Tyrosine Phosphatase SHP-2 Is Involved in Regulation of Platelet-derived Growth Factor-induced Migration*

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SHP-2 is a ubiquitously expressed Src homology-2-containing cytosolic tyrosine phosphatase that binds to and becomes tyrosine-phosphorylated by the activated platelet-derived growth factor receptor-β (PDGFR-β). Removal of the binding site on the receptor, by mutation of Tyr1009 to Phe1009 (denoted Y1009F), led to loss of PDGF-stimulated phosphatase activity in cells expressing the mutated receptor, and these cells failed to form membrane edge ruffles and to migrate toward PDGF. Furthermore, treatment with phosphatase inhibitors phenylarsine oxide (PAO) and orthovanadate led to loss of PDGF-stimulated phosphatase activity and attenuated PDGF-stimulated migration of wild type PDGFR-β cells. Treatment of wild type PDGFR-β cells with combinations of PAO or orthovanadate and phosphatidylinositol 3-kinase inhibitors wortmannin or LY294002 resulted in a synergistic inhibition of PDGFR-β-mediated cell migration. PDGF stimulation of wild type PDGFR-β cells led to induction of p125 focal adhesion kinase (FAK) activity at low concentrations of the growth factor and a decrease at higher concentrations. In the mutant Y1009F cells and in wild type PDGFR-β cells treated with PAO and orthovanadate, FAK activity was not increased in response to PDGF. These results suggest that SHP-2 activity is involved in regulation of FAK activity and thereby of cell migration through PDGFR-β, independently of phosphatidylinositol 3-kinase.

Migration of cells is an integral part of many different physiological and pathological processes. Embryonic development, angiogenesis, wound healing, and tumor spreading require cell motility. Signal transduction through growth factor receptors with intrinsic protein-tyrosine kinase activity leads to induction of directed cellular migration, chemotaxis. Activation of the tyrosine kinase, upon growth factor binding and dimerization of receptors, results in receptor autophosphorylation (1). Phosphorylated tyrosine residues present binding sites for signal transduction molecules containing one or two copies of so-called Src homology 2 domains, a conserved stretch of about 240 amino acid residues (2). Binding of signal transduction molecules to the receptor leads to initiation of signaling cascades, through activation of enzymatic activities intrinsic to or associated with the signal transduction molecules. The signaling cascade is eventually established as a cellular response, such as migration, proliferation, and differentiation.

Platelet-derived growth factor (PDGF) acts on a wide spectrum of cells, including mesenchymal cells (3). There are two receptors for PDGF, denoted the PDGF α- and β-receptors (4, 5). The receptors are similar in structure, with an extracellular part organized in five immunoglobulin-like domains, a single transmembrane stretch and an intracellular part containing the kinase domain. The kinase domain is interrupted by the insertion of a noncatalytic kinase insert. More than 10 auto-phosphorylation sites have so far been identified in the PDGF β-receptor (6). Several of these have been shown to interact with Src homology 2 domain-containing proteins, which have been implicated in transduction of signaling cascades leading to cellular migration. Thus, two autophosphorylation sites (Tyr740 and Tyr751) in the kinase insert of the receptor bind the regulatory subunit (p85) of phosphatidylinositol (PI) 3-kinase. PI 3-kinase activity has been shown to be a prerequisite for PDGF-induced cellular migration (7, 8). The two autophosphorylation sites in the carboxyl-terminal tail (Tyr1009 and Tyr1021) mediate binding of phospholipase C-γ (PLC-γ), which is implicated in regulation of chemotaxis (8, 9). In addition, phosphorylated Tyr1009 binds the phosphotyrosine phosphatase SHP-2 (also denoted PTP1D, Syp, and SH-PTP2) (10, 11). SHP-2 activity has been implicated in cytoskeletal organization (12), which may indicate a function for SHP-2 in cellular migration. Numerous studies have shown that SHP-2 becomes tyrosine-phosphorylated in growth factor-stimulated cells and stably associated with activated growth factor receptors (13, 14). Tyrosine phosphorylation of SHP-2 has been reported to induce its catalytic activity (15), although mutation of the phosphorylatable tyrosine residues in SHP-2 does not preclude its activation (16). SHP-2 may act as a negative regulator of PDGF receptor function, by dephosphorylating the receptor or its cognate substrates (17). On the other hand, SHP-2 is also implicated in positive regulation of PDGF function, since tyrosine phosphorylation of SHP-2 presents a binding site for the adaptor protein Grb2, which is known to mediate Ras activation through complex formation with the nucleotide exchange factor Sos (16, 18). However, the role of Grb2/Sos binding to SHP-2 is not clear, and mutation of the Grb2-binding site on SHP-2 does not interfere with PDGF-induced Erk-2 activation (19). The recent report on targeted inactivation of the SHP-2 gene showed that loss of SHP-2 was embryonally lethal and led...
to failure of development of the vascular system (19). The failure to migrate toward PDGF failed to develop due to failure of development of the vascular system (19).

