Structural Determinants in the Platelet-derived Growth Factor α-Receptor Implicated in Modulation of Chemotaxis*

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Activation of the platelet-derived growth factor (PDGF) β-receptor leads to cell growth and chemotaxis. The PDGF α-receptor also mediates a mitogenic signal, but fails to induce cell migration in certain cell types. To examine this difference in signal transduction, a series of point-mutated PDGF α-receptors were analyzed. Porcine aortic endothelial cells expressing mutant PDGF α-receptors, in which tyrosine residues 768, 993, or 1018 were changed to phenylalanine residues migrated toward PDGF, whereas wild-type α-receptors and mutant α-receptors changed at tyrosine residues 720, 944, or 988 failed to migrate. All mutant receptors were mitogenically active and their capacity to activate phosphatidylinositol 3'-kinase and phospholipase C-γ was not different from that of the wild-type receptor. Tyr-768 was found to be the abnormally phosphorylated in PDGF-stimulated cells; in the Y768F mutant, there was a considerable increase in phosphorylation of Ser-767. Tyr-993 was not phosphorylated, but mutation of this tyrosine residue to a phenylalanine residue resulted in increased efficiency of phosphorylation on Tyr-988. Tyr-1018 is known to be an autophosphorylation site (7). Nine and three autophosphorylation sites have so far been identified in the PDGF β- and α-receptors, respectively. Several of these have been shown to interact in a specific manner with certain SH2 domain-containing proteins (reviewed in Ref. 8). For the PDGF β-receptor, two autophosphorylation sites in the juxtamembrane domain (Tyr-579 and Tyr-581) mediate the binding of Src family kinases. There are four autophosphorylation sites in the kinase insert which bind Grb2 (Tyr-716), the regulatory subunit (p85) of phosphatidylinositol 3'-kinase (PI 3-kinase; Tyr-740 and Tyr-751), Nck (Tyr-751), and the GTPase activating protein (GAP) of Ras (Tyr-771). The two autophosphorylation sites in the carboxy-terminal tail (Tyr-1009 and Tyr-1021) mediate binding of the SH2 domain-containing phosphatase PTP1D/Syp and phospholipase C-γ (PLC-γ), respectively. The adapter proteins Shc and Shb seem to interact with multiple tyrosine residues (9, 37). In the case of the PDGF α-receptor, tyrosine residues 754, 988, and 1018 have been identified as autophosphorylation sites (10, 11). Among them, Tyr-1018 has been shown to mediate association of PLC-γ (11). Tyr-731 and Tyr-742 are important for the binding of PI 3-kinase (12), and are likely to be phosphorylated sites, although this has not been directly shown.

Both PDGF α- and β-receptors mediate mitogenic signals upon ligand stimulation, probably through multiple parallel signal transduction pathways (13–15). On the other hand, the migratory response of PDGF β-receptor expressing cells is critically dependent on activation of PI 3-kinase (16, 17). Although the PDGF α-receptor also activates PI 3-kinase, this receptor mediates migration in a cell type-specific manner. Thus, Swiss 3T3 cells and human granulocytes expressing endogenous PDGF α-receptors (18, 19) and 32D cells expressing the PDGF α-receptors after transfection (12) allows chemotaxis toward PDGF-AA. In other cell types, such as human foreskin fibroblasts (20), human monocytes (19), rat and baboon vascular smooth muscle cells (21), PDGF-AA fails to induce chemotaxis. In these cell types, which express both PDGF α- and β-receptors, PDGF-AA has also been shown to inhibit chemotaxis induced by PDGF-AB or -BB (19, 22). The mechanisms underlying the cell type-specific effect of the PDGF α-receptor on chemotaxis is still unknown.
Expression of the PDGF-β receptor after transfection in por-
cine aortic endothelial (PAE) cells, which lack endogenous
PDGF receptors, allows the cells to migrate toward PDGF-BB,
whereas PAE cells expressing the PDGF-α receptor do not
migrate toward PDGF-AA or -BB (23), thus offering a suitable
model for analysis of the molecular mechanisms responsible for
α-receptor-induced suppression of chemotaxis.

Here, we show that mutations of three tyrosine residues in
the α-receptor allows the transduction of a chemotactic re-
sponse of PAE cells expressing the mutant α-receptors. Char-
acterization of the pattern of autophosphorylation in these
mutants implicated the presence of multiple pathways medi-
ated via the PDGF α-receptor which control cellular
chemotaxis.

EXPERIMENTAL PROCEDURES

Cell Culture and Mutagenesis—Site-directed mutagenesis was
performed on a cDNA encoding the full-length human PDGF α-receptor
(24) using the Altered sites in vitro Mutagenesis System (Promega Corp.). The following dinucleotides were used for the mutagenesis:
5′-GAGACCAGACGAGGAGTGGTTATTTCTGCTT-3′ (Y729F), 5′-
GAGGATTTGTTATCTG-3′ (Y944F), 5′-GAGATTCTGAGAACTGTC-3′ (Y949F), and 5′-
GAGACCTGCTATCATCCTCCTC-3′ (Y1018F). All mutations were
confirmed by DNA sequencing. The wild-type and the mutated cDNAs
were inserted into the eukaryotic expression vectors pZip Neo or into pCDNA. The constructs were transfected into PAE cells (25) by electroporation and subsequently selected for neomycin resist-
ance as described previously (24). PAE cells expressing the wild-type
PDGF-β receptors have been described earlier (24).125I-PDGF-BB bind-
ing assays and Scatchard analysis were performed according to Mori
et al. (27). For serum starvation, subconfluent cultured cells were incu-
bated in Ham’s F-12 medium containing 0.5% fetal calf serum for 16
h.

