were placed into the top wells of the chamber. In each experiment, at least 6 of the 48 wells of the chamber were used for each condition examined. The chamber was incubated for 4 h at 37 °C in a 5% CO₂ atmosphere. Following incubation, the chamber was disassembled, and the cells on the upper surface of the filter were removed. The cells on the lower surface were fixed and stained with Diff-Quick (Baxter Healthcare Corp., FL). Chemotaxis was quantified by counting the number of cells on the lower surface of the filter in each well using a grid containing 100 non-overlapping fields. The total number of cells present in 100 fields was approximately 5-20 in resting or nonresponding cells and between 80 and 150 in responding cells. The response being measured was primarily chemotaxis, since including PDGF in the top and bottom chamber reduced the number of cells migrating through the filter by approximately 70%.

RESULTS

Characterization of αPDGFR Mutant Expressing Cell Lines—Activation of the αPDGFR results in its tyrosine phosphorylation at a number of residues. Eight major phosphorylation sites have been identified, and their phosphorylation leads to the recruitment of SH2 domain-containing signaling enzymes such as Src family members (Src), the phosphotyrosine phosphatase SHP-2, phosphatidylinositol 5-kinase (PI3K), and phospholipase C-γ1 (PLCγ). To evaluate systematically the role of each of the signaling molecules for αPDGFR-mediated cellular responses in a single cell line, we created a series of αPDGFR mutants in which the tyrosine residues required for the association of one of the above listed signaling enzymes was mutated to phenylalanine (subtraction mutants) (Fig. 1A). A second set of αPDGFR mutants (add-back mutants) included the F7 receptor, in which all 7 tyrosine phosphorylation sites were mutated, as well as a panel of receptors that have the tyrosine residue(s) required for binding of one of the associated proteins restored (Fig. 1B). The resulting receptors were expressed in Ph cells, a 3T3-like cell line that expresses...
normal levels of the βPDGFR but no endogenous αPDGFR (5).

To analyze the expression levels of the introduced αPDGFRs, lysates were prepared from resting or PDGF-stimulated cells, and the samples were subjected to an anti-αPDGFR Western blot. As shown in Fig. 2A (subtraction panel) and Fig. 3A (add-back panel), all cell lines expressed comparable levels of the introduced receptor, which we have previously estimated to be similar to the level of the endogenous αPDGFR (24). Furthermore, the WT αPDGFR coimmunoprecipitated with PLCγ, the p85 subunit of PI3K, and SHP-2 in a PDGF-dependent manner. No associated proteins were detected in receptor immunoprecipitates from stimulated cells expressing an empty vector (23). Mutation of tyrosine 1018 largely reduced binding of PLCγ, the F31/42 mutant failed to associate with PI3K (p85), and the F720 mutant did not bind SHP-2 upon PDGF stimulation (Fig. 2B). We and others (3, 4) have previously found that the WT receptor associates with Src family members, and this event is dependent on tyrosines 572 and 574, which are in the juxtamembrane domain of the receptor. Although the F720 and F720 mutants have been reported elsewhere, they are included in the present study for completeness.

FIG. 2: Expression levels, tyrosine phosphorylation, and binding characteristics of the subtraction panel of αPDGFR mutants. A, expression levels. Resting Ph cells expressing various constructs were lysed, and 30 μg of cell lysate were resolved on a 7.5% PAGE gel, transferred to Immobilon, and subjected to an αPDGFR Western blot (top panel). The top band is the mature, glycosylated species, and the bottom band is the immature form of the receptor. The bottom panel is a RasGAP Western blot performed on the same samples and indicates that there were similar amounts of cell lysate present in all samples. The parental Ph cells express no αPDGF-Rs (3). B, PDGF-dependent tyrosine phosphorylation and association with signaling molecules. Quiescent Ph cells expressing either the WT receptor or the various phosphorylation site mutants were left resting (-) or stimulated with 50 ng/ml PDGF-AA (+) for 5 min. The cells were lysed, and the lysates were immunoprecipitated with an antisera recognizing the αPDGFR (27F). Immunoprecipitates representing approximately 1.5 x 10⁶ cells were resolved by SDS-PAGE, transferred to Immobilon, and subjected to Western blot analysis. Immunoblotting with anti-αPDGFR antisera revealed that there were similar amounts of receptor present in all of the samples. The receptor blot was stripped and reprobed with a mixture of anti-phosphotyrosine antisera (4G10/PY20, 1:1). Western blot analysis of αPDGFR immunoprecipitates was also performed using antisera against PLCγ, p85, and SHP-2 to detect coimmunoprecipitation of these signaling molecules with the activated αPDGFR.