We have used PDGF-stimulated porcine aortic endothelial (PAE) cells expressing the PDGF β-receptor or receptor mutants Y1009F and Y1021F, to examine the potential role for SHP-2 in migration. PAE cells expressing a Y1009F mutant PDGF β-receptor, which lacks the SHP-2 binding site, failed to migrate toward PDGF. Moreover, treatment with the tyrosine phosphatase inhibitors PAO and vanadate led to attenuation of migration of PDGF-BB-stimulated PAE cells expressing the wild type PDGF-R-β. Our data indicate a role for SHP-2 in migration via a pathway that acts parallel to the PI 3-kinase pathway and involves regulation of focal adhesion kinase activity.

MATERIALS AND METHODS

Cell Culture—PAE cells expressing the wild type PDGF-R-β or mutant PDGF-R-β Y1009F and Y1021F have been described previously (22). Cells were maintained in Ham's F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Sigma) and antibiotics at 37 °C and 5% CO2. For starvation, cells were kept in serum-free medium supplemented with 0.1% serum bovine albumin.

Antibodies and Other Reagents—The rabbit antiserum PDGF-R-3, specifically recognizing the PDGF-R-β, has been described earlier (23). Anti-SHP-2 monoclonal antibody and anti-Grb2 rabbit antiserum were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The monoclonal anti-phosphotyrosine antibody PY20 and the monoclonal anti-FAK (2A7) antibody were from Transduction Laboratories. The rabbit antiserum against PLC-γ, raised against a synthetic peptide corresponding to a part of human PLC-γ was kindly provided by Dr. Lars Ronnstrand (Ludwig Institute for Cancer Research, Uppsala, Sweden). Peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse antibodies were purchased from Amersham Pharmacia Biotech (Amersham Pharmacia Biotech; the filter was blocked in 3% bovine serum albumin, and sodium orthovanadate was purchased from Sigma. PDGF-BB was purchased from Peprotech Inc.

Immunoprecipitation and Immunoblotting—Serum-starved cells were treated with 50 ng/ml PDGF-BB for 8 min, rinsed with ice-cold phosphate-buffered saline (PBS) containing 0.1 mM Na3VO4 and 1 mM dithiothreitol and lysed in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 0.5 mM Na3VO4, 1% aprotinin (Trasytol, Bayer), 10 μg/ml leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride (Sigma). Cell lysates were incubated with rabbit antiserum or monoclonal antibodies for 1 h at 4 °C and then incubated with 0.5 ml of inositol-free Ham's F-12 medium containing 0.25% fetal calf serum and 2 μCi of [32P]ATP for 10 min at room temperature. Samples were analyzed by SDS-PAGE. After electrophoresis the gel was treated in 2% glutaraldehyde to fix the proteins to the gel and then with 0.5 M KOH at 55 °C for 45 min, to hydrolyze phosphorylation on serine. The gel was dried and analyzed by autoradiography.

Chemotaxis Assay—The assay was performed in a mini-Baydon chamber as described (24) using micropore nitrocellulose filters (8 μm thick, 8-μm pore) coated with type-1 collagen solution at 100 μg/ml (Vitrogen 100; Collagen Corp.). Cells were trypsinized and resuspended at a concentration of 5 × 105 cells/ml in serum-free medium containing 0.25% bovine serum albumin. The cell suspension was placed in the upper well of the microchamber, and 25 μl of serum-free medium without or with various concentrations of PDGF-BB placed below the filter in the lower chamber. For each set of experiments, random migration in the presence of serum-free medium containing 0.25% bovine serum albumin served as control and is referred to as 100% migration. All experiments were performed in triplicates for every concentration of PDGF-BB. The assays were read in blind.