The rabbit antisera PDGFR-3, specifically recognizing
the PDGF-β receptor (26) and PDGFR-7, recognizing the PDGF α-re-
teceptor, have been described earlier (23). The rabbit antiserum YSD
and SQT were raised against synthetic peptides corresponding to amino
acid residues 754–769 and 1042–1061, respectively, of the human
PDGF α-receptor. The mixed monoclonal antibodies against bovine
PLCγ were purchased from Upstate Biotechnology Inc. The monoclonal
antibody against RasGap was purchased from Santa Cruz Biotechnol-
yogy. The monoclonal anti-phosphotyrosine antibody PY20 was from
Afferini Research Products Ltd. Peroxidase-conjugated sheep anti-
mouse immunoglobulins were from Amersham Corp.

[3H]Thymidine Incorporation Assay—The ability of PDGF-BB to
stimulate DNA synthesis, measured by the incorporation of [3H]thymi-
dine, in the different transfected cell lines was performed as described
previously (26).

Chemotaxis Assay—The migration assay was performed in a modi-
fied Boyden chamber essentially as described before (19). Microscope
filters (thickness; 150 μm), pore size, 8 μm) coated with type-I collagen
solution at 100 μg/ml (Vitrogen 100; Collagen Corp.) were used in the assay.
Cells were trypsinized and resuspended in Ham’s F-12 medium supplemented with 10% FCS at the final concentration of 3 × 10^4/ml.
The cell suspension was placed in the upper chamber and the medium
containing various doses of PDGF-BB in Ham’s F-12, 10% FCS was
placed below the filter in the lower chamber. For each set of experi-
ments, the migration of the respective cell-clone in Ham’s F-12, 10%
FCS above and below the filter (stimulated random migration or che-
mokinesis) served as control and is referred to as 100% migration. All
experiments were performed in duplicate for each concentration of
PDGF-BB and at least two independent cell clones from wild-type or
each type of mutated receptors were analyzed. All the assays were read
in blind.

Immunoprecipitation and Immunoblotting—Starved cells were
treated with PDGF-BB for 1 h on ice, rinsed with ice-cold phos-
phate-buffered saline (PBS), and then lysed in the Nonidet P-40 lysis
buffer(20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 2.5 mM
EDTA, 500 μM Na3VO4, 1 μg aprotinin (Trasylol; Bayer) and 1 μg
phenylmethylsulfonyl fluoride (Sigma). Immunoprecipitation with rab-it antiserum or monoclonal antibody was performed at 4°C, followed by
incubation with protein A-Sepharose CL-4B (Pharmacia) for 30 min at
4°C. The reaction was terminated by adding 30% trichloroacetic acid. The
clarified cell lysates were immunoprecipitated using PDGFR-3 or
PDGFR-7. The Sepharose-immune complexes were subjected to PI 3-
kinae assay essentially as described by Wennström et al. (16). Briefly,
the immunoprecipitates were washed three times with PBS containing
1% Nonidet P-40, once with PBS, once with 0.1% Triton X-100 and
then with chilled water and once with 20% trichloroacetic acid. The
lysates were washed three times with PBS, followed by 200 mM LiCl
and 100 μM NaCl, 1 mM EDTA on ice. The Sepharose beads were
washed in 50 μl of 20 mM Tris-HCl, pH 7.5, 7.100 μM NaCl and
then incubated at room temperature for 10 min. Ten μl of [γ-32P]ATP
and MgCl2 (final concentration, 10 μM) were added and the samples
were further incubated for 10 min. Reactions were stopped by addition
of chloroform, methanol, 11.6 M HCl (50:100:1), phospholipids were
extracted with chloroform and the organic phase was washed with
methanol, 1 M HCl (3:1). Incubations were performed in vacuo,
dissolved in chloroform, spotted on Silica Gel 60 plates (Merck)
impregnated with 1% potassium oxalate, and resolved by chromatography
during 30 min. The radioactive compounds were visualized by autoradiography.

Measurement of Inositol Phosphate Formation—Measurement of
total inositol phosphates was performed according to Kazawa et al. (28).
Cells were labeled with [3H]inositol (1.5 μCi/ml) in serum-free,
inositol-free Ham’s F-12 medium for 48 h. The labeled cells were
treated twice with an assay buffer (5 mM Hepes, pH 7.4, 150 mM NaCl,
5 mM KCl, 5.5 mM glucose, 1.8 mM MgSO4, and 1 μM CaCl2) and then
incubated in the same buffer containing 0.01% bovine serum albu-
min and 200 μM LiCl for 20 min at 37°C. The cells were then incubated
without or with 50 ng/ml PDGF-BB or with 5% FCS, for 15 min at
37°C. The reaction was terminated by adding 30% trichloroacetic acid.
The acid supernatant was recovered, treated with diethyl ether to remove
the organic layer, and neutralized with 0.1 μl NaOH. Dimethyl-
propylamine was applied to an anion exchange column containing 1 M
Dowex AG1-X8 (100–200 mesh, formate form). The resin was washed with 8 column
volumes of H2O and then with 20 volumes of a solution containing 5 M
disodium tetraborate and 60 mM sodium formate. Finally, total inositol
mono-, bis-, and tetrakisphosphates were eluted from the resin by
adding of 0.1 M formic acid containing 1 mM ammonium formate and
subjected to scintillation counting.