FIG. 1. Schematic diagram illustrating the series of αPDGFR mutants used in this study. The cytoplasmic domain of the αPDGFR is shown as a schematic in which the tyrosine phosphorylation sites are represented as black squares. Signaling enzymes predicted to stably associate with the receptor mutants are indicated by geometric shapes and are identified at the top of the schemes. The nomenclature of the “subtraction panel” (A) and “add-back panel” (B) of αPDGFR mutants is indicated to the right of each receptor representation. In the subtraction panel, the names indicate which of the tyrosine residues have been replaced with phenylalanine, and in the add-back panel the name of each mutant denotes a pair of residues whose mutations in the F7 construct has been repaired. Tyr-572 and -574 are located in the juxtamembrane domain (JM) of the receptor and are required for Src binding to the receptor; Tyr-720, -731, and -742 are in the kinase insert (KI) of the receptor and are responsible for SHP-2 and PI3K binding, respectively; Tyr-988 and -1018 are located in the Tail of the receptor, and are involved in the binding of a yet unidentified protein and PLCγ, respectively.

increased the phosphotyrosine content of all the receptors, with the exception of the kinase-inactive receptor mutant (R627). The level of receptor autophosphorylation was not substantially decreased, even in mutants missing up to 7 phosphorylation sites. This may reflect the presence of additional phosphorylation sites or the ability of the receptor to autophosphorylate at cryptic sites. A similar phenomenon is observed with mutants of the βPDGFR (22).
The amount of Src in each of the samples (lower panel) is largely dependent on the tyrosine phosphorylation sites ining characteristics of the add-back panel of the receptor-associated proteins were detected in receptor immunoprecipitates isolated from resting or PDGF-stimulated cells. Western blot analysis of an aliquot of the samples was routinely performed to determine the amount of receptor in each sample. In cells expressing the WT aPDGFR, PDGF triggered a substantial increase in the PI3K activity present in receptor immunoprecipitates. Mutating tyrosines 731 and 742 severely impaired association of PI3K with the aPDGFR, whereas PI3K activity was recruited to WT levels by all other mutants in the subtraction panel (Fig. 4A). The reduced response in cells expressing the F988 receptor in this experiment may be explained by the fact that there was less receptor immunoprecipitate present in this particular sample (see Fig. 2B). In the add-back panel, only the Y31/42 receptor was able to recruit PI3K activity in response to PDGF stimulation (Fig. 4B). These data indicate that tyrosines 731 and 742 are necessary and sufficient for binding of PI3K to the activated aPDGFR and are consistent with the findings of other groups that these tyrosines are required for association with PI3K.

We also examined PLCγ activation by measuring PDGF-AA-dependent accumulation of inositol phosphates. Similar to the findings of other groups (8), PLCγ activation was very weakly induced by PDGF-AA, even though PLCγ was tyrosine-phosphorylated and recruited to the aPDGFR (3, 4). In contrast, the βPDGFR was able to detectable accumulation of PLCγ products, as was a chimera of βPDGFR (Ref. 26 and data not shown). Attempts to optimize PLCγ activation by altering the dose of PDGF or the duration of exposure to PDGF did not improve the response. We conclude that if PLCγ is activated by PDGF-AA, it is below the level of detection in our assays.

In summary, the data in Figs. 2–4 show that the aPDGFR recruits some signaling enzymes such as PI3K, Src, and to a lesser extent PLCγ with high fidelity and in this respect is comparable to the βPDGFR. Association of SHP-2 is more complicated, as there appear to be multiple and possibly cryptic binding sites.

Src and PI3K Contribute to aPDGFR-mediated Erk Activation—Activation of extracellular regulated kinase 1 and 2 (Erk1/2) is thought to play a critical role in growth factor-induced cellular responses. We have previously found that the F72/74 receptor is substantially better than the WT receptor in
We next tested Erk activation in the add-back panel of cell lines. In the F7 cells, PDGF-induced Erk activation was completely abolished (Fig. 5). Restoration of the binding sites for either PI3K or Src partially rescued PDGF-dependent activation of Erk, whereas the Y720, Y988, and Y1018 mutants were not able to induce Erk activation. In summary, these findings indicate that PI3K and/or Src contribute to αPDGFR-mediated Erk activation.