Determination of PTP Activity of SHP-2—Anti-PDGFR-β immunoprecipitates immobilized on protein A-Sepharose beads were washed twice with lysis buffer (see “Immunoprecipitation”) without vanadate and twice in the assay buffer containing 25 mM imidazole (pH 7.5), 0.1 mg/ml bovine serum albumin. PDGF-BB was purchased from Amersham Pharmacia Biotech; the filter was blocked in 3% bovine serum albumin, and sodium orthovanadate was purchased from Sigma. PDGF-BB was purchased from Peprotech Inc.

Inositol Phosphate Assay—Cells were grown in 24-well plates to subconfluency. They were washed in inositol-free Ham’s F-12 medium and then incubated with 0.5 ml of inositol-free Ham’s F-12 medium containing 0.25% fetal calf serum and 2 μCi of [32P]ATP. After 10 min, cells were washed in 1 ml of lysis buffer containing 5% FBS, 1 μg/ml leupeptin, and 2 μg/ml aprotinin. The cells were harvested and sonicated in 0.5 M KOH at 55 °C for 15 min, followed by extraction of total inositol phosphates from the heparin-Sepharose column with 0.5 M KOH and 0.5 M HCl. The assays did not work well when using anti-SHP-2 immunoprecipitated material or when many samples were processed simultaneously.

In vitro migration was measured in vitro, using anti-receptor antiserum for immunoprecipitation from cell lines pretreated or not with phoshatase inhibitors and stimulated with or without PDGF-BB. The immobilized immunoprecipitates were used for lipid kinase reactions using phosphatidylinositol as an exogenous substrate, and the reactions were analyzed as described by Wennström et al. (7).

Assay for FAK Activity—Serum-starved cells pretreated or not with phoshatase inhibitors and stimulated with or without PDGF-BB. The immobilized immunoprecipitates were used for lipid kinase reactions using phosphatidylinositol as an exogenous substrate, and the reactions were analyzed as described by Wennström et al. (7).

PI 3-Kinase Assay—PI 3-kinase activity was measured in vitro, using anti-receptor antiserum for immunoprecipitation from cell lines pretreated or not with phoshatase inhibitors and stimulated with or without PDGF-BB. The immobilized immunoprecipitates were used for lipid kinase reactions using phosphatidylinositol as an exogenous substrate, and the reactions were analyzed as described by Wennström et al. (7).
polyacrylamide gels. After fixation in methanol/acetic acid, the gel was treated with 1 M KOH for 1 h at 55 °C, fixed again, dried, and exposed on RX films (Fuji).

RESULTS

Characterization of PAE Cell Lines Expressing Wild Type and Mutant PDGFR-β—It has previously been shown that phosphorylated Tyr1009 and surrounding amino acid residues in the PDGFR-β present a binding site for SHP-2 (10, 11). In order to characterize the role of SHP-2 in PDGFR-induced cellular responses, we examined the properties of PAE cells expressing the wild type PDGFR-β, the mutant Y1009F (lacking the binding site for SHP-2), and, for comparison, the mutant Y1021F (lacking the binding site for PLC-γ). These cell lines have previously been reported to express similar numbers of receptors and to transduce mitogenic signals with similar efficiencies (22). Fig. 1A shows that PDGF-BB stimulation of the different cell lines led to similar fold induction of receptor kinase activity. An immune complex kinase assay on SHP-2 immunoprecipitations showed that SHP-2 was tyrosine-phosphorylated and in complex with the PDGFR-β after PDGF-BB stimulation of PAE cells expressing the wild type receptor or the Y1021F mutant but not in cells expressing the Y1009F mutant PDGFR-β (Fig. 1B). Immunoprecipitation of SHP-2, followed by immunoblotting with anti-phosphotyrosine antibody as indicated. Immunoactive proteins were detected by enhanced chemiluminescence. IP, immunoprecipitation; IB, immunoblotting. E, accumulation of inositol phosphates in the different cell lines was estimated by labeling cells with [3H]myoinositol, followed by ion exchange chromatography on Dowex AG-1-X8 resin. The data show means ± S.E. of at least three experiments. *, significantly different from unstimulated at p < 0.05 (Student’s t test).

