

# Mutation of a Src phosphorylation site in the PDGF $\beta$ -receptor leads to increased PDGF-stimulated chemotaxis but decreased mitogenesis

Klaus Hansen<sup>1</sup>, Matilda Johnell<sup>2</sup>,  
Agneta Siegbahn<sup>2</sup>, Charlotte Rorsman,  
Ulla Engström, Christer Wernstedt,  
Carl-Henrik Heldin and Lars Rönstrand<sup>3</sup>

Ludwig Institute for Cancer Research, Box 595, Biomedical Center, S-751 24 Uppsala, Sweden and <sup>2</sup>Department of Clinical Chemistry, University Hospital, S-751 85 Uppsala, Sweden

<sup>1</sup>Present address: Danish Cancer Society, Division for Cancer Biology, Strandboulevarden 49, DK-2100 Copenhagen, Denmark

<sup>3</sup>Corresponding author

**Ligand induced activation of the  $\beta$ -receptor for platelet-derived growth factor (PDGF) leads to activation of Src family tyrosine kinases. We have explored the possibility that the receptor itself is a substrate for Src. We show that Tyr934 in the kinase domain of the PDGF receptor is phosphorylated by Src. Cell lines expressing a  $\beta$ -receptor mutant, in which Tyr934 was replaced with a phenylalanine residue, showed reduced mitogenic signaling in response to PDGF-BB. In contrast, the mutant receptor mediated increased signals for chemotaxis and actin reorganization. Whereas the motility responses of cells expressing wild-type  $\beta$ -receptors were attenuated by inhibition of phosphatidylinositol 3'-kinase, those of cells expressing the mutant receptor were only slightly influenced. In contrast, PDGF-BB-induced chemotaxis of the cells with the mutant receptor was attenuated by inhibition of protein kinase C, whereas the chemotaxis of cells expressing the wild-type  $\beta$ -receptor was less affected. Moreover, the PDGF-BB-stimulated tyrosine phosphorylation of phospholipase C- $\gamma$  was increased in the mutant receptor cells compared with wild-type receptor cells. In conclusion, the characteristics of the Y934F mutant suggest that the phosphorylation of Tyr934 by Src negatively modulates a signal transduction pathway leading to motility responses which involves phospholipase C- $\gamma$ , and shifts the response to increased mitogenicity.**

