Journal of Cell Science 111, 607-614 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS1516

PDGF α-receptor mediated cellular responses are not dependent on Src family kinases in endothelial cells

R. Hooshmand-Rad[1,*], K. Yokote[1,†], C.-H. Heldin[1] and L. Claesson-Welsh[2]

[1]Ludwig Institute for Cancer Research, Box 595, Biomedical Center, S-751 24 Uppsala, Sweden
[2]Department of Medical and Physiological Chemistry, Biomedical Center, Box 575, S-75123 Uppsala, Sweden
*Author for correspondence
[†]Present address: Second Department of Internal Medicine, Chiba University School of Medicine, 1-8-1 Inohana Chuo-ku, Chiba City, Chiba 260, Japan

Accepted 15 December 1997: published on WWW 9 February 1998

SUMMARY

Two novel autophosphorylation sites in the juxtamembrane region of the PDGF α-receptor, Tyr-572 and Tyr-574, were identified. A Y572/574F mutant PDGF α-receptor was generated and stably expressed in porcine aortic endothelial cells. In contrast to the wild-type receptor, the mutant receptor was unable to associate with or activate Src family tyrosine kinases. Tyrosine phosphorylated synthetic peptides representing the juxtamembrane sequence of the receptor dose-dependently inhibited the binding of Src family tyrosine kinases to the autophosphorylated PDGF α-receptor. The mutant receptor showed similar PDGF-induced kinase activity and ability to mediate mitogenicity, actin reorganization and chemotaxis as the wild-type receptor. Thus activation of Src family kinases by the PDGF α-receptor is not essential for PDGF-induced mitogenicity or actin reorganization.

Key words: PDGF α-receptor, Src, Mitogenicity

INTRODUCTION

PDGF is a homo- or hetero-dimeric protein composed of A- and B-polypeptide chains which binds to two structurally related tyrosine kinase receptors, designated α- and β-receptors (Claesson-Welsh et al., 1989; Matsui et al., 1989; Yarden et al., 1986). The PDGF α-receptor is able to bind both the A- and B-chains of PDGF, whereas the β-receptor binds only the B-chain (Heldin et al., 1988). The receptors are expressed on cells of mesenchymal origin, such as fibroblasts, glia cells and capillary endothelial cells, and on epithelial and neuronal cells (Heldin and Westermark, 1996).

The PDGF ligand-receptor pairs have been implicated in embryonal development and wound healing as well as several pathological conditions for example fibrosis, atherosclerosis, glomerulonephritis and malignancies (Heldin and Westermark, 1996; Raines et al., 1990). Mice carrying null mutations of either PDGF ligands or receptors have helped clarify the functional roles of these ligand-receptor pairs. Mice deficient for PDGF-B show renal, cardiovascular, and hematological abnormalities (Levéen et al., 1994). The phenotype of the PDGF β-receptor deficient mice is very similar (Soriano, 1994). On the other hand PDGF-A deficient mice show severe defects in alveologenesis (Boström et al., 1996). Deletion of the PDGF α-receptor gene is, however, embryonically lethal in mice, giving rise to skeletal defects and increased apoptosis along the migration pathway of cranial neural crest cells (Soriano, 1997).

Upon PDGF binding receptors dimerize, inducing receptor autophosphorylation and phosphorylation of intracellular substrates (Ullrich and Schlessinger, 1990). An important role for receptor autophosphorylation is to present binding sites for signal transduction molecules containing Src homology 2 (SH2) domains (Pawson, 1995). SH2 domains consist of approximately 100 amino acids which bind phosphorylated tyrosine residues within the context of a stretch of three to six amino acid residues specific for each SH2 domain, contained in activated receptors and cytoplasmic phosphoproteins. Several of the autophosphorylation sites in the PDGF receptors have been shown to interact in a specific manner with certain SH2 domain-containing proteins (Claesson-Welsh, 1994). Among the nine autophosphorylation sites identified in the PDGF β-receptor, two in the juxtamembrane domain (Tyr-579 and Tyr-581) mediate the binding of Src family kinases (Mori et al., 1993). In the PDGF α-receptor, tyrosine residues 720, 754, 762, 768, 988 and 1,018 have been identified as autophosphorylation sites (Bazenet et al., 1996; Eriksson et al., 1995; Rupp et al., 1994; Yokote et al., 1996). Tyr-1018 and tyr-988 have been shown to mediate association with PLC-γ (Eriksson et al., 1995) and Tyr-720 with SHP-2 (Bazenet et al., 1996). Although not shown to be phosphorylation sites, Tyr-731 and Tyr-742 are important for the binding of PI3-kinase (Yu et al., 1991).

Here we identify two novel autophosphorylation sites, Tyr-572 and Tyr-574, in the juxtamembrane region of the PDGF α-receptor, and show that they are required for binding and activation of Src family tyrosine kinases. In spite of reports on the important role of Src in PDGF-mediated mitogenicity

(Barone and Courtneidge, 1995; Twamley-Stein et al., 1993), the Y572/574F mutant, which is unable to activate Src, was found to mediate a mitogenic response to PDGF similar to the wild-type receptor.

MATERIALS AND METHODS

Cell culture and mutagenesis

Site-directed mutagenesis was performed on a cDNA encoding the full-length human PDGF α-receptor (Claesson-Welsh et al., 1989) using the Altered sites in vitro Mutagenesis System (Promega Corporation). An oligonucleotide with the sequence 5′-GATGGACATGAATTTATTTTTGTGGAC-3′ was used to introduce the Y572/574F mutation and one with the sequence 5′-GATGGACATGAATTTATTTATGTGGAC-3′ was used to introduce the Y572F mutation. The mutation was confirmed by nucleotide sequencing. Wild-type and mutated cDNAs were inserted into the eukaryotic expression vector pcDNA3 (Invitrogen). The constructs were transfected into porcine aortic endothelial (PAE) cells (Miyazono et al., 1987), by electroporation and subsequently selected for neomycin resistance as previously described (Claesson-Welsh et al., 1988; Mori et al., 1993). ^{125}I-PDGF-BB binding assay was performed according to the method of Mori et al. (1993), to screen for positive clones.

Antibodies

The rabbit antiserum PDGFR-7, recognizing the C-terminal tail of the PDGF α-receptor (Eriksson et al., 1992), and SSD-2, raised against the juxtamembrane region of the PDGF β-receptor (Mori et al., 1993), have been described earlier. The rabbit antiserum against carboxy-terminal sequences of Src, Fyn and Yes (cst.1) was prepared as described by Kypta et al. (1990). The monoclonal phosphotyrosine antibody PY20 was from Affiniti Research Products Ltd. Peroxidase-conjugated sheep anti-mouse Ig and peroxidase-conjugated swine anti-rabbit Ig were from Amersham Corp. and Dakopatts, respectively.