FIG. 1. PDGF-BB-stimulated receptor tyrosine kinase activity, tyrosine phosphorylation of SHP-2, and inositol phosphate accumulation in cells expressing the wild type or mutant PDGFR-β. A and B, PAE cells expressing the wild type, Y1009F, or Y1021F PDGF β-receptor (β-R) were incubated in the absence (−) or presence (+) of 50 ng/ml PDGF-BB for 8 min at 37 °C. Cells were lysed and immunoprecipitated with anti-receptor antisera PDGFR-3 (A) or anti-SHP-2 antibody (B), and samples were subjected to kinase assays as described under “Materials and Methods,” followed by SDS-PAGE and autoradiography. C and D, the different PAE cell lines expressing wild type or mutant receptors were stimulated as above and processed for immunoprecipitation with the anti-SHP-2 antibody or anti-PDGFR-β antisera as indicated. The samples were separated by SDS-PAGE and transferred to nitrocellulose and blotted with anti-SHP-2 antibody or anti-phosphotyrosine antibody as indicated. Immunoreactive proteins were detected by enhanced chemiluminescence. IP, immunoprecipitation; IB, immunoblotting. E, accumulation of inositol phosphates in the different cell lines was estimated by labeling cells with [3H]myoinositol, followed by ion exchange chromatography on Dowex AG-1-X8 resin.
remained in the Y1009F cells could indicate that there are additional binding sites for SHP-2 on the PDGFR-β. Although the sensitive immune complex kinase assay (Fig. 1B) showed complex formation between the wild type PDGFR-β and SHP-2, we were not able to demonstrate such complexes in PDGFR-treated, intact cells (Fig. 1D).

It has been shown previously that PLC-γ associates with Tyr(P)1021 and, to a lesser extent, with Tyr(P)1009 (22). PDGFR-BB stimulation of the different cell lines led to a 1.5-fold increase in formation of inositol phosphate (Fig. 1E) in cells expressing the wild type PDGFR-β. In the Y1009F cells, inositol phosphate formation was significantly increased in response to PDGF stimulation, although not to the levels seen in the wild type receptor cells. In contrast, no inositol phosphate formation in response to PDGF stimulation could be detected in cells expressing the Y1021F mutant receptor. These data show that the Y1009F mutant receptor fails to associate with SHP-2 and mediate its tyrosine phosphorylation but that it is still able to mediate activation of PLC-γ.

**Phosphotyrosine Phosphatase Activity in PDGF-BB-stimulated PAE Cells Expressing the Wild Type and Mutant PDGFR-β and Effects of Phosphatase Inhibitors**—We examined the effect of SHP-2 tyrosine phosphorylation and its catalytic activity. Phosphatase (PTP) activity was measured using anti-receptor immunoprecipitates from the different PAE cell lines treated or not with PDGF-BB. The receptor precipitates were incubated in the presence of a 32P-labeled synthetic phosphopeptide, which served as a substrate for the PTP activity. The PTP activity in unstimulated cells was used as a control and was set to 100% as described under “Materials and Methods.” Each value is the mean of duplicate determinations, and the results are representative of three separate experiments. A, anti-PDGFR-β immunoprecipitates prepared from the cells treated with PAO and vanadate and stimulated or not with PDGF-BB (50 ng/ml for 8 min at 37 °C) were subjected to kinase assay as described under “Materials and Methods,” followed by SDS-PAGE and autoradiography. DMSO, Me2SO.