**Keywords:** chemotaxis/c-Src/PDGF  $\beta$ -receptor/  
phospholipase C- $\gamma$ /phosphorylation

## Introduction

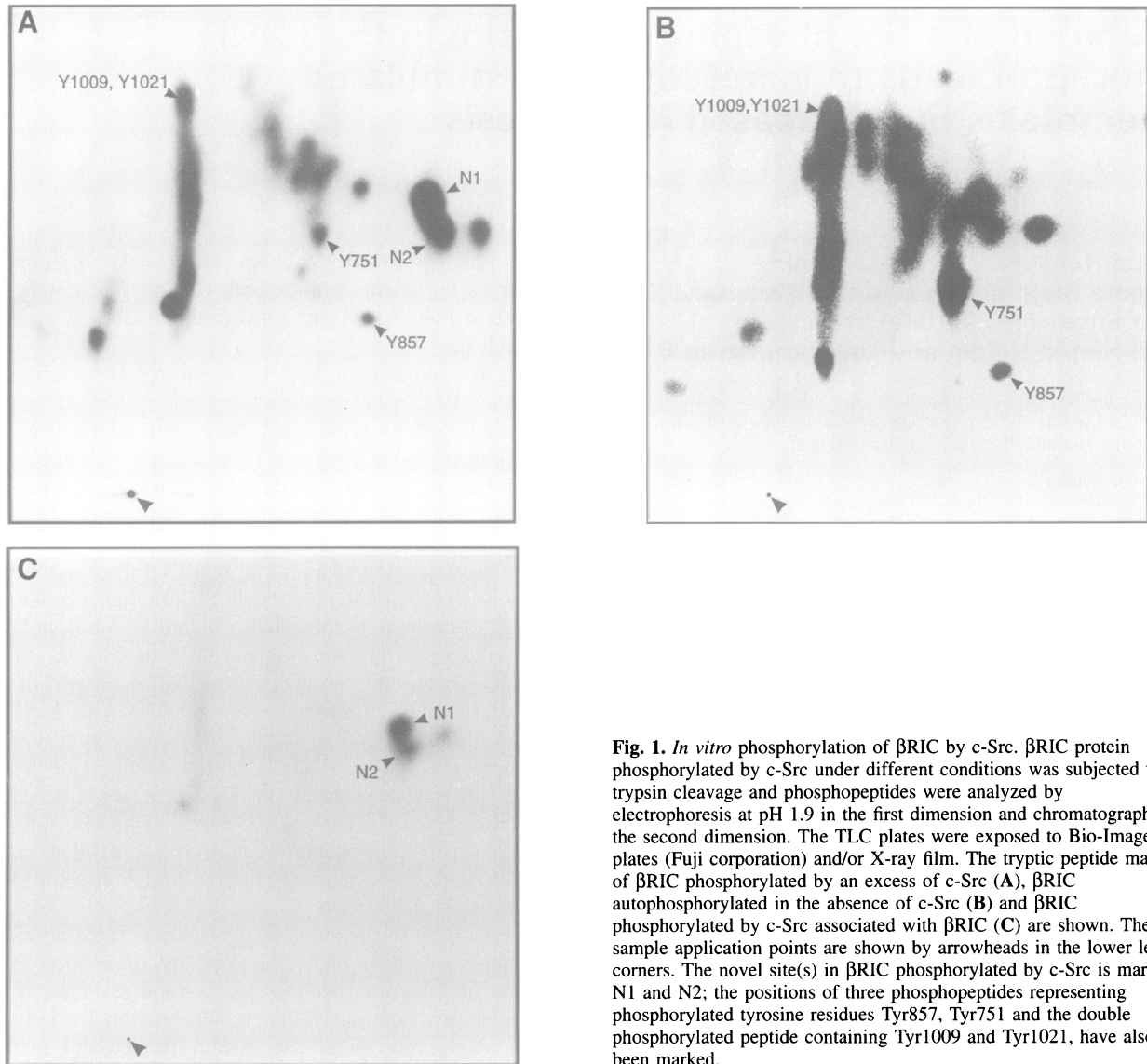
Platelet-derived growth factor (PDGF) stimulates growth, chemotaxis and actin reorganization of connective tissue cells and other cells (for reviews, see Heldin and Westermark, 1990; Raines *et al.*, 1990). PDGF is a family of homo- or heterodimers of disulfide-bonded A- and B-polypeptide chains. The isoforms exert their effects on cells by binding to two structurally related tyrosine kinase receptors. Ligand binding induces dimerization of the

receptors. Since the A-chain binds only  $\alpha$ -receptors, whereas the B-chain binds both  $\alpha$ - and  $\beta$ -receptors, the different PDGF isoforms form different dimeric receptor complexes.

Dimerization is a key event in receptor activation (Heldin, 1995) and leads to autophosphorylation of specific tyrosine residues in the intracellular parts of the receptors. The phosphorylated tyrosine residues, and their immediate environment, define high affinity docking sites for signaling molecules containing Src homology 2 (SH2) domains (Pawson, 1995). Nine autophosphorylation sites have been determined so far in the PDGF  $\beta$ -receptor; they interact in specific manners with SH2 domains containing signal transduction molecules, including phospholipase C- $\gamma$  (PLC- $\gamma$ ; binds with high affinity to Tyr1021 of the PDGF  $\beta$ -receptor), the GTPase activating protein of Ras (GAP; Tyr771), phosphatidylinositol 3'-kinase (PI3'-kinase; binds to Tyr740 and Tyr751), the protein tyrosine phosphatase Syp (PTP1D; Tyr1009), members of the Src family of tyrosine kinases (Tyr579), as well as the adaptor proteins Nck (Tyr751), Grb2/Sem5 (Tyr716) and Shc (binds to several phosphorylated tyrosine residues in the PDGF  $\beta$ -receptor with medium affinity) (reviewed by Claesson-Welsh, 1994).

In addition to the autophosphorylation, tyrosine kinase receptors are also phosphorylated by other kinases on serine or threonine, as well as tyrosine residues. It has been shown previously that transformation by v-Src of cells expressing either the insulin-like growth factor-1 receptor (Peterson *et al.*, 1994) or the receptor for epidermal growth factor (EGF) (Wasilenko *et al.*, 1991; Stover *et al.*, 1995) led to increased phosphorylations of these receptors on tyrosine residues. In both cases, the phosphorylation catalyzed by v-Src increases the catalytic potential of the receptor tyrosine kinases. Recently, it has also been shown that v-Src or c-Src can phosphorylate two tyrosine residues in the catalytic subdomain VIII in the focal adhesion kinase (FAK) (Calalb *et al.*, 1995); phosphorylation of these residues was found to be required for maximal kinase activity of FAK.