Covalent Coupling of Antibody to Protein A-Sepharose—The anti-
serum of interest was incubated with an equal volume of protein
A-Sepharose (1:1 slurry) for 1 h at 4°C. The antibody-protein A-Sepharose
was washed three times with PBS, twice in 0.1 M sodium borate, pH 9.0,
and then resuspended in the same buffer. Dimethylpimelimidate was
applied to an anion exchange column containing 1 M Dowex AG1-X8
(100–200 mesh, formate form). The resin was washed with 8 column
volumes of H2O and then with 20 volumes of a solution containing 5 M
disodium tetraborate and 60 mM sodium formate. Finally, total inositol
mono-, bis-, and tetrakisphosphates were eluted from the resin by
adding of 0.1 M formic acid containing 1 M ammonium formate and
subjected to scintillation counting.

Phosphorylation Labeling, Phosphopeptide Mapping, Edman Degradation,
and Phosphomono Acid Analysis—These procedures were
performed essentially according to Rönnstrand et al. (29). Briefly,
serum-starved cells were labeled by incubation in phosphate-free Ham’s
F-12 medium supplemented with 0.5% diazoylated fetal calf serum and 4
μCi/ml [32P]orthophosphate for 3 h at 37°C. Cells were treated with
100 ng/ml PDGF-BB in the same medium for 1 h at 4°C, and lysed in a
Tris-lysine buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Triton
X-100, 0.5% deoxycholate, 5 mM EDTA, 100 μM Na3VO4, 1 μg
Trasylol,
and 1 mM phenylmethylsulfonyl fluoride). Immunoprecipitation was performed using PDGF-R-7, the samples were separated by SDS-PAGE and then transferred onto nitrocellulose membrane. For in situ trypsin digestion, the radioactively labeled PDGF α-receptor was cut out after exposure to film, and incubated with 0.5% polyvinylpyrrolidone 40, 0.6% acetic acid for 30 min at 37 °C. The filter pieces were rinsed three times with water and then incubated for 12 h at 37 °C with 400 µl of 50 mM ammonium bicarbonate containing 1 µg of modified sequencing-grade trypsin (Promega). The supernatant was lyophilized, oxidized in performic acid for 1 h on ice, again lyophilized, resuspended in 50 µl of ammonium bicarbonate, and incubated with 1 µg of trypsin for another 12 h at 37 °C. The samples were lyophilized, dissolved in a pH 1.9 buffer consisting of 88% formic acid, acetic acid, water (50:35:6:794), and analyzed by two-dimensional phosphopeptide mapping on a cellulose plate, using electrophoretic separation at pH 1.9 in the first dimension, followed by ascending chromatography (isobutyric acid, pyridine, glacial acetic acid, H2O, 1-butanol, 65:53:29:2) in the second. Radioactive phosphopeptides were visualized by autoradiography. When necessary, phosphopeptides on plates were scraped off and then eluted with the same buffer as for the mapping. In case of immunoprecipitation of trypptic fragments, lyophilized trypptic digests were dissolved in 50 mM ammonium bicarbonate and incubated for 2 h at 4 °C with YSD-antiserum covalently coupled to protein A-Sepharose. The Sepharose was washed three times with 50 mM ammonium bicarbonate, 0.05% Triton X-100, twice with 150 mM ammonium bicarbonate, 0.5% Triton X-100, and twice with distilled water. The immunoprecipitates were then eluted with 1% diethylamine, pH 11.9. The purified peptides were coupled to a Sequelon-AA membrane (Milligen/Biosearch, Burlington, MA) according to the manufacturer's instructions, and Edman degradation was run in an Applied Biosystems gas-phase sequencer. For cyanogen bromide cleavage, the piece of nitrocellulose membrane with autophosphorylated receptors was rinsed with water and incubated with 30% formic acid containing 100 mg/ml cyanogen bromide for 12 h at 25 °C. The sample was lyophilized, dissolved in 50 mM ammonium bicarbonate, and immunoprecipitated with SQT-antiserum. The eluted material was oxidized, subjected to trypptic digestion, and analyzed by two-dimensional phosphopeptide mapping. For phosphoamino acid analysis, peptides were hydrolyzed in 6 N hydrochloric acid for 1 h at 110 °C, separated by two-dimensional electrophoresis on a cellulose plate, and then examined by autoradiography.

RESULTS

Characterization of PAE Cell Lines Expressing Y720F, Y768F, Y944F, Y988F, Y993F, and Y1018F Mutants of the PDGF α-Receptors—Signal transduction by receptor tyrosine kinases is dependent on interactions between autophosphorylated tyrosine residues in the receptors and SH2-domain containing intracellular signaling proteins. In the closely related PDGF α- and β-receptors, certain tyrosine residues are positioned in regions conserved between the two receptors, whereas other tyrosine residues are unique for each receptor (3). We reasoned that the uniquely positioned tyrosine residues could be involved in α-receptor-specific signaling, e.g. transduction of a negative effect on chemotaxis. Thus, the codons for the unique tyrosine residues 720, 768, 944, 988, 993, and 1018 of the wild-type human PDGF α-receptor cDNA were changed individually to phenylalanine codons by site-directed mutagenesis, generating Y720F, Y768F, Y944F, Y988F, Y993F, and Y1018F receptor mutants. PAE cells which lack endogenous PDGF receptors, were stably transfected with cDNAs for the different mutants as well as with wild-type PDGF α-receptor cDNA. PAE cells expressing the wild-type PDGF β-receptors were prepared as described previously (27). The numbers of receptors expressed per PAE cell, estimated by 125I-PDGF-BB binding experiments, were similar for the different wild-type and mutant receptor expressing cell lines (Table I). Furthermore, as judged from the results of immunocomplex kinase assays, all the different mutant receptors were ligand-stimulatable kinases. Thus, the fold-induction of kinase activity in response to PDGF was the same for the wild-type and mutant receptors (Fig. 2). To further characterize the mutant receptor expressing cell lines, [3H]thymidine incorporation assays were performed (Table II). The mutant expressing cell types as well as the wild-type α-receptor cells all responded mitogenically to PDGF-BB to a similar extent. PAE cells expressing the wild-type PDGF β-receptor showed a slightly higher mitogenic response. Mutations at Certain α-Receptor-Unique Tyrosine Residues Enable the Mutant Receptors to Mediate Chemotaxis—PAE cells expressing the PDGF α-receptor after transfection respond mitogenically to PDGF-BB, but fail to migrate toward PDGF-BB or PDGF-AA (23). To examine the effect of mutations at uniquely positioned tyrosine residues in the PDGF α-receptor on the ability to mediate a chemotactic response, PAE cells expressing the wild-type or mutant PDGF α-receptors were examined using a modified Boyden chamber assay. For comparison, PAE cells expressing the wild-type PDGF β-receptors were also examined. Cells were suspended in Ham's F-12 medium containing 10% FCS and placed above an 150-µm thick filter in the Boyden chamber. Various doses of PDGF-BB in Ham's F-12, 10% FCS were added below the filters and the