**FIG. 2.** PDGF-BB-dependent increase in PTP activity in different cell types and effect of PTP inhibitors on PTP activity and the receptor kinase activity. Serum-starved PAE cells expressing the wild-type, Y1009F, or Y1021F PDGFR-β and wild type PDGFR-β cells pretreated with PAO (0.6 μM) and vanadate (Van; 60 μM) for 4 h were incubated with or without 50 ng/ml PDGF-BB for 8 min at 37 °C. Cells were lysed and immunoprecipitated with anti-receptor antibody. A, immunoprecipitates were assayed for phosphotyrosine phosphatase activity using synthetic 32P-labeled phosphopeptide as a substrate. PTP activity in unstimulated cells served as control and was set to 100% as described under “Materials and Methods.” Each value is the mean of duplicate determinations, and the results are representative of three separate experiments. B, anti-PDGFR-β immunoprecipitates prepared from the cells treated with PAO and vanadate and stimulated or not with PDGF-BB (50 ng/ml for 8 min at 37 °C) were subjected to kinase assay as described under “Materials and Methods,” followed by SDS-PAGE and autoradiography. DMSO, Me2SO.

PDGF-induced Migration Requires SHP-2 and FAK Activity

![Figure 2](image_url)

**FIG. 3.** Effects of PAO and vanadate on PDGF-BB-stimulated tyrosine phosphorylation of SHP-2 and its association with Grb2. Serum-starved PAE cells expressing PDGFR-β were incubated in the absence or presence of 0.1% dimethyl sulfoxide (DMSO), 0.6 μM PAO, or 60 μM orthovanadate for 4 h at 37 °C and then stimulated with 50 ng/ml PDGF for 8 min. Cell lysates were immunoprecipitated with the anti-SHP-2 antibody. The samples were separated by SDS-PAGE and immunoblotted with antibody to phosphotyrosine (PY-20), the anti-SHP-2 antibody, or the anti-Grb2 antibody, respectively. Immunoreactive proteins were detected by enhanced chemiluminescence. Ip, immunoprecipitation; Ib, immunoblotting.

**FIG. 3A.** PTP activity in unstimulated cells was used as a control and was set to 100%. As shown in Fig. 2A, PDGF-BB-dependent increases in receptor-associated PTP activities in PAE cells expressing the wild type and Y1021F PDGFR-β were 322 ± 44% and 471 ± 71%, respectively. In contrast, the PDGF-BB-dependent increase in receptor-associated PTP activity in PAE cells expressing the Y1009F mutant was 120 ± 15%. These data indicate that SHP-2 is the dominating PDGFR-β receptor-associated phosphotyrosine phosphatase.
and tyrosine phosphorylation, and thereby Grb2-binding, of SHP-2 remains after treatment of cells with PAO or orthovanadate. In contrast, PDGF-β-receptor-associated phosphatase activity is reduced to that in unstimulated cells, indicating that the catalytic activity of SHP-2 is inhibited by PAO and vanadate.

Migration of PDGFR-β Wild Type and Mutant PAE Cells toward PDGF-BB and Effects of Phosphatase Inhibitors—To examine the potential role of SHP-2 in PDGF-BB-stimulated chemotaxis, PAE cells expressing the wild-type (open circles), Y1021F (closed circles), and Y1009F (open squares) PDGFR-β were analyzed for their migration toward different concentrations of PDGF-BB. B–E, the effects of phosphatase inhibitors and PI 3-kinase inhibitors on migration of PAE cells expressing PDGF-β-receptor were measured by preincubation with increasing concentrations of PAO (B), orthovanadate (C), wortmannin (D), and LY294002 (E) for 30 min. Migration toward 10 ng/ml PDGF-BB was measured in a mini-Boyden chamber for 4 h in the presence of the drugs. Random migration (PDGF-BB on both sides of the filter) is referred to as 100% migration. Migrating cells were estimated as described under “Materials and Methods.” The data show means ± S.E. of at least three experiments. *, significantly different from control at $p < 0.05$ (Student’s $t$ test).

FIG. 4. Chemotaxis of different cell types and effects of PTP inhibitors and PI 3-kinase inhibitors on PDGF-BB-induced chemotaxis. A, PAE cells expressing the wild-type (open circles), Y1021F (closed circles), and Y1009F (open squares) PDGFR-β were analyzed for their migration toward different concentrations of PDGF-BB. B–E, the effects of phosphatase inhibitors and PI 3-kinase inhibitors on migration of PAE cells expressing PDGF-β-receptor were measured by preincubation with increasing concentrations of PAO (B), orthovanadate (C), wortmannin (D), and LY294002 (E) for 30 min. Migration toward 10 ng/ml PDGF-BB was measured in a mini-Boyden chamber for 4 h in the presence of the drugs. Random migration (PDGF-BB on both sides of the filter) is referred to as 100% migration. Migrating cells were estimated as described under “Materials and Methods.” The data show means ± S.E. of at least three experiments. *, significantly different from control at $p < 0.05$ (Student’s $t$ test).