**PI3K Is Required for αPDGFR-induced DNA Synthesis**—To investigate the role of each of the signaling molecules for PDGF-AA-induced DNA synthesis, we compared this response in Ph cells expressing either the WT αPDGFR or the various mutant receptors. Quiescent cells were stimulated with increasing doses of PDGF-AA, pulsed with [3H]thymidine, and harvested, and the incorporated radioactivity was quantitated. As shown in Fig. 6, stimulation of WT receptor expressing cells with PDGF-AA resulted in a dose-dependent increase in [3H]thymidine uptake, which was maximally 3-fold at 25 ng/ml PDGF-AA. The inability of the αPDGFR to interact with either SHP-2 or PLCγ, as well as mutation of tyrosine 988, did not affect the ability of the receptor to mediate ligand-induced DNA synthesis (Fig. 6A). In contrast, mutation of the binding sites for PI3K fully abolished PDGF-dependent DNA synthesis, as the F31/42 receptor showed no increase in [3H]thymidine incorporation, and its response was similar to that of a kinase-inactive receptor (R627). Activation of the F7274 receptor, however, led to enhanced DNA synthesis as compared with the WT receptor. This may be explained by the observation that Src is involved in Cbl-mediated down-regulation of the activated αPDGFR and thus the prolonged half-life of the F7274 receptor results in enhanced ligand-induced signaling and DNA synthesis.2 Consistent with the observation that the F31/42 mutant was unable to mediate DNA synthesis, the restoration of the PI3K-binding site to the F7 mutant was sufficient to salvage the ability of the receptor to mediate PDGF-dependent [3H]thymidine incorporation. Although its response was reduced at low doses of PDGF, the Y31/42 mutant was able to trigger DNA synthesis similar to the WT receptor at high doses of PDGF (Fig. 6B). In contrast, restoration of the binding sites for Src, SHP-2, or PLCγ, as well as tyrosine 988, to the F7 receptor did not rescue αPDGFR-mediated DNA synthesis. These findings indicate that of the receptor-associated proteins tested, PI3K is the major contributor to PDGF-AA-dependent DNA synthesis.

**Multiple Signaling Molecules Contribute to PDGF-dependent Chemotaxis**—Since the Ph cells expressing the introduced αPDGFR undergo DNA synthesis and chemotaxis, we were able to compare and contrast the involvement of signaling enzymes for both of these responses in the same cell type. To determine which of the signaling molecules are critical for αPDGFR-mediated chemotaxis, we compared the ability of the WT and mutant receptors to stimulate PDGF-induced migration of Ph cells. To this end, quiescent cells expressing the various receptors were trypsinized, washed, and subjected to a chemotaxis assay in the presence of buffer or 10 ng/ml PDGF-AA. As shown in Fig. 7A, stimulation of the WT αPDGFR with PDGF-AA led to a dramatic increase in cell migration to approximately 11-fold the basal level. This response was due to primarily chemotaxis, instead of chemokinesis, since adding PDGF to both upper and lower chambers, instead of only the bottom chamber, reduced the response by approximately 70%. Like the WT receptor, the F720 and F988 mutants efficiently triggered PDGF-dependent chemotaxis. Mutation of the Src-binding sites, however, completely abolished the ability of the αPDGFR to mediate PDGF-induced chemotaxis as the F7274 mutant triggered no increase in cell migration upon PDGF

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stimulation ($p < 0.001$). Furthermore, mutation of the binding sites for PI3K and PLC\(_\gamma\) reduced PDGF-induced chemotaxis significantly by 53 ($p < 0.01$) and 44% ($p < 0.05$), respectively. The restoration of either binding site to the F7 receptor was not sufficient to rescue PDGF-dependent chemotaxis (Fig. 7B). Interestingly, although the failure to activate Src by the F72/74 mutant completely blocked PDGF-induced chemotaxis, activation of Src alone was not enough to mediate a chemotactic response by the Y72/74 aPDGFR. Thus, unlike DNA synthesis, the chemotactic response was not rescued by engaging any one of the signaling enzymes that are recruited to the aPDGFR.