![Fig. 1. Schematic illustration of the location of tyrosine residues in the intracellular domains of the PDGF α- and β-receptors and their interacting SH2 domain-containing proteins.](http://www.jbc.org/)

![Table I. Number of receptors on wild-type and tyrosine residue-mutated PDGF α-receptors expressed on PAE cells.](http://www.jbc.org/)
Experimental Procedures.

Acid-precipitable radioactivity was measured as described under “Experimental Procedures,” followed by SDS-PAGE, and autoradiography. The samples were subjected to kinase assays as described in “Experimental Procedures,” and analyzed under “Experimental Procedures,” and autoradiography.

**TABLE II**

**Chemotaxis of cells expressing the wild-type PDGF β-receptors, the wild-type, or mutant PDGF α-receptors.**

| Receptor type          | N[\(^3\)H]Thymidine incorporation (\% of non-stimulated control; mean ± S.D.) |
|------------------------|--------------------------------------------------------------------------------|
| Wild-type β            | 262 ± 37                                                                       |
| Wild-type α            | 192 ± 17                                                                       |
| Y720F α                | 183 ± 22                                                                       |
| Y768F α                | 186 ± 5                                                                        |
| Y944F α                | 170 ± 2                                                                       |
| Y988F α                | 198 ± 6                                                                       |
| Y993F α                | 191 ± 17                                                                       |
| Y1018F α               | 194 ± 12                                                                       |

Stimulation of [\(^3\)H]thymidine incorporation by PDGF-BB in PAE cells expressing either the wild-type PDGF β-receptor, the wild-type or tyrosine residue-mutated PDGF α-receptors. After 48 h of serum starvation, cells were incubated with 0.5 mCi/ml [\(^3\)H]thymidine and 10 ng/ml PDGF-BB at 37°C for 24 h. Trichloroacetic acid-precipitable radioactivity was measured as described under “Experimental Procedures.”

**FIG. 2.** Ligand-stimulated kinase activity of the wild-type β, wild-type α, and the tyrosine residue-mutated PDGF α-receptors. PAE cells expressing the wild-type and mutant PDGF receptors were incubated without (−) or with (+) 100 ng/ml PDGF-BB for 30 min at 4°C. The cells were lysed and immunoprecipitated with anti-receptor antisera. The precipitated material was characterized with regard to in vitro phosphorylation of phosphatidylinositol (PI) as assessed by liquid chromatography. As seen in Fig. 4A, the wild-type α- and β-receptors and the chemotactic mutant PDGF α-receptors, showed similar extents of phosphatidylinositol phosphate formation upon PDGF-BB stimulation.

It has been suggested that the SH2 domain-containing proteins PLC-γ and RasGAP are involved in modulation of the chemotactic response (17, 31). PLC-γ in a positive and RasGAP in a negative manner. We examined complex formation and tyrosine phosphorylation of these signaling proteins upon re-
ceptor activation. The different cell types were stimulated with PDGF-BB for 30 min on ice and then lysed. The lysates were immunoprecipitated either with anti-PLC-γ antiserum or with anti-receptor antiserum, separated by SDS-PAGE, transferred onto nitrocellulose membranes; the membranes were then immunoblotted with the anti-phosphotyrosine antibody PY20. Upon ligand stimulation of wild-type PDGF β-receptor, wild-type α-receptor, and the mutant Y768F and Y993F α-receptors, PLC-γ became tyrosine phosphorylated and was found in complex with the autophosphorylated receptors. Phosphorylation of PLC-γ was not detected upon activation of mutant Y1018F PDGF α-receptors, in agreement with the recent characterization of Tyr-1018 as the major PLC-γ binding site in the PDGF α-receptor (11). Eriksson et al. (11) also showed that a higher extent of complex formation between the receptor and PLC-γ is observed upon activation of the PDGF α-receptor compared to the β-receptor; however, tyrosine phosphorylation and consequently activation of the catalytic activity of PLC-γ, is consid-

### Table: Fold-increase of PIP2 upon PDGF stimulation

|                | wild-type β | wild-type α | Y768F α | Y993F α | Y1018F α |
|----------------|-------------|-------------|---------|---------|---------|
| Fold-increase of PIP2 upon PDGF stimulation | 15.8        | 11.7        | 10.8    | 12.0    | 8.4     |