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Migration of PDGFR-β Wild Type and Mutant PAE Cells toward PDGF-BB and Effects of Phosphatase Inhibitors—To examine the potential role of SHP-2 in PDGF-BB-stimulated chemotaxis, PAE cells expressing wild type, Y1009F, or Y1021F PDGFR-β were examined in a mini-Boyden chamber. Cells were loaded on one side of an 8-μm micropore nitrocellulose filter, and PDGF-BB at different concentrations was loaded on the other side. The ability of cells to move through the filter, toward the chemottractant, was measured by Giemsa staining of the filter and counting of the cells. As shown in Fig. 4A, PDGF-BB stimulated a dose-dependent increase in cell migration, with a maximal migration occurring at 100 ng/ml PDGF-BB. PAE cells expressing the Y1021F mutant receptor also migrated efficiently but with a different dose optimum, 50 ng/ml PDGF-BB, as compared with the wild type receptor cells. In contrast, PAE cells expressing the Y1009F mutant PDGFR-β migrated only very inefficiently at high doses of PDGF-BB (Fig. 4A). These results indicate that receptor binding of SHP-2 is a requirement for PDGF-BB-stimulated migration. We further examined the effects of PAO and orthovanadate on PDGF-BB-stimulated migration of PAE cells expressing the wild type PDGFR-β. As seen in Fig. 4B, inclusion of PAO during the migration assay led to a dose-dependent decrease of PDGF-BB-stimulated (10 ng/ml) migration. Orthovanadate treatment also led to a decrease in PDGF-BB-stimulated migration (Fig. 4C). 1 μM PAO and 300 μM or-
thovanadate efficiently suppressed PDGF-BB-stimulated and basal cellular migration (Fig. 4, B and C). The results are in accordance with a role for SHP-2 in PDGF-BB-stimulated migration.

Cellular migration involves reorganization of the actin cytoskeleton, and veil-like actin-dense structures at the cell margin, denoted membrane edge ruffles, have previously been shown to be an integral part of cellular motility (20). We examined PAE cells expressing the wild type PDGFR-β or the Y1009F and Y1021F mutants, respectively, for their ability to respond to PDGF-BB stimulation with formation of membrane edge ruffles. Fig. 5 shows that edge ruffles were formed both on cells expressing wild type PDGFR-β and on the Y1021F cells but that no ruffles were detected on the PDGF-BB-stimulated Y1009F cells. About 90% of the PDGF-treated wild type receptor and Y1021F receptor cells exhibited membrane edge ruffles, whereas 4% of the Y1009F cells showed these structures. These data agree with the notion that cellular migration is dependent on the formation of these membrane extensions.

Synergistic Effects of SHP-2 and PI 3-Kinase Inhibitors—It has been shown previously that activation of PI 3-kinase is a prerequisite for PDGF-induced migration of cells (7, 8). In agreement, Fig. 4, D and E, show a dose-dependent decrease in PDGFR-β-mediated migration of cells toward PDGF-BB when treated with the PI 3-kinase inhibitors wortmannin and LY294002. The efficiency of inhibition of migration of cells treated with LY294002 was less than for wortmannin-treated cells, possibly since LY294002 appears to be a more specific PI 3-kinase inhibitor. We tested whether activation of PI 3-kinase was disturbed in cells expressing the SHP-2 binding site mutant receptor and in cells treated with the phosphatase inhibitors. PAE cells expressing the Y1009F receptor, as well as wild type PDGFR-β cells treated or not with PAO, were stimulated with PDGF-BB and tested for PI 3-kinase activation in vitro. This was performed by immunoprecipitation with anti-receptor antiserum and incubation of the precipitate in the presence of 

\[ ^{32}P\text{-ATP}\]

and [γ-ATP]. Fig. 6, A and B, shows that PDGF treatment induced phosphorylation of PI to similar extents in cells expressing the wild type and mutant Y1009F PDGFR-β and that the presence of PAO had no effect. These data indicate that PI 3-kinase and SHP-2 regulate migration through dis-
distinct signal transduction pathways.