**Coexpression of Single Add-back aPDGFR Mutants in Ph Cells Is Sufficient to Mediate PDGF-dependent Chemotaxis**—The experiments shown in Fig. 7 indicate that Src, PI3K, and PLC\(\gamma\) contribute to aPDGFR-mediated chemotaxis of Ph cells. Although multiple signal relay enzymes bind to activated PDGFRs, it is not clear whether these molecules are required to associate with one receptor molecule in order to send biological signals. Since none of the add-back mutants was able to trigger this response, we tested whether coexpressing the add-back mutants would restore PDGF-dependent chemotaxis. Thus, in these cells, more than one signaling molecule (Src, PI3K, or PLC\(\gamma\)) is able to associate with aPDGFRs; however, they cannot bind to one receptor molecule. The design of this experiment was to keep the total level of receptor comparable to that of the WT receptor shown in the 1st lane of Fig. 8A. Since multiple receptors were being expressed, we decreased the level of expression of each of the receptors. Consequently, cells expressing only one receptor (2nd lane of Fig. 8A) had less receptor than cells harboring 2 or 3 types of receptor mutants. Importantly, the overall level of receptor expressed even in the triple expressers (far right lane of Fig. 8A) was not markedly different from the level seen in WT receptor-expressing cells. Since we did not have antibodies that distinguish between the receptor mutants, we were not able to determine the relative expression level in coexpressing cells. However, we verified that the coexpressing cells were able to recruit PLC\(\gamma\), p85, or Src, which was diagnostic for the presence of each of the 3 add-back receptors. We found that PLC\(\gamma\), p85, and Src were present in the immunoprecipitates only from cells infected with viruses for all 3 of the add-back mutants (Fig. 8B and data not shown). This analysis was used to verify the presence of the appropriate receptors in the other cell lines as well.

To compare the chemotactic response of these cell lines, we employed the Boyden chamber assay used in Fig. 7. Although cells expressing any one of the add-back mutants failed to chemotax in response to PDGF-AA (Fig. 7), when 2 or more of these receptors were coexpressed, the cells became responsive to PDGF-AA ($p < 0.01$) (Fig. 8C). The cells that coexpressed the Y72/74, Y31/42, and Y1018 mutants seemed to respond best; however, the response of the cells expressing only two of the three receptors was not statistically significant from the triple expressers ($p > 0.05$). These data indicate that binding and activation of multiple signaling molecules is necessary and sufficient to trigger aPDGFR-mediated chemotaxis in Ph cells and that these signal relay enzymes are not required on the same receptor.
**DISCUSSION**

We have addressed the importance of signaling enzymes recruited to the cPDGFR for two different cellular responses in a single, physiologically relevant cell line. PDGF-AA-dependent DNA synthesis required stable association with only one signaling enzyme, PI3K. In contrast, several signal relay molecules contribute to cPDGFR-mediated chemotaxis. These include Src, and to a lesser extent PI3K and PLCγ. In addition, our results indicate that these signaling enzymes do not need to be recruited to the same receptor for Ph cells to respond chemotactically to PDGF-AA.

**Effect of the Half-life of the Receptor on Downstream Signal-\(\text{ing} \) Events and Biological Responses—To identify the signal relay mechanisms that are critical for cPDGFR-mediated DNA synthesis and chemotaxis, we altered the ability of the receptor to recruit receptor-associated signaling molecules by specific tyrosine to phenylalanine substitutions. A potential problem in the evaluation of the cPDGFR mutants in this system is that mutation of tyrosines 572 and 574 reduces the ligand-induced degradation of the receptor. We have previously shown that the prolonged half-life of the F72/74 receptor leads to increased Erk activation and DNA synthesis. However, a prolonged receptor half-life alone is not sufficient to mediate increased responses, as the F7 receptor has a long half-life, yet it is unable to mediate efficient Erk activation, DNA synthesis, or chemotaxis (Fig. 5B). In contrast, the half-life of the Y72/74 receptor is comparable to the WT receptor, and this mutant activates Erk better than the WT receptor (Fig. 5). Thus, although the various cPDGFR mutants show variabilities in the rate of their ligand-induced degradation, their ability to initiate cellular responses appears to depend much more heavily on the recruitment of signaling enzymes rather than on the half-life of the receptor.