**Fig. 4.** A, thin-layer chromatography of PI 3-kinase reaction products from unstimulated or ligand stimulated cells. Cells expressing the wild-type PDGF β-receptors, wild-type PDGF α-receptors, Y768F, Y993F, or Y1018F mutant PDGF α-receptors were incubated without (-) or with (+) 50 ng/ml PDGF-BB for 5 min at 37 °C. After incubation, the cells were lysed and immunoprecipitated with anti-receptor antiserum. The immunoprecipitates were subjected to PI 3-kinase assays and the PI 3-kinase reaction products were analyzed by thin-layer chromatography followed by autoradiography. The positions of phosphatidylinositol phosphate and the origin (ORI) are indicated. B, tyrosine phosphorylation of PLC-γ after PDGF-BB stimulation of PAE cells expressing wild-type or tyrosine residue-mutated PDGF receptors. Cells expressing the wild-type PDGF β-receptors, wild-type PDGF α-receptors, Y768F, Y993F, or Y1018F mutant PDGF α-receptors were incubated without (-) or with (+) 100 ng/ml PDGF-BB for 30 min at 4 °C. After incubation, the cells were lysed and immunoprecipitated with anti-PLC-γ monoclonal antibody (left panel) or with anti-receptor antiserum (right panel). The immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The blots were probed with either anti-phosphotyrosine monoclonal antibody PY20 or with anti-PLC-γ antibody and then visualized using ECL Western blotting detection system (Amersham). Migration positions of the PDGF β-receptors (βR), PDGF α-receptors (αR), and PLC-γ are indicated. Density of the detected bands were measured by densitometric scanning. The relative densities of the tyrosine phosphorylated PLC-γ-tyrosine phosphorylated PDGF receptor for the wild-type β, wild-type α, Y768F α, Y993F α, and Y1018F α-receptors were 1.1, 0.074, 0.095, 0.098, and 0.000, respectively. C, formation of inositol phosphates in PAE cells expressing wild-type or tyrosine-residue mutated PDGF receptors. Cells labeled with myo-[3H]inositol (1.5 μCi/ml) for 48 h were incubated in presence of vehicle (open bars), 50 ng/ml PDGF-BB (solid bar), or 5% FCS (shaded bars) for 15 min at 37 °C. The samples were quenched by addition of 30% trichloroacetic acid. The total inositol phosphates were separated by anion exchange chromatography and the amount measured by scintillation counting. The values represent means ± S.D. of triplicate determinations.
erably more efficient in activated PDGF β-receptor cells than in PDGF α-receptor cells. There was no difference in the efficiency of tyrosine phosphorylation of PLC-γ between the wild-type α-receptor and the Y768F and Y993F mutant α-receptors as assessed by densitometric scanning of the immunoblots in Fig. 4B. In order to examine whether the efficiency of induction of the enzymatic activity of PLC-γ differed between the wild-type and the chemotactic mutant α-receptors, PDGF-induced formation of inositol phosphates was measured (Fig. 4C). Cells were labeled with myo-[3H]inositol for 48 h, incubated with or without 50 ng/ml PDGF-BB for 15 min at 37 °C, and the samples were extracted by addition of 30% trichloroacetic acid to the cell monolayer. Total inositol phosphates were collected using AG 1-X8 formate resin. Upon PDGF-BB stimulation, the total amount of radioactively labeled inositol phosphates increased to 160% of the control value in the wild-type β-receptor expressing cells, while the wild-type α-receptor expressing cells showed only a slight increase. The difference in the level of increase in total inositol phosphates between the PDGF β- and α-receptors is consistent with results previously described for primary human fibroblasts (11) and rat vascular smooth muscle cells (32). Like in the wild-type α-receptor, the extent of accumulation of total inositol phosphates was negligible in the Y768F, Y993F, or Y1018F chemotactic mutant α-receptors. Thus, it is not likely that PLC-γ catalytic activity is involved in mediation of chemotaxis of the mutant α-receptor cells.

Upon ligand stimulation of the wild-type PDGF β-receptor expressing cells, RasGAP was tyrosine phosphorylated and in complex with the receptor, as judged from communoprecipitation of the receptor when a RasGAP antiserum was used. However, neither activation of the wild-type nor the chemotactic mutant PDGF α-receptors induced phosphorylation or association of RasGAP with the receptors (data not shown). This is in agreement with previous reports by Heideran et al. (33) and Bazenet et al. (34), in which RasGAP is described to be a substrate for the PDGF β-receptor but not for the α-receptor.

In conclusion, these observations argue against modulation of PDGF α-receptor-mediated tyrosine phosphorylation or activation of PI 3-kinase, RasGAP, or PLC-γ, as a reason for the gain of chemotactic ability of the Y768F, Y993F, and Y1018F mutant α-receptors.