Immunoprecipitation and immunoblotting

Serum-starved cells were treated with PDGF-BB for 30 minutes on ice followed by 5 minutes incubation at 37°C, rinsed with ice-cold phosphate-buffered saline (PBS), and then lysed in NP-40 lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 2.5 mM EDTA, 100 μM Na_3VO_4, 1% aprotinin (Trasylol; Bayer) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Immunoprecipitation and immunoblotting were performed as previously described (Mori et al., 1993). The blots were reprobed after removal of the first probe by incubation in 5 mM sodium phosphate, pH 7.5, 2% SDS and 2 mM 2-mercaptoethanol for 30 minutes at 60°C. In some experiments, the PDGF receptor was collected on wheat germ lectin Sepharose 6 MB (Pharmacia Biotech), by incubation for 2 hours at 4°C; after washing, samples were subjected to SDS-PAGE.

Peptide synthesis and peptide inhibition experiment

Peptides were synthesized by Fmoc chemistry as previously described (Mori et al., 1993). For peptide inhibition experiments, the cell lysate was incubated with various concentrations of synthetic peptides at 4°C for 1 hour, then antiserum against Src (cst.1) was added and the incubation was continued for another hour. After three washes with NP-40 lysis buffer, the samples were separated by SDS-PAGE and immunoblotting was performed using phosphotyrosine antibody PY20.

In vitro kinase assay and enolase assay

After ligand treatment, the cells were lysed and immunoprecipitation was performed with antisera against the PDGF α-receptor (PDGFR-7), PDGF β-receptor (PDGFR-3), or Src (cst.1). The immunoprecipitated samples were subjected to kinase assays, analyzed by SDS-PAGE and autoradiography, as described by Mori et al. (1993). Enolase assay was performed as described by Kypta et al. (1990).

[^{32}P]Orthophosphate labeling, phosphopeptide mapping, Edman degradation, and phosphoamino acid analysis

The procedures were performed essentially according to the method of Rönnstrand et al. (1992). Briefly, serum-starved cells were labeled by incubation in phosphate-free Ham's F-12 medium supplemented with 0.5% dialyzed fetal calf serum and 4 mCi/ml [^{32}P]orthophosphate for 3 hours at 37°C. Cells were treated with 100 ng/ml PDGF BB in the same medium for 1 hour at 4°C, and lysed in a lysis buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 5 mM EDTA, 100 μM Na_3VO_4, 1% Trasylol and 1 mM PMSF. Immunoprecipitation was performed using PDGFR-7 antiserum, whereafter the samples were separated by SDS-PAGE and then transferred onto nitrocellulose membrane. For in situ tryptic 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 minutes at 37°C. The filter pieces were rinsed three times with water and then incubated for 12 hours at 37°C with 400 μl of 50 mM ammonium bicarbonate containing 1 μg of modified sequencing grade trypsin (Promega Corporation). The supernatant was lyophilized, oxidized in performic acid for 1 hour on ice, again lyophilized, resuspended in 50 μl of ammonium bicarbonate, and incubated with 1 μg of trypsin for another 12 hours at 37°C. The samples were lyophilized, dissolved in a pH 1.9 buffer consisting of 88% formic acid-acetic acid-water (50:156:1794), 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:H_2O:*n*-butanol, 65:5:3:2:29) 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 the case of immunoprecipitation of tryptic fragments, lyophilized tryptic digests were dissolved in 50 mM ammonium bicarbonate and incubated for 2 hours at 4°C with SSD-2 antiserum covalently coupled to Protein A-Sepharose beads. The beads were then washed three times in 50 mM ammonium bicarbonate, 0.05% Triton X-100, twice in 150 mM ammonium bicarbonate, 0.05% Triton X-100 and twice in distilled water. The immunoprecipitated peptides were then eluted with 1% diethylamine (pH 11.9) and 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 phosphoamino acid analysis, peptides were subjected to hydrolysis in 6 M hydrochloric acid for 1 hour at 110°C, followed by separation by two-dimensional electrophoresis on a cellulose plate, and analysis by autoradiography.

[^{3}H]thymidine incorporation assay

The ability of PDGF to stimulate DNA synthesis in the different transfected cell lines, measured by the incorporation of [^{3}H]thymidine, was performed as previously described (Mori et al., 1993).

Actin reorganization

The assay was a modification of the procedure described by Wennström et al. (1992). Briefly, cells cultured on glass coverslips in six-well plates were serum-starved and thereafter stimulated or not with 20 ng/ml PDGF-BB for 5 minutes at 37°C. The cells were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. The cells were rinsed three times with PBS and incubated with TRITC-labeled phalloidin (Sigma)

for 30 minutes. The cells were again rinsed three times with PBS and mounted in Fluoromount-G (Southern Biotechnology).

Chemotaxis assay

The chemotactic responses of wild-type and Y572/574F mutant α-receptor expressing PAE cells were measured using a Boyden chamber as described (Auerbach et al., 1991). A Costar nucleopore filter (8 μm thick, 8 μm pore) was coated with type-1 collagen (100 μg/ml) and dried. The lower chambers were filled with Ham's F12 medium containing 0.1% fetal calv serum (FCS) supplemented with increasing concentrations of PDGF-BB. Ham's F12 containing 10% FCS was used as a control. The precoated filter was then placed between the lower and upper chambers. Cultured cells were harvested and resuspended at a final concentration of 0.5×10^6 cells/ml in Ham's F12 containing 0.1% FCS. This cell suspension was added to the upper chambers and incubated at 37°C for 4 hours. Thereafter the filter was fixed in 99% ethanol and stained with Giemsa solution. Cells that had migrated through the filter were counted.

RESULTS

Characterization of a PAE cell line expressing a Y572/574F mutant of the PDGF α-receptor

In the PDGF β-receptor, two tyrosine residues in the juxtamembrane domain, Tyr-579 and Tyr-581, become phosphorylated upon ligand stimulation and are involved in binding Src family members (Mori et al., 1993). The juxtamembrane region of the PDGF α- and β-receptors are structurally similar (Fig. 1). To investigate the role of the corresponding tyrosine residues in the PDGF α-receptor, Tyr-572 and Tyr-574 codons of the cDNA encoding the wild-type human PDGF α-receptor were changed to phenylalanine codons by site-directed mutagenesis, generating the Y572/574F mutant receptor. The wild-type as well as the Y572/574F PDGF mutant α-receptor were stably introduced into porcine aortic endothelial (PAE) cells.