To substantiate this assumption, PAE cells expressing the wild type PDGFR-β were treated with suboptimal concentrations of inhibitors for PI 3-kinase (wortmannin or LY294002) or SHP-2 (PAO or orthovanadate), individually or in combination. As seen in Table I, low doses of either of these drugs had no inhibitory effect or a weak inhibitory effect on PDGF-induced migration. In contrast, treatment with combinations of the drugs at these low concentrations led to suppression of migration. The combination of PAO and wortmannin, as well as the combination of orthovanadate and LY294002, led to a specific reduction in stimulated migration, indicating that PI 3-kinase and SHP-2 act through independent and synergistic molecular mechanisms.

Regulation of Focal Adhesion Kinase Activity in PDGF-BB-stimulated PDGFR-β Wild Type and Mutant PAE Cells—

PDGF is known to induce activation of p125 FAK (28). Targeted inactivation of the FAK gene leads to loss of cellular migration (29). To examine whether SHP-2 participates in regulation of PDGF-induced FAK activation, lysates of PDGF-stimulated PAE cells expressing the wild type or Y1009F or Y1021F mutant PDGFR-β were immunoprecipitated with a monoclonal antibody reactive with FAK. The immunoprecipitates were incubated in kinase buffer and [γ-32P]ATP. Fig. 7 shows that FAK kinase activity was induced in the wild type receptor cells in a dose-dependent manner, with a maximal response seen at 5 ng/ml PDGF (Fig. 7D). At higher concentrations, FAK activity declined, in agreement with data in previous reports showing dose-dependent fluctuations in PDGF-induced tyrosine phosphorylation of p125 FAK (28). In cells expressing the Y1021F mutant, a similar concentration-dependent induction of FAK kinase activity was seen (Fig. 7B). The -fold induction of FAK kinase activity was consistently slightly lower in the Y1021F cells, possibly implicating PLC-γ in FAK activation. PDGF treatment largely failed to affect FAK kinase activity in the Y1009F mutant receptor cells (Fig. 7C).

DISCUSSION

In this paper, we show that PDGF-induced migration of cells expressing PDGFR-β is dependent on the association of SHP-2 with the PDGF receptor. Cells expressing a mutant Y1009F PDGFR-β, in which the binding site for SHP-2 is removed, failed to migrate toward PDGF. PDGF receptor immunoprecipitates from these cells did not contain any phosphatase activity, but we did detect increased accumulation of inositol phosphate in the PDGF-stimulated Y1009F cells, although not to the levels seen in wild type receptor cells. For comparison, we examined cells expressing another PDGFR-β mutant, Y1021F, which does not bind PLC-γ. These cells migrated efficiently toward PDGF. Immunoprecipitated Y1021F recep-
How is SHP-2 involved in PDGF-induced cellular migration? PDGF-dependent changes in FAK activity were attenuated in cells expressing the mutant Y1009F mutant PDGFR-β, indicating that SHP-2 could be critical for regulation of FAK kinase activity. Moreover, cells treated with the phosphotyrosine phosphatase inhibitors phenylarsine oxide and orthovanadate migrated only very inefficiently toward PDGF-BB and failed to respond to PDGF-BB stimulation with induction of FAK activity. p125 FAK was originally identified as an abundantly tyrosyl-phosphorylated protein in v-Src-transformed cells (30). FAK associates with integrins in focal contact sites and clustering of integrins via binding to their extracellular matrix ligands leads to activation of FAK. Targeted knock-out of the fak gene in mice generates mutant embryos with a general defect in mesoderm development, and cells from the embryos show reduced motility in vitro (29). The number of focal contacts is increased in the mutant embryos, indicating that FAK activity is required for turnover of focal contacts. How FAK activity regulates the turnover of focal contacts remains to be shown. We suggest that regular cycles of activation and inactivation of FAK kinase activity could be a mechanism for the turnover of focal contacts. SHP-2 has been shown to regulate p125 FAK activity in insulin-stimulated cells (31). Moreover, a direct correlation between the level of FAK tyrosine phosphorylation and assembly of focal adhesion in focal contacts has been reported (32). In suspended cells, complex formation between SHP-2 and FAK has been identified (33). The complex formation appears to diminish in conjunction with attachment, and we have not been able to identify PDGF-induced SHP-2-FAK complex formation by immunoblotting in our cell model. In this work, we have measured the level of FAK activity by in vitro kinase activity of immunoprecipitated FAK in the different conditions. It was striking that the pattern of deregulated FAK activity was similar between cells expressing the mutant Y1009F PDGFR-β and cells expressing the wild type PDGFR-β treated with phosphatase inhibitors. Under these conditions, we failed to detect changes in the net amount of phosphorysine in FAK, as measured by immunoblotting using anti-phosphotyrosine antibodies (data not shown), which implies that the level of phosphorysine in FAK may not reflect FAK kinase activity, as suggested previously (34).