Tyr-762, Tyr-768, and Ser-767 Are in Vivo Phosphorylation Sites in the PDGF α-Receptor—In order to investigate the molecular mechanisms for the gain of chemotactic capacity of the mutant PDGF α-receptors, we first examined whether Tyr-768 is an autophosphorylation site in the PDGF α-receptor. For this purpose, PAE cells expressing the wild-type or Y768F PDGF α-receptors were labeled with [32P]orthophosphate, incubated with PDGF-BB, lysed, and immunoprecipitated with the anti-α-receptor antisera and with a polyclonal antibody which specifically recognizes Tyr-768 and the surrounding amino acid residues (YSD antiserum). The immunoprecipitates were eluted and subjected to Edman degradation. As shown in Fig. 5C, upper panel, radioactive peaks were detected at cycles 3, 8, and 9 in the wild-type receptor fragment, corresponding to Tyr-762, Ser-767, and Tyr-768. Phosphoamid acid analysis of the immunoprecipitated peptide revealed phosphorylation strongly on tyrosine and weakly on serine. In the corresponding analysis of the Y768F PDGF α-receptor (Fig. 5C, lower panel), peaks of phosphorylation were seen at cycles 3 and 8, which corresponded to Tyr-762 and Ser-767. Radioactivity in cycle 9 most likely represents trailing from cycle 8. The result of phosphoamid acid analysis of the immunoprecipitated Y768F peptide revealed a dramatic increase in the ratio of serine phosphorylation to tyrosine phosphorylation compared to that of the corresponding peptide from the wild-type receptor. The ratios of the radioactive content of the Tyr-768-containing spot in the wild-type receptor fragmentation chromatography was run extensively to obtain high resolution of the Tyr-768-containing spot in the wild-type receptor map, which resulted in loss of some phosphopeptides from the TLC plate. When the peptides were run for a shorter time in the ascending chromatography, at least two phosphopeptide spots were found in the upper right corner of the Y768F receptor map but not in the wild-type receptor map (data not shown). One of the two peptides was phosphorylated on serine and radioactivity appeared at cycle 8, the other showed tyrosine and serine phosphorylation and radioactivity appeared at cycles 3 and 8, as judged from phosphoamid acid analysis and Edman degradation (data not shown). These phosphopeptides most likely represented the peptides containing Tyr-762 and Phe-768.

In order to confirm phosphorylation on tyrosine residues 762 and 768, and to further examine serine phosphorylation in the peptide, trypsin digests from the in vivo [32P]-labeled wild-type and Y768F PDGF α-receptors were immunoprecipitated with an antiserum which specifically recognizes Tyr-768 and the surrounding amino acid residues (YSD antiserum). The immunoprecipitates were eluted and subjected to Edman degradation. As shown in Fig. 5C, upper panel, radioactive peaks were detected at cycles 3, 8, and 9 in the wild-type receptor fragment, corresponding to Tyr-762, Ser-767, and Tyr-768. Phosphoamid acid analysis of the immunoprecipitated peptide revealed phosphorylation strongly on tyrosine and weakly on serine. In the corresponding analysis of the Y768F PDGF α-receptor (Fig. 5C, lower panel), peaks of phosphorylation were seen at cycles 3 and 8, which corresponded to Tyr-762 and Ser-767. Radioactivity in cycle 9 most likely represents trailing from cycle 8. The result of phosphoamid acid analysis of the immunoprecipitated Y768F peptide revealed a dramatic increase in the ratio of serine phosphorylation to tyrosine phosphorylation compared to that of the corresponding peptide from the wild-type receptor. The ratios of the radioactive content of cycle 8 to cycle 3 were 0.26 for the wild-type peptide and 1.57 for the Y768F peptide, which suggested that substitution of Tyr-768 to a phenylalanine residue resulted in considerable enhancement of phosphorylation on Ser-767.

Replacement of Tyr-993 with a Phenylalanine Residue Enhances Autophosphorylation at Tyr-988—We next analyzed whether Tyr-993 is autophosphorylated in vivo. In a two-dimensional analysis of a tryptic digest of the Y993F α-receptors generated after in vivo [32P] labeling as described above, there was no difference as compared to the pattern of the wild-type α-receptor tryptic digest suggesting that Tyr-993 is not an autophosphorylation site (Fig. 6A). This is in agreement with previous observations (11). However, a spot of high intensity showed up in the Y993F map (Fig. 6A, arrow). Extraction of the corresponding radioactive phosphopeptide followed by Edman
Fig. 5. A, two-dimensional tryptic phosphopeptide maps of the wild-type and Y768F PDGF α-receptors. Cells expressing the wild-type α or Y768F PDGF α-receptors were labeled in vivo with [32P]orthophosphate, stimulated with PDGF-BB, and immunoprecipitated with anti-receptor antiserum. The immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The bands corresponding to the PDGF α-receptors were cut out, digested with trypsin in situ, oxidized, and again extensively digested with trypsin. The resulting digests were separated electrophoretically at pH 1.9, followed by ascending chromatography. Radioactive phosphopeptides were visualized by autoradiography. The position of a phosphopeptide spot present in the wild-type α-receptor map but not in the Y768F α-receptor map is indicated by an arrowhead. In each case, the origin is marked with an open triangle. B, Edman degradation elution profile and phosphoamino acid analysis (inset) of the phosphopeptide indicated by an arrowhead in the wild-type PDGF α-receptor map in A. The phosphopeptide was extracted from TLC plate, subjected to Edman degradation, and the [32P] radioactivity in the fractions generated in each cycle was measured. The amino acid sequence of the tryptic peptide containing Tyr-768 is presented along with the fraction numbers. Migration positions of serine and tyrosine in the phosphoamino acid analysis are indicated as S and Y, respectively. C, Edman degradation and phosphoamino acid analysis of the tryptic peptides from the wild-type (upper panel) and Y768F (lower panel) PDGF α-receptors immunoprecipitated with YSD antiserum. The amino acid sequence of the YSD-immunoprecipitated peptide is presented along with the fraction numbers. Results of the phosphoamino acid analysis on the peptides are shown on the right-hand side of each panel.
degradations showed a peak of radioactivity at the seventh cycle (Fig. 6B). The peptide was mainly phosphorylated on tyrosine as judged from phosphoamino acid analysis (Fig. 6B, inset). There are four possible tryptic fragments from the PDGF \(\alpha\)-receptor containing a tyrosine residue at the seventh position from the amino terminus: one fragment from the juxtamembrane domain containing Tyr-613 (Fig. 6B, a), one from the kinase insert containing Tyr-742 (Fig. 6B, b), and two from the carboxyl-terminal tail containing Tyr-988 and Tyr-1018, respectively (Fig. 6B, c and d). Tyr-1018 has previously been shown to be autophosphorylated in vivo (14). Two-dimensional analysis of a tryptic digest from the Y1018F mutant receptor, after in vivo labeling, allowed identification of two spots representing the Tyr-1018-containing peptide (Fig. 6A, arrows). The location of the intense spot in the Y993F map was clearly different from the Tyr-1018-containing peptide spots.