In order to estimate the expression levels of the receptors, PAE cells expressing wild-type or mutant receptors were stimulated or not with PDGF, lysed and glycoproteins collected using wheat germ lectin Sepharose 6 MB. The samples were separated by SDS-PAGE, transferred onto nitrocellulose membrane and immunoblotted with PDGFR-7 (PDGF α-receptor anti-serum). As shown in Fig. 2A, the wild-type and Y572/574F mutant PDGF α-receptors were expressed to similar extents. The same filter was stripped and reprobed with the phosphotyrosine antibody PY20. Fig. 2B shows that ligand-binding stimulated autophosphorylation of wild-type and mutant receptors to similar degrees.

That the Y572/574F mutant PDGF α-receptor has intact kinase activity was shown by in vitro kinase assay performed on immunoprecipitated receptors (Fig. 2C). In contrast, the corresponding mutant of the PDGF β-receptor, Y579/581F, was found to have markedly decreased kinase activity in comparison to the wild-type β-receptor (Fig. 2C), consistent with previous observations (Mori et al., 1993; Vaillancourt et al., 1995).

PDGF α-receptor: S-P-D-G-H-E-^{572}Y-I-^{574}Y-V-D-P-M-Q

PDGF β-receptor: S-S-D-G-H-E-^{579}Y-I-^{581}Y-V-D-P-M-Q

Fig. 1. Comparison of the amino acid sequence in the juxtamembrane regions of the PDGF α- and β-receptors. Numbers indicate the position of amino acids. The sequence of the peptide used to make the SSD-2 antiserum is underlined.

Tyr572 and Tyr574 are in vivo phosphorylation sites in the PDGF α-receptor

We next examined the in vivo phosphorylation of Tyr-572 and Tyr-574 in response to PDGF stimulation. PAE cells expressing the wild-type, Y572F or Y572/574F mutant PDGF α-receptors were labeled with [^{32}P]orthophosphate, incubated with PDGF, lysed and immunoprecipitated with PDGFR-7. The immunoprecipitated material was separated by SDS-PAGE, transferred onto nitrocellulose membrane and exposed to X-ray film. Thereafter, the phosphorylated PDGF α-receptor band

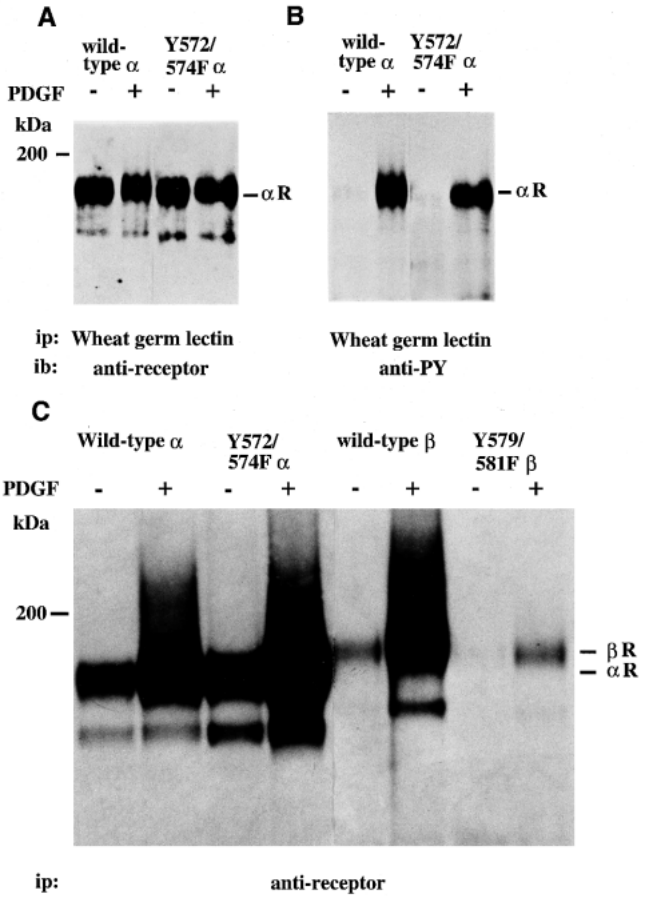

Fig. 2. Y572/574F mutant PDGF α-receptor undergoes autophosphorylation. (A) PAE cells expressing wild-type or Y572/574F mutant PDGF α-receptors were incubated with (+) or without (–) 100 ng/ml of PDGF, lysed and precipitated using wheat germ lectin Sepharose. The samples were separated by SDS-PAGE, transferred onto nitrocellulose membrane and analyzed by immunoblotting with anti-receptor antiserum (PDGFR-7). The position of the PDGF α-receptor is indicated (αR). (B) The blot shown in A was stripped, and reprobed with phosphotyrosine antibody PY20. (C) PAE cells expressing wild-type PDGF α-receptor, Y572/574F mutant PDGF α-receptor, wild-type β-receptor or Y579/581F mutant PDGF β-receptor were treated as in A and cell lysates were incubated with anti-receptor antiserum and subjected to in vitro kinase assay. The samples were separated by SDS-PAGE and then analyzed by autoradiography. ip, immunoprecipitation; ib, immunoblotting.

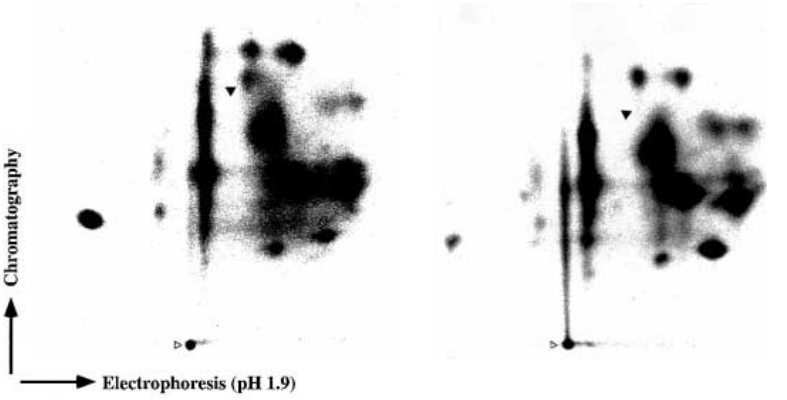

Fig. 3. Two-dimensional tryptic phosphopeptide maps of the wild-type and Y572/574F mutant PDGF α-receptors. PAE cells expressing wild-type or Y572/574F mutant PDGF α-receptors were labeled in vivo with [^{32}P]orthophosphate, stimulated with PDGF and immunoprecipitated with anti-receptor antiserum (PDGFR-7). The immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The wild-type and mutant 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 Y572/574F mutant α-receptor map is indicated by a closed triangle. In each case, the origin is marked with an open triangle.