Other PDGFR-β-coupled signal transduction molecules have been shown to be critical for membrane edge ruffling and chemotaxis of cells toward PDGF. Thus, PI 3-kinase has been implicated in regulation of actin and in migration induced by different growth factors, most likely via regulation of the monomeric GTP-binding protein Rac (35), which is linked to rearrangement of the actin cytoskeleton (36). Moreover, PI 3-kinase has been shown to regulate FAK activity in PDGF-stimulated cells (37), which would be of consequence for cellular migration. By treating cells with low doses of PI 3-kinase inhibitor wortmannin or LY294002 in combination with the phosphotyrosine phosphatases PAO and orthovanadate, we could show that migration was regulated synergistically by PI 3-kinase and SHP-2, indicating that these signaling molecules regulate independent pathways. It is likely that these pathways converge at some point, e.g., by affecting FAK function, but our data indicate that they originate independently and that SHP-2 does not regulate PI 3-kinase activity.

Furthermore, PLC-γ has been shown to have a regulatory role in PDGF-stimulated migration (8, 9). Using a thick filter assay, where the migration distance is measured, we have previously examined the effect of a double mutation at Tyr1009 and Tyr1021 replacing the tyrosine residues with phenylalanine residues, thereby creating the mutant receptor Y1009F/Y1021F. PAE cells expressing Y1009F/Y1021F migrate as efficiently as the wild type PDGFR-β cells (7), which implies that PLC-γ has a regulatory role that balances the loss of SHP-2. The complexity of this regulation is indicated by the data reported by Hansen et al. (9), who described a mutant PDGFR-β Y943F, which allows increased tyrosine phosphorylation of PLC-γ and mediates cellular migration with increased efficiency. How PLC-γ exerts its modulatory role in chemotaxis is not clear. Different second messengers downstream of PLC-γ, such as Ca²⁺, or members of the protein kinase C family could be involved in mediation of this effect. Treatment of the Y934F mutant receptor cells with the protein kinase C inhibitor bisindolylmaleimide attenuated chemotaxis toward PDGF, in contrast, wild type PDGFR-β cells were able to...
migrate in the presence of the protein kinase C inhibitor (9). In accordance, our data show that loss of PLC-γ binding and inositol phosphate formation does not affect PDGF-induced migration (cf. Fig. 4A). Thus, the role of PLC-γ in growth factor-induced chemotaxis appears to be dependent on the balance of negative and positive signal transduction pathways affecting cellular migration.

In a recent study by Yu et al. (33), fibroblasts isolated from mouse embryos with targeted inactivation of the shp-2 gene were shown to display an increased number of focal adhesions, deregulated tyrosine phosphorylation of FAK, and decreased cellular motility on fibronectin. Our data agree with and extend the study by Yu et al., by showing that SHP-2 has a critical role, possibly by regulating FAK kinase activity, in growth factor-stimulated actin reorganization and chemotaxis.

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Note Added in Proof—In agreement with our data Rönstrand et al. (Rönstrand, L., Arvidsson, A., and Heldin, C. H. (1999) Oncogene, in press), describe reduced migration of cells expressing a PDGFR-β mutant lacking several SHP-2 binding sites.

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