The aim of the present study was to explore the possibility that the interaction between the PDGF  $\beta$ -receptor and members of the Src family, leading to a subsequent activation of the Src kinase activity, causes a phosphorylation of the receptor by Src. Here we present evidence that a tyrosine residue in the second part of the kinase domain of the PDGF  $\beta$ -receptor (Tyr934) becomes phosphorylated by Src kinases in response to PDGF stimulation in cells expressing the human PDGF  $\beta$ -receptor. The functional consequence of this phosphorylation was investigated by studying cells stably expressing a mutated receptor in which Tyr934 was replaced with a phenylalanine by site-directed mutagenesis.



**Fig. 1.** *In vitro* phosphorylation of  $\beta$ IRIC by c-Src.  $\beta$ IRIC protein phosphorylated by c-Src under different conditions was subjected to trypsin cleavage and phosphopeptides were analyzed by electrophoresis at pH 1.9 in the first dimension and chromatography in the second dimension. The TLC plates were exposed to Bio-Imager plates (Fuji corporation) and/or X-ray film. The tryptic peptide maps of  $\beta$ IRIC phosphorylated by an excess of c-Src (A),  $\beta$ IRIC autophosphorylated in the absence of c-Src (B) and  $\beta$ IRIC phosphorylated by c-Src associated with  $\beta$ IRIC (C) are shown. The sample application points are shown by arrowheads in the lower left corners. The novel site(s) in  $\beta$ IRIC phosphorylated by c-Src is marked N1 and N2; the positions of three phosphopeptides representing phosphorylated tyrosine residues Tyr857, Tyr751 and the double phosphorylated peptide containing Tyr1009 and Tyr1021, have also been marked.

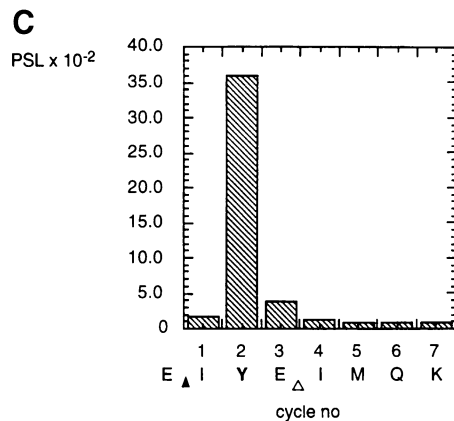
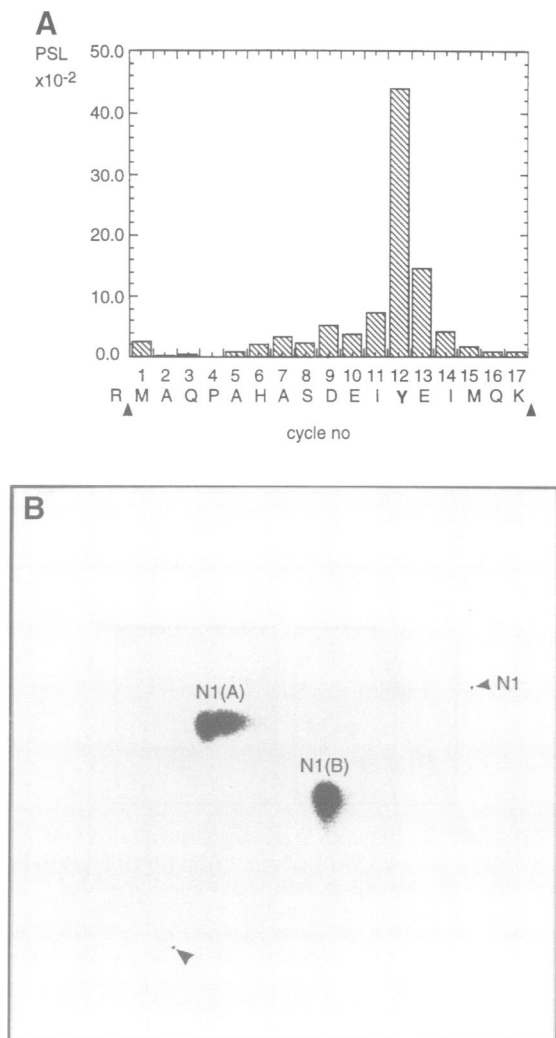
## Results