was excised from the membrane, subjected to in situ tryptic digestion and the digest was analyzed by two-dimensional phosphopeptide mapping (Fig. 3). Phosphopeptide maps of the wild-type and the Y572/574F mutant receptor were similar except for a single phosphopeptide spot missing in the mutant receptor map (indicated by arrowheads). The data suggest that the mutant receptor lacks at least one phosphorylation site. To further confirm that the missing spot corresponds to a fragment of the juxtamembrane domain of the receptor, tryptic digests of [^{32}P]orthophosphate-labeled wild-type and mutant receptors were immunoprecipitated with an antiserum (SSD-2) raised against a synthetic peptide corresponding to the human PDGF β-receptor amino acid residues 573-586, a region which is highly similar to amino acid residues 566-580 in the PDGF α-receptor (Fig. 1). The immunoprecipitated material was thereafter subjected to Edman degradation, and the ^{32}P radioactivity in the generated fragments was measured in each cycle. As shown in Fig. 4A, most of the ^{32}P radioactivity was collected at cycles 12 and 14 in the wild-type PDGF α-receptor, corresponding to Tyr-572 and Tyr-574, respectively. As expected, the radioactive peak at cycle 12 corresponding to Tyr-572 was drastically diminished in the Y572F mutant whereas the peak at cycle 14 remained intact (Fig. 4B). In contrast, no radioactivity was detected in either of these cycles in the fragment derived from the Y572/574F mutant (Fig. 4C). Together with the phosphopeptide mapping results, we conclude that Tyr-572 and Tyr-574 are in vivo phosphorylation sites in the PDGF α-receptor.

Tyr-572 and Tyr-574 in the PDGF α-receptor are binding sites for the Src family tyrosine kinases

In the PDGF β-receptor, phosphorylated Tyr-579 and Tyr-581 in the juxtamembrane domain serve as binding sites for Src family tyrosine kinases (Mori et al., 1993). We therefore next investigated the ligand-induced interaction between Src family tyrosine kinases and wild-type and Y572/574F mutant PDGF α-receptors. The cells were treated as described above and the lysates were immunoprecipitated with an antiserum (cst.1) which recognizes the Src family members. The immunoprecipitates were separated and then analyzed by

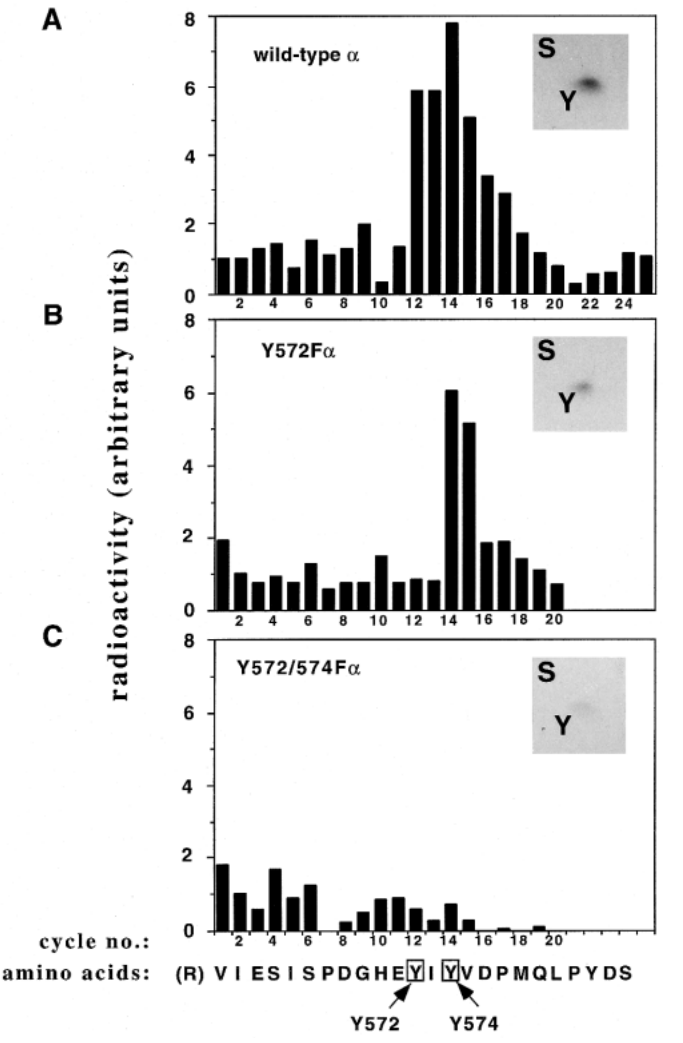

Fig. 4. Analysis of phosphorylation in the juxtamembrane region of wild-type and Y572/574F PDGF α-receptors. Tryptic peptides from wild-type (A) and mutant (B) PDGF α-receptors were immunoprecipitated with SSD-2 antiserum and subjected to Edman degradation. The radioactivity released in each cycle was determined. The amino acid sequence of the SSD-2 immunoprecipitated PDGF α-receptor juxtamembrane fragment peptide is presented along with the cycle numbers. The results of phosphoamino acid analyses of the peptides are shown to the right hand side of each panel.