We argued that Tyr-988 could be phosphorylated to an increased extent in the Y993F PDGF \(\alpha\)-receptor, because of the closeness of these residues. To examine this possibility, the following experiment was performed. The wild-type, Y988F, Y993F, and Y1018F PDGF \(\alpha\)-receptors were labeled in vivo, autophosphorylated, and immunoprecipitated according to the procedure described above. The receptor proteins were then chemically cleaved with cyanogen bromide resulting in peptide fragments of relatively large sizes. A group of fragments from the carboxyl-terminal tail of the receptors (amino acid residues 981 to 1072) were collected by immunoprecipitation using a specific antiserum raised against amino acid residues 1042 to 1061 of the \(\alpha\)-receptor (SQT antiserum). The immunoprecipitated fragments were further digested by incubation with trypsin and then analyzed by two-dimensional phosphopeptide mapping. The tryptic map derived from the immunoprecipi-
Phosphorylation.

Spot number 1. The spots 3, 4, and 5 were lacking in the Y988F α-receptor map, suggesting that these spots in the wild-type α-receptor map represented peptides containing Tyr-988. Interestingly, the radioactive content of spots 4 and 5 from the Y993F α-receptor was 50% higher as compared with the corresponding spots derived from the wild-type α-receptor. The radioactive contents of spots 1 and 2 were the same in the two cases. These results indicate that Tyr-988 is phosphorylated with increased stoichiometry following mutation of Tyr-993 to a phenylalanine residue, without affecting the phosphorylation efficiency on Tyr-1018.

DISCUSSION

It is well established that PDGF β-receptor expressing cells migrate efficiently toward PDGF-BB. Data based on β-receptors lacking the PI 3-kinase binding site (16, 17), as well as treatment with the PI 3-kinase inhibitor wortmannin and expression of a mutated PI 3-kinase p85 subunit, indicate that PI 3-kinase activity is crucial for β-receptor-mediated chemotaxis. The ligand-stimulated PDGF α-receptor also activates PI 3-kinase, but fails to induce chemotaxis in certain cell types. We show that point-mutated α-receptors can acquire the capacity to mediate chemotaxis of PAE cells, in which the wild-type α-receptor is chemotactically inactive. Our data imply that the lack of wild-type α-receptor-mediated chemotaxis in the PAE cells and other cell types is not due to a lack of ability to activate the signal transduction pathways leading to directed migration. Rather, one or more negative signals are also activated by the α-receptor which suppress the migratory response. Since PI 3-kinase was active in the ligand-stimulated chemo- tactic mutants, the negative signals are likely to act downstream of PI 3-kinase. PLC-γ, which has been described to be involved in positive regulation of chemotaxis in certain cell types (16, 17) is most likely not responsible for modulation of α-receptor-mediated chemotaxis, since the Y1018F mutant, which is unable to bind and activate PLC-γ, was able to mediate chemotaxis. RasGAP has been inferred to negatively modulate PDGF α-receptor-mediated chemotaxis (17). Neither the wild-type, nor the chemotactically active mutant α-receptors, associated with or phosphorylated RasGAP, argued against a role for RasGAP in α-receptor signaling.

The PDGF α-receptor can thus emit both positive and negative signals influencing the migratory response. The capacity to emit these signals appear to depend on the cellular environment. Interestingly, the negative signals can affect migration initiated by other ligand-receptor complexes. We have previously shown that activation of the PDGF α-receptor leads to inhibition of PDGF β-receptor-mediated chemotaxis, in a dose-dependent manner (19). Koyama et al. (21, 22) described that activation of the PDGF α-receptor in vascular smooth muscle cells led to inhibition of chemotaxis induced by the PDGF β-receptor, or by receptors for fibronectin and smooth muscle cell-derived growth factor. It is likely that the negative signals acting on the α-receptor-induced chemotaxis and on chemotaxis induced by other types of receptors are the same.

Koyama et al. (21, 22) showed that PDGF α-receptor-induced inhibition of fibronectin-mediated chemotaxis was attenuated by treatment of the vascular smooth muscle cells with the protein kinase C (PKC) inhibitor staurosporine suggesting that

| Receptor types | Spot No. | Intensity of the spots |
|---------------|----------|------------------------|
| Wild-type α  | 100      | 177                    |
| Y988F α      | 100      | 186                    |
| Y993F α      | 100      | 191                    |
| Y1018F α     | 38       | 15                     |

Fig. 7. A, two-dimensional tryptic phosphopeptide maps of cyanogen bromide-deaved fragments derived from the carboxy-terminal tail of wild-type, Y988F, Y993F, and Y1018F PDGF α-receptors. Cells expressing the wild-type, Y988F, Y993F, or Y1018F PDGF α-receptors were labeled in vivo with [32P]orthophosphate, stimulated with PDGF-BB, and immunoprecipitated with anti-receptor antisemur. The immuno precipitates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The band corresponding to the PDGF α-receptor was cut out, deaved with cyanogen bromide, and immunoprecipitated with anti-receptor antisemur. The immuno precipitated fragment was oxidized and digested with trypsin. The resulting digests were separated electrophoretically at pH 1.9, followed by ascending chromatography. Radioactive phosphopeptides were visualized by Bio-imaging analyzer BAS 2000 (Fujip photo film). The origin of each sample has been trimmed away in the figure, but is located outside the lower-left corner of each panel. B, quantification of radioactivity in the phosphopeptide spots in the tryptic maps in A. The intensities of the phosphopeptide spots (designated spots 1 to 5) in A were measured using BAS 2000. In order to compare the intensity values of the spots between the different receptor maps, the value for spot number 1 was fixed to 100 in each map. Intensities of other spots were calculated relative to the values for spot number 1. Arrows indicate the recorded major changes in phosphorlylation.