**PI3K Is Critical for cPDGFR-mediated DNA Synthesis**—The characterization of the cPDGFR mutants revealed that the cPDGFR recruits and presumably activates multiple signaling enzymes, yet only PI3K is required for cell cycle progression. This was found by both the subtraction approach, in which deletion of the PI3K-binding sites in the F31/42 receptor resulted in complete lack of DNA synthesis, and the add-back
pressing single add-back mutants were lysed, and 30 μg of cell lysate were resolved on a SDS-7.5% PAGE gel, transferred to Immobilon, and subjected to Western blot analysis with anti-PDGFR (Fig. 6). Moreover, pretreatment of cells with the PI3K inhibitor wortmannin also abolished PDGF-dependent DNA synthesis in cells expressing either the WT or the Y31/42 receptor (data not shown). Although these two different approaches identified PI3K as the critical signaling enzyme for PDGF-AA-dependent DNA synthesis in Ph cells, our findings contrast the previously published data which indicated that PI3K is not required for mitogenic signaling by the aPDGFR (9, 10). These studies were performed using either human aPDGFR constructs expressed in 32D hematopoietic cells, or fms/aPDGFR chimeric receptors expressed in NIH3T3 cells. The extracellular transmembrane and a portion of the juxtamembrane domain of the chimeric receptor is the colony-stimulating factor-1 (fms). The differences between our results and these findings may be due to cell type-specific effects or to the use of chimeric receptors as opposed to full-length aPDGFRs. A potentially critical characteristic of the fms/aPDGFR chimera is that the portion of the aPDGFR that includes the Src-binding site is missing. Consequently, it is possible that the chimeric receptors do not activate Src. Since Src is a negative regulator of aPDGFR signaling, 2 the finding that the chimeric fms/aPDGFR does not require PI3K for mitogenesis may relate to the lack of the repressive influence of Src in this receptor.

It is interesting to note that in our system, mitogenic signaling by the two PDGFR subtypes is not identical. Whereas the aPDGFR triggers DNA synthesis via activation of PI3K only, the βPDGFR initiates multiple, redundant mitogenic pathways, including PI3K and PLCγ. This result is in contrast to our previous studies in which PDGFR (8). However, since PLCγ is tyrosine-phosphorylated upon aPDGFR engagement (3) and is required for maximal PDGF-AA-dependent chemotaxis (Fig. 7 and 8), it nonetheless appears to contribute to signal relay by the aPDGFR.

**Multiple Signaling Enzymes Contribute to aPDGFR-mediated Chemotaxis—** By comparing the ability of the various aPDGFR mutants to trigger PDGF-dependent chemotaxis, we found that multiple signaling enzymes contribute to this cellular response. Although Src was absolutely required for aPDGFR-induced chemotaxis, mutation of the binding sites for either PI3K or PLCγ resulted in a partial inhibition of chemotaxis. However, when both PI3K and PLCγ were absent but Src was present (Y72/74 receptor) the chemotactic response was completely abolished. These results indicate that PI3K and PLCγ contribute to PDGF-induced chemotaxis via independent pathways. Furthermore, Src alone is not sufficient to trigger aPDGFR-induced chemotaxis. In addition, since the F72/74 receptor activates PI3K similar to the WT receptor, and Src is not required for efficient tyrosine phosphorylation of PLCγ (3, 4), these signaling enzymes appear to contribute to aPDGFR-mediated chemotaxis either independently of Src or they act upstream of Src.

Consistent with our findings, PI3K and PLCγ have been shown to contribute to aPDGFR-mediated chemotaxis in other systems. Inhibition of these signaling molecules by receptor mutants (10, 16) or inhibitors of PI3K or PLCγ also partially inhibited the chemotactic response by both the α and βPDGFR. With regard to Src, however, Hooshmand-Rad et al. (4) demonstrated that Src family members are not required for aPDGFR-mediated chemotaxis in porcine aortic endothelial (PAE) cells. However, a comparison of Src-mediated effects in PAE and Ph cells reveals a number of additional cell type-dependent differences including tyrosine...
phosphorylation of signaling molecules and DNA synthesis (3, 4), suggesting that Src plays distinct roles in αPDGF receptor-mediated chemotaxis when it is expressed in different cell types such as PAE cells and Ph cells.

Src, PI3K, and PLCγ Do Not Need to Associate with the Same Receptor to Trigger αPDGF-dependent Chemotaxis—Whereas Src, PI3K, and PLCγ contribute to maximal αPDGF-dependent chemotaxis, none of these signaling enzymes alone is able to trigger an efficient chemotactic response. In addition, coexpression of single add-back mutants revealed that the chemotactic response can be rescued even if each receptor is able to recruit only one signaling molecule. Thus, whereas multiple receptors can contribute to maximal chemotaxis for these cellular events, which ultimately lead to directed cell movement.

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