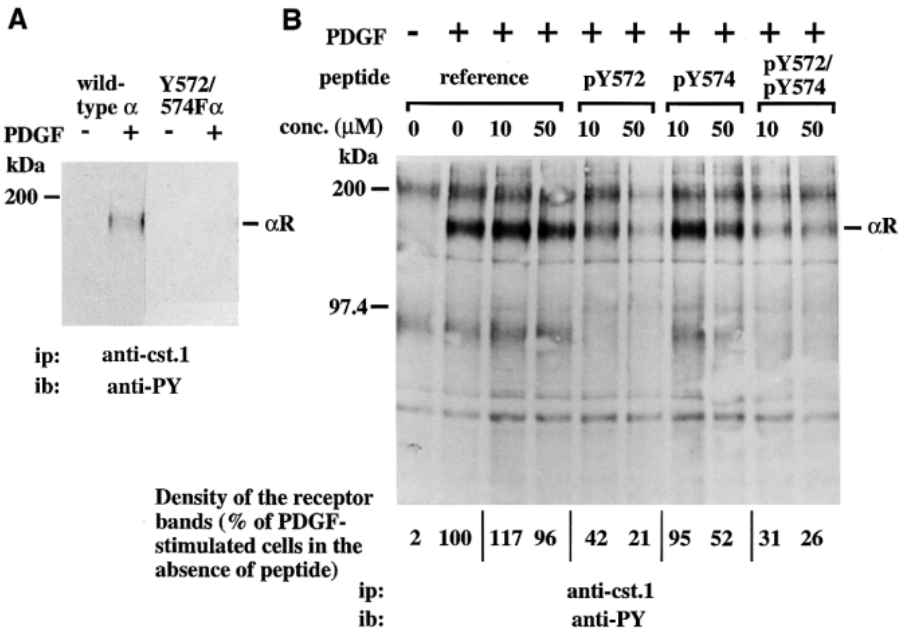

Fig. 5. Tyr-572 and Tyr-574 are binding sites for Src family tyrosine kinases in the PDGF α-receptor. (A) PAE cells expressing the wild-type or Y572/574F mutant PDGF α-receptors were incubated with (+) or without (–) 100 ng/ml of PDGF, lysed and immunoprecipitated using an antiserum against Src family members (cst.1). The samples were separated by SDS-PAGE, transferred onto nitrocellulose membrane and analyzed by immunoblotting with phosphotyrosine antibody PY20. The position of the PDGF α-receptor is indicated (αR). (B) PAE cells expressing the wild-type PDGF α-receptor were treated with (+) or without (–) 100 ng/ml of PDGF, lysed and then incubated with indicated concentrations of synthetic peptides corresponding to the juxtamembrane region of the PDGF α-receptor, either unphosphorylated, phosphorylated on Tyr-572, phosphorylated on Tyr-574, or phosphorylated on both Tyr-572 and Tyr-574 for 1 hour, then cst.1 antiserum was added and incubation was continued for an additional hour. The samples were separated by SDS-PAGE and analyzed by immunoblotting with phosphotyrosine antibody PY20. The position of the autophosphorylated PDGF α-receptor is indicated (αR). ip, immunoprecipitation; ib, immunoblotting.

immunoblotting with phosphotyrosine antibody PY20, to examine co-precipitation of the receptors. As shown in Fig. 5A, the association of Src molecules to the Y572/574F mutant receptor was severely reduced compared to that of the wild-type receptor.

In order to examine the involvement in binding Src family members of Tyr-572 and Tyr-574 individually, inhibition of complex formation was performed using phosphorylated peptides. Peptides corresponding to the juxtamembrane region of the human PDGF α-receptor were synthesized, in which Tyr-572 and Tyr-574 were phosphorylated individually or simultaneously (Table 1). PAE cells expressing the wild-type PDGF α-receptor were treated with PDGF, and the cell lysate was incubated with the synthetic peptides for 1 hour prior to immunoprecipitation with cst.1 anti-serum. The samples were separated and analyzed by PY20 immunoblotting. As shown in Fig. 5B, phosphorylated peptides inhibited complex formation between Src family tyrosine kinases and wild-type PDGF α-receptor dose-dependently. The efficiency of inhibition using 10 μM peptide was highest in the doubly phosphorylated (pY572/pY574) peptide; the pY572 peptide also efficiently inhibited complex formation, whereas the pY574 peptide was less efficient. Thus, phosphorylated Tyr-572 and Tyr-574 both serve as binding sites for Src family members.

Table 1. Synthetic peptides used for the inhibition experiment

Peptide	Amino acid sequence
	572 574
Reference	I-S-P-D-G-H-E-Y - I - Y -V-D-P-M-Q-L-P-Y
pY572	I-S-P-D-G-H-E-pY- I - Y -V-D-P-M-Q-L-P-Y
pY574	I-S-P-D-G-H-E-Y - I -pY -V-D-P-M-Q-L-P-Y
pY572/pY574	I-S-P-D-G-H-E-pY- I -pY -V-D-P-M-Q-L-P-Y

pY indicates phosphorylated tyrosine residue.
Numbers indicate the positions of the tyrosine residues.

Activation of Src family tyrosine kinases is abolished in the Y572/574F mutant PDGF α-receptor

PDGF-induced activation of Src family tyrosine kinases was examined using enolase as an exogenous substrate. Cst.1 immunoprecipitates from PAE cells expressing the wild-type or the Y572/574F mutant PDGF α-receptor were subjected to in vitro kinase assay in the presence of enolase, followed by separation and analysis by autoradiography. Incorporation of ^{32}P radioactivity into enolase, as well as into Src itself, increased upon PDGF-stimulation in the wild-type receptor but not in the Y572/574F mutant (Fig. 6). Thus, binding to ligand-stimulated receptor is a prerequisite for Src kinases to be activated in PDGF α-receptor mediated signaling pathways, measured as phosphorylation of Src itself as well as phosphorylation of an exogenous substrate.

PDGF-BB-stimulated [^{3}H]thymidine incorporation and actin reorganization in cells expressing wild-type or Y572/574F mutant PDGF α-receptor

The ability of the wild-type and mutant receptors to transduce mitogenic signals was examined by [^{3}H]thymidine incorporation assay (Fig. 7). Two clones each of wild-type and Y572/574F mutant receptor expressing cells, representative of several other clones, are shown. Both types of receptors were able to respond to stimulation by PDGF with a similar dose-dependency. Non-transfected PAE cells do not respond to PDGF with increased [^{3}H]thymidine incorporation (Rönnstrand et al., 1992).

Stimulation of PAE cells expressing the PDGF α-receptor with PDGF induces reorganization of the actin filament system. This includes the appearance of veil-like actin-containing structures around the edges of the cell, i.e. edge ruffles, and a loss of actin stress fibers, visualized as bundles of actin spanning the cytoplasm. Upon PDGF stimulation, PAE cells expressing the Y572/574F mutant receptor showed a

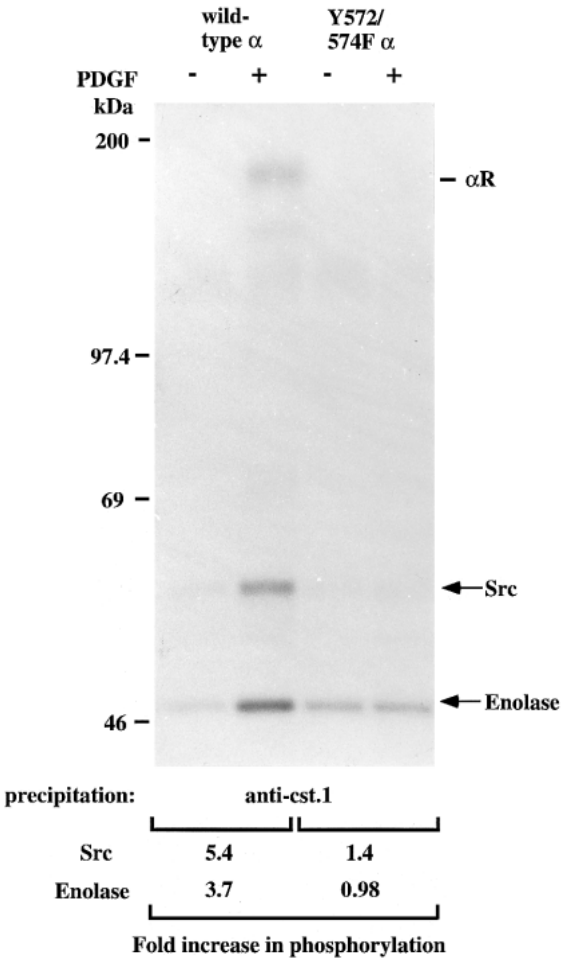

Fig. 6. Activation of Src family kinases is abolished in Y572/574F mutant PDGF α-receptor. PAE cells expressing wild-type or Y572/574F mutant α-receptors were incubated with (+) or without (–) 100 ng/ml of PDGF, lysed and immunoprecipitated using cst.1 antiserum. The immunoprecipitates were subjected to kinase assay in the presence of an exogenous substrate, enolase. The samples were separated by SDS-PAGE and analyzed by autoradiography. The positions of Src family kinases (Src), enolase, and the PDGF α-receptor (αR), are indicated. The intensities of the Src and enolase bands were measured using a Bioimager apparatus (Fuji) and given as fold increases.