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PKC is a mediator of PDGF α-receptor negative signaling. However, another PKC inhibitor H-7, and the PKC activators 12-O-tetradecanoylphorbol-13-acetate and SC-9, had no effect in their study, which together with the rather low specificity of staurosporine for PKC (35), may suggest involvement of a different serine/threonine kinase in the negative signaling.

Diliberto et al. (36) showed that PDGF-BB (activating both α- and β-receptors) was more potent than PDGF-AA (activating only α-receptors) in stimulating Ca2+ fluxes in Balb/C3T3 fibroblasts. In addition, preincubation of the cells with PDGF-AA lead to inhibition of the PDGF-BB-induced increase in the intracellular Ca2+ levels, in part through PKC-dependent mechanisms. It is an interesting possibility, which remains to be tested, that differences in the modulation of intracellular Ca2+ levels account for the differences in chemotactic signaling between the wild-type α-receptor and α-receptor mutants.

Our data indicate that several mechanisms are operating in negative modulation of PDGF α-receptor negative signaling. Phosphorylation of Tyr-768, which is surrounded by the motif PASY768KKK, appears to allow negative modulation of chemotaxis, maybe by binding a specific signal transduction molecule. This signal transduction molecule would be activated by the α-receptor but not by the β-receptor, since there is no tyrosine residue with a similar environment in the β-receptor. It is, however, also possible that the gain of chemotactic ability of the Y768F α-receptor mutant was due to the increased phosphorylation of Ser-767 in the mutant receptor expressing cells, which could lead to positive modulation of chemotaxis. Analysis of a double mutant, simultaneously changed at Ser-767 and Tyr-768, will allow us to differentiate between these two alternatives. Tyr-762 was also shown to be an autophosphorylation site in our study. Although this residue is a positional homologue of Tyr-771 in the β-receptor, which is the binding site for RasGAP, the α-receptor does not bind RasGAP. Distinct surrounding motifs for the two tyrosine residues, RSLY762DRP in the α-receptor and SSNY771MAP in the β-receptor, probably account for the difference in binding of RasGAP. The α-receptor Tyr-993 is not phosphorylated, but we could show that there was increased phosphorylation of Tyr-988 in the Y993F mutant receptor expressing cells. Tyr-988 is surrounded by the motif DNA/Y988I/GV which is not found in the β-receptor. It is possible that the increased phosphorylation of Tyr-988 in the Y993F mutant receptor cells allows positive modulation of chemotaxis, by a more efficient recruitment of a specific signal transduction molecule.

The mechanism by which the Y1018F α-receptor mutant allowed chemotaxis is not clear. It is not likely that the loss of PLC-γ binding resulted in the chemotactic phenotype, since PLC-γ has been shown to act positively (17) or to be without effect (16) on β-receptor-mediated chemotaxis. Moreover, activation of the catalytic activity of PLC-γ is not prominent in ligand-stimulated wild-type or mutant α-receptor expressing PAE cells (Fig. 5) or in other cell types expressing endogenous receptors, such as vascular smooth muscle cells (32, 35) or human foreskin fibroblasts (11). It is possible that a signal transduction molecule distinct from PLC-γ can also bind to phosphorylated Tyr-1018 and mediate the negative signal for chemotaxis.

The different potential pathways for modulation of PDGF α-receptor-induced chemotaxis are summarized in Fig. 8. We suggest that autophosphorylated tyrosine residues 768 and 1018 serve to mediate negative regulation of chemotaxis, downstream of PI 3-kinase. In contrast, it is possible that Tyr-988 mediates a signal for positive regulation. The negative signaling for modulation of chemotaxis appears to act on the level of directed migration only, since both membrane edge ruffling,

FIG. 8. Schematic illustration of possible modes of modulation of chemotactic signaling by the PDGF α-receptor. Numbers indicate the positions of tyrosine residues. (P) indicates that the tyrosines are phosphorylatable. +, positive modulation; −, negative modulation; P13-K, phosphatidylinositol 3-kinase. Mutation of the phosphorylatable tyrosine residues 768 or 1018 to phenylalanine residues relieves a putative negative regulatory mechanisms, whereas mutation of Tyr-993 causes an increased autophosphorylation of Tyr-988 possibly resulting in an enhanced chemotactic signaling.

which can be regarded as an integral part of the cellular motility response (16), as well as random migration, were induced to the same level in ligand-stimulated wild-type and mutant α-receptor PAE cells, as in the wild-type PDGF β-receptor expressing cells. This is in agreement with the observation by Koyama et al. (21) that PDGF-AA inhibited PDGF-BB-induced chemotactic activity but not random migration of baboon smooth muscle cells. Identification of the signal transduction molecules that interact with the phosphorylation sites determined in this work, Tyr-768 and Tyr 988, and elucidation whether molecules other than PLC-γ interact with phosphorylated Tyr-1018, may reveal the basis for the cell-type specific pattern of α-receptor negative signaling. Such efforts should also broaden our understanding of how directed movement of cells is controlled.

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