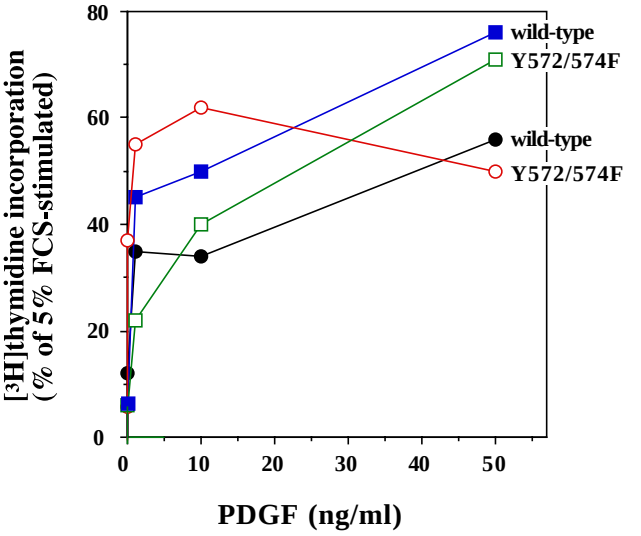

Fig. 7. Stimulation of [^{3}H]thymidine incorporation by PDGF in two clones each of PAE cells expressing wild-type or Y572/574F mutant PDGF α-receptors. After 48 hours of serum starvation, cells expressing the wild-type (closed square/circle) or Y572/574F mutant (open square/circle) PDGF α-receptors were incubated with [^{3}H]thymidine and the indicated concentration of PDGF or 5% fetal calf serum (FCS) for 24 hours. After the incubation, trichloroacetic acid-precipitable radioactivity was measured.

similar rearrangement of the actin filament system as cells with wild-type receptors (Fig. 8). Non-transfected PAE cells have previously been shown not to respond to PDGF in actin reorganization assays (Eriksson et al., 1992). Therefore, Src appears not to play a crucial role in PDGF α-receptor mediated mitogenesis or reorganization of the actin cytoskeleton.

DISCUSSION

In this study, we have identified two novel autophosphorylation sites, Tyr-572 and Tyr-574, in the juxtamembrane region of the PDGF α-receptor and shown that these sites are required for the binding and activation of Src family tyrosine kinases. We found that the Y572/574F mutant PDGF α-receptor, despite the lack of association to and activation of Src family tyrosine kinases, is fully capable of responding mitogenically to PDGF.

Tyr-579 and Tyr-581 have previously been shown to be autophosphorylation sites in the PDGF β-receptor and to serve as binding sites for Src family tyrosine kinases (Mori et al., 1993). However, the autophosphorylation of the PDGF β-receptor was dramatically decreased when both of these tyrosine residues were replaced by phenylalanine residues (Mori et al., 1993; Vaillancourt et al., 1995). Thus, it has not been possible to definitely elucidate the role of Src family kinases in PDGF signaling using PDGF β-receptor Src-binding mutants. In contrast, the autophosphorylation of the Y572/574F mutant PDGF α-receptor was similar to that of the wild-type receptor (Fig. 2C).

Microinjection studies have shown that Src family kinases are involved in mitogenic signaling of PDGF receptors (Barone and Courtneidge, 1995; Twamley-Stein et al., 1993). In our study, the Y572/574F mutant PDGF α-receptor, which is unable to bind to or activate Src family tyrosine kinases, still mediated DNA synthesis upon ligand stimulation. A possibility which remains to be elucidated is that Src family members are activated downstream in signaling pathways initiated at PDGF receptors. Recently, Myc has been proposed as a downstream mediator of Src-dependent, PDGF-mediated, mitogenic signaling (Barone and Courtneidge, 1995; Eisenman and Cooper, 1995). Due to relatively high background levels of c-*myc* mRNA in serum-starved PAE cells it was difficult to detect an induction upon PDGF-stimulation (data not shown). Therefore the role of Src in PDGF-induced c-*myc* induction in PAE cells remains to be clarified.

Src has been shown to be able to activate members of the Raf-1 family of Ser/Thr kinases (Marais et al., 1995; Stokoe and McCormick, 1997), which in turn activate MEK and MAP

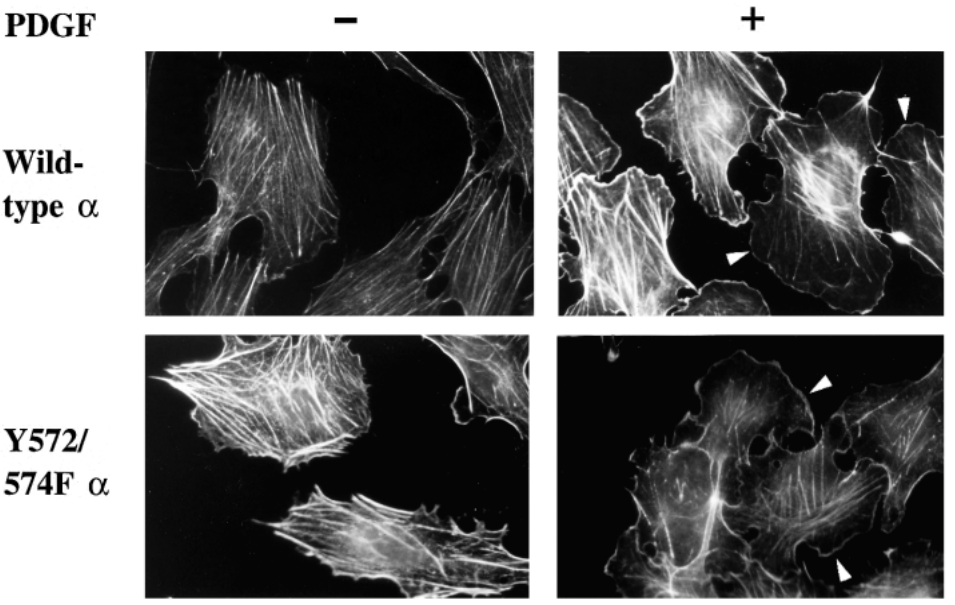

Fig. 8. PDGF-induced actin reorganization in wild-type and Y572/574F mutant PDGF α-receptor expressing cells. Serum-starved cells were either stimulated (+) or not (–) with 20 ng/ml PDGF. Actin filaments were visualized using TRITC-labeled phalloidin. Edge ruffles are indicated by arrowheads.

kinases. The Y572/574F mutant was, however, found to be able to induce MAP kinase activity upon PDGF stimulation to a degree similar to the wild-type PDGF α-receptor (data not shown). In v-Src transformed cells, the adaptor protein Shc is tyrosine phosphorylated (McGlade et al., 1992), and may thus mediate Ras activation via recruitment of the Grb2-Sos complex (Egan et al., 1993). It has also been shown that transformation by v-Src could be reversed by inhibiting Ras function (Smith et al., 1986). Therefore, we considered the possibility that the Src family kinases are involved in PDGF-induced Ras activation via phosphorylation of Shc. However, the phosphorylation level of Shc was found to be similar in PAE cells expressing the wild-type and Y572/574F mutant PDGF α-receptor (data not shown), which is in line with the adequate level of MAP kinase activation and mitogenic response of the mutant receptor.

Members of the Src family have been shown to associate with and phosphorylate PLC-γ in vitro (Liao et al., 1993; Nakanishi et al., 1993). We therefore investigated the phosphorylation and activation of PLC-γ in the Y572/574F mutant PDGF α-receptor expressing cells. We observed that PLC-γ phosphorylation levels were comparable in the wild-type and mutant PDGF α-receptors (data not shown). This suggests that binding of PLC-γ to Tyr-1018 and Tyr-988 in the PDGF α-receptor (Eriksson et al., 1995) is sufficient for phosphorylation, and that this phosphorylation takes place independent of Src kinase activity in response to PDGF stimulation.

Src kinases have been reported to activate PI3-kinase (Cantley et al., 1991) which is involved in PDGF-mediated actin reorganization and chemotaxis (Wennström et al., 1994). The C-terminal Src kinase (Csk) phosphorylates Src family tyrosine kinases and downregulates their activity in vitro (Okada et al., 1991). Elevated Src activity in Csk-deficient cells leads to impaired organization of actin stress fibers, and decreased cell attachment (Nada et al., 1994). However, no significant difference in PDGF-induced actin reorganization in PAE cells expressing wild-type or Y572/574F mutant PDGF α-receptors was found (Fig. 8), nor was the chemotactic response to PDGF perturbed in this mutant (data not shown), implicating that Src is not critical in PDGF α-receptor mediated actin reorganization and chemotaxis.

In conclusion, we have found two new autophosphorylation sites in the PDGF α-receptor which are responsible for Src binding upon PDGF stimulation. Src binding to the PDGF α-receptor, however, seems not to be necessary for receptor kinase activity, nor for the ability of the receptor to recruit and phosphorylate other substrates such as Shc and PLC-γ. The intact mitogenic response of the Y572/574F mutant receptor is consistent with the finding that MAP kinase activity is undisturbed in this mutant. Future studies will be aimed at determining the precise role of Src in PDGF α-receptor signaling. An important tool in these studies would be gene targeted cells lacking expression of Src, Fyn and Yes. The use of such cells would help answer the question of whether Src family members act further down-stream in PDGF-mediated signal transduction pathways.

We are grateful to Ulla Engström for providing peptides, Christer Wernstedt and Ulf Hellman for help with amino acid sequencing and preparation of oligonucleotides, Lars Rönnstrand for the SSD-2 antibodies and Ingegärd Schiller for secretarial assistance. We thank Andrius Kazlauskas and members of his group for sharing their unpublished results.

REFERENCES

Auerbach, R., Auerbach, W. and Polakowski, I. (1991). Assays for angiogenesis: A review. *Pharmac. Ther.* **51**, 1-11.

Barone, M. V. and Courtneidge, S. A. (1995). Myc but not Fos rescue of PDGF signalling block caused by kinase-inactive Src. *Nature* **378**, 509-512.

Bazenet, C. E., Gelderloos, J. A. and Kazlauskas, A. (1996). Phosphorylation of tyrosine 720 in the platelet-derived growth factor alpha receptor is required for binding of Grb2 and SHP-2 but not for activation of Ras or cell proliferation. *Mol. Cell Biol.* **16**, 6926-6936.

Boström, H., Willetts, K., Pekny, M., Levéen, P., Lindahl, P., Hedstrand, H., Pekna, M., Hellström, M., Gebre-Medhin, S., Schalling, M., Nilsson, M., Kurland, S., Törnell, J., Heath, J. K. and Betsholtz, C. (1996). PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* **85**, 863-873.

Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A.,

Kapeller, R. and Soltoff, S. (1991). Oncogenes and signal transduction. *Cell* **64**, 281-302.

Claesson-Welsh, L., Eriksson, A., Morén, A., Severinsson, L., Ek, B., Östman, A., Betsholtz, C. and Heldin, C.-H. (1988). cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. *Mol. Cell. Biol.* **8**, 3476-3486.

Claesson-Welsh, L., Eriksson, A., Westermark, B. and Heldin, C.-H. (1989). cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B-type PDGF receptor. *Proc. Nat. Acad. Sci. USA* **86**, 4917-4921.

Claesson-Welsh, L. (1994). Platelet-derived growth factor receptor signals. *J. Biol. Chem.* **269**, 32023-32026.

Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M. and Weinberg, R. A. (1993). Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363**, 45-51.

Eisenman, R. N. and Cooper, J. A. (1995). Beating a path to Myc. *Nature* **378**, 438-439

Eriksson, A., Nånberg, E., Rönnstrand, L., Engström, U., Hellman, U., Rupp, E., Carpenter, G., Heldin, C.-H. and Claesson-Welsh, L. (1995). Demonstration of functionally different interactions between phospholipase C-γ and the two types of platelet-derived growth factor receptors. *J. Biol. Chem.* **270**, 7773-7781.

Eriksson, A., Siegbahn, A., Westermark, B., Heldin, C.-H. and Claesson-Welsh, L. (1992). PDGF α- and β-receptors activate unique and common signal transduction pathways. *EMBO J.* **11**, 543-550.

Heldin, C.-H., Bäckström, G., Östman, A., Hammacher, A., Rönnstrand, L., Rubin, K., Nistér, M. and Westermark, B. (1988). Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. *EMBO J.* **7**, 1387-1393.

Heldin, C.-H. and Westermark, B. (1996). Role of platelet-derived growth factor *in vivo*. In *The Molecular and Cellular Biology of Wound Repair* (ed. R. A. F. Clark), pp. 249-273. Plenum Press, New York.

Kypta, R. M., Goldberg, Y., Ulug, E. T. and Courtneidge, S. A. (1990). Association between the PDGF receptor and members of the *src* family of tyrosine kinases. *Cell* **62**, 481-492.

Levéen, P., Pekny, M., Gebre-Medhin, S., Swolin, B., Larsson, E. and Betsholtz, C. (1994). Mice deficient for PDGF B show renal, cardiovascular and hematological abnormalities. *Genes Dev.* **8**, 1875-1887.

Liao, F., Shin, H. S. and Rhee, S. G. (1993). In vitro tyrosine phosphorylation of PLC-γ1 and PLC-γ2 by src-family protein tyrosine kinases. *Biochem. Biophys. Res. Commun.* **191**, 1028-1033.

Marais, R., Light, Y., Paterson, H. F. and Marshall, C. J. (1995). Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *EMBO J.* **14**, 3136-3145.

Matsui, T., Heidaran, M., Miki, T., Toru, M., Popescu, N., La Rochelle, W., Kraus, M., Pierce, J. and Aaronson, S. A. (1989). Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. *Science* **243**, 800-803.

McGlade, J., Cheng, A., Pelicci, G., Pelicci, P. G. and Pawson, T. (1992). Shc proteins are phosphorylated and regulated by the v-Src and v-Fps protein-tyrosine kinases. *Proc. Nat. Acad. Sci. USA* **89**, 8869-8873.

Miyazono, K., Okabe, T., Urabe, A., Takaku, F. and Heldin, C.-H. (1987). Purification and properties of an endothelial cell growth factor from human platelets. *J. Biol. Chem.* **262**, 4098-4103.

Mori, S., Rönnstrand, L., Yokote, K., Engström, Å., Courtneidge, S. A., Claesson-Welsh, L. and Heldin, C.-H. (1993). Identification of two juxtamembrane autophosphorylation sites in the PDGF β-receptor; involvement in the interaction with Src family tyrosine kinases. *EMBO J.* **12**, 2257-2264.

Nada, S., Okada, M., Aizawa, S. and Nakagawa, H. (1994). Identification of major tyrosine-phosphorylated proteins in Csk-deficient cells. *Oncogene* **9**, 3571-3578.

Nakanishi, O., Shibasaki, F., Hidaka, M., Homma, Y. and Takenawa, T. (1993). Phospholipase C-γ1 associates with viral and cellular src kinases. *J. Biol. Chem.* **268**, 10754-10759.

Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T. and Nakagawa, H. (1991). CSK: a protein-tyrosine kinase involved in regulation of src family kinases. *J. Biol. Chem.* **266**, 24249-24252.

Pawson, T. (1995). Protein modules and signalling networks. *Nature* **373**, 573-580.

Raines, E. W., Bowen-Pope, D. F. and Ross, R. (1990). Platelet-derived growth factor. In *Handbook of Experimental Pharmacology. Peptide Growth Factors and Their Receptors* (ed. M. B. Sporn and A. B. Roberts), pp. 173-262. Springer-Verlag, Heidelberg.

Rupp, E., Siegbahn, A., Rönnstrand, L., Wernstedt, C., Claesson-Welsh, L. and Heldin, C.-H. (1994). A unique autophosphorylation site in the platelet-derived growth factor α receptor from a heterodimeric receptor complex. *Eur. J. Biochem.* **225**, 29-41.

Rönnstrand, L., Mori, S., Arvidsson, A.-K., Eriksson, A., Wernstedt, C., Hellman, U., Claesson-Welsh, L. and Heldin, C.-H. (1992). Identification of two C-terminal autophosphorylation sites in the PDGF β-receptor: involvement in the interaction with phospholipase C-γ. *EMBO J.* **11**, 3911-3919.

Smith, M. R., DeGudicubus, S. J. and Stacey, D. W. (1986). Requirement for c-ras proteins during viral oncogene transformation. *Nature* **320**, 540-543.

Soriano, P. (1994). Abnormal kidney development and hematological disorders in PDGF β-receptor mutant mice. *Genes Dev.* **8**, 1888-1896.

Soriano, P. (1997). The PDGFα receptor is required for neural crest cell development and for normal patterning of the somites. *Development* **124**, 2691-2700.

Stokoe, D. and McCormick, F. (1997). Activation of c-Raf-1 by Ras and Src through different mechanisms: activation *in vivo* and *in vitro*. *EMBO J.* **16**, 2384-2396.

Twamley-Stein, G. M., Pepperkok, R., Ansorge, W. and Courtneidge, S. A. (1993). The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH 3T3 cells. *Proc. Nat. Acad. Sci. USA* **90**, 7696-7700.

Ullrich, A. and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212.

Vaillancourt, R. R., Heasley, L. E., Zamarripa, J., Storey, B., Valius, M., Kazlauskas, A. and Johnson, G. L. (1995). Mitogen-activated protein kinase activation is insufficient for growth factor receptor-mediated PC12 cell differentiation. *Mol. Cell. Biol.* **15**, 3644-3653.

Wennström, S., Landgren, E., Blume-Jensen, P. and Claesson-Welsh, L. (1992). The platelet-derived growth factor β-receptor kinase insert confers specific signaling properties to a chimeric fibroblast growth factor receptor. *J. Biol. Chem.* **267**, 13749-13756.

Wennström, S., Siegbahn, A., Yokote, K., Arvidsson, A.-K., Heldin, C.-H., Mori, S. and Claesson-Welsh, L. (1994). Membrane ruffling and chemotaxis transduced by the PDGF β-receptor require the binding site for phosphatidylinositol 3′ kinase. *Oncogene* **9**, 651-660.

Yarden, Y., Escobedo, J. A., Kuang, W.-J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Francke, U., Friend, V. A., Ullrich, A. and Williams, L. T. (1986). Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. *Nature* **323**, 226-232.

Yokote, K., Mori, S., Siegbahn, A., Rönnstrand, L., Wernstedt, C., Heldin, C.-H. and Claesson-Welsh, L. (1996). Structural determinants in the platelet-derived growth factor α-receptor implicated in modulation of chemotaxis. *J. Biol. Chem.* **271**, 5101-5111.

Yu, J.-C., Heidaran, M. A., Pierce, J. H., Gutkind, J. S., Lombardi, D., Ruggiero, M. and Aaronson, S. A. (1991). Tyrosine mutations within the α platelet-derived growth factor receptor kinase insert domain abrogate receptor-associated phosphatidylinositol-3 kinase activity without affecting mitogenic or chemotactic signal transduction. *Mol. Cell. Biol.* **11**, 3780-3785.

Note added in proof

In a recent paper (submitted) Gelderloos, Rosenkranz, Bazenet and Kazlauskas described results very similar to ours, regarding the intact biological signalling capacity of the Y572/574F mutant PDGFα-receptor.