### *In vitro* phosphorylation of the PDGF $\beta$ -receptor by c-Src

In order to determine whether c-Src, after activation by the PDGF  $\beta$ -receptor, phosphorylates the receptor itself, the intracellular part of the PDGF  $\beta$ -receptor ( $\beta$ IRIC) expressed in a baculovirus system was used as a substrate for Src in an *in vitro* kinase assay.  $\beta$ IRIC was first pre-phosphorylated in the presence of 1 mM unlabeled ATP. In order to distinguish between  $\beta$ IRIC autophosphorylation and phosphorylation by Src, the kinase activity of  $\beta$ IRIC was inhibited by *N*-ethylmaleimide before further incubation with Src in the presence of [ $^{32}$ P]ATP. Analysis by SDS-PAGE revealed phosphorylation of  $\beta$ IRIC as well as of Src (data not shown). When the phosphorylated  $\beta$ IRIC protein was subjected to tryptic digestion followed by two-dimensional analysis and compared with the autophosphorylated  $\beta$ IRIC protein, several common phosphopeptides representing autophosphorylated regions in a PDGF-stimulated  $\beta$ -receptor were observed, and, in addition, two new peptides denoted N1 and N2 (compare Figure 1A and B).

In order to study the phosphorylation of  $\beta$ IRIC by Src under more physiological conditions, Src was first allowed to bind to the pre-phosphorylated  $\beta$ IRIC, and the excess Src was thereafter removed by washing, before addition of [ $^{32}$ P]ATP. Under these conditions, phosphorylation of both  $\beta$ IRIC and Src was also seen (data not shown); analysis of the tryptic map of the  $\beta$ IRIC component revealed mainly the phosphopeptides N1 and N2 (Figure 1C). In conclusion, N1 and N2 appear to represent sites in  $\beta$ IRIC which were phosphorylated preferentially by Src, but not involved in autophosphorylation.

In order to identify the localization in  $\beta$ IRIC of the phosphorylated residues in N1 and N2, the phosphopeptides were eluted from the thin layer chromatography (TLC) plate and subjected to phosphoamino acid analysis and Edman degradation. Tyrosine was the only phosphorylated amino acid recovered (data not shown). Upon Edman degradation, the radioactivity from phosphopeptide N1 was released in cycle 12 (Figure 2A), suggesting that the phosphorylated tyrosine is located 12 amino acid residues downstream of a lysine or an arginine residue. Four such tyrosine residues are present in the  $\beta$ IRIC sequence (Table I).



**Fig. 2.** Analysis of c-Src-induced phosphorylation of  $\beta$ RIC. (A) To determine the site of phosphorylation in the  $\beta$ RIC protein represented by phosphopeptide N1 (Figure 1C), the peptide was eluted from the TLC plate, coupled to a Sequelon<sup>TM</sup>-AA membrane through C-terminal coupling and subjected to Edman degradation by the use of a gas phase sequencer. The release of radioactivity in the individual cycles was determined and is indicated as pixel values (PSL). The deduced sequence in the PDGF  $\beta$ -receptor (see below) is shown below the figure, with the tryptic cleavage sites C-terminal to lysine residues at positions 0 and 17 indicated by arrowheads. (B) The phosphopeptide N1 (Figure 1A) was also subjected to cleavage with endoproteinase Glu-C, followed by analysis of generated peptides by two-dimensional separation on a TLC plate. The original position of the N1 phosphopeptide and the sample application point are indicated by arrowheads. The novel phosphopeptides, named N1A and N1B, are also indicated. (C) Phosphopeptide N1A was eluted from the TLC plate of (B), coupled to Sequelon<sup>TM</sup>-AA membrane and subjected to Edman degradation. The radioactivity released in the individual cycles was determined. The deduced sequence of the analyzed peptide is shown under the figure. Arrowheads indicate cleavage sites for endoproteinase Glu-C.

**Table I.** Sequences of the four possible tryptic peptides of the PDGF  $\beta$ -receptor with a tyrosine residue in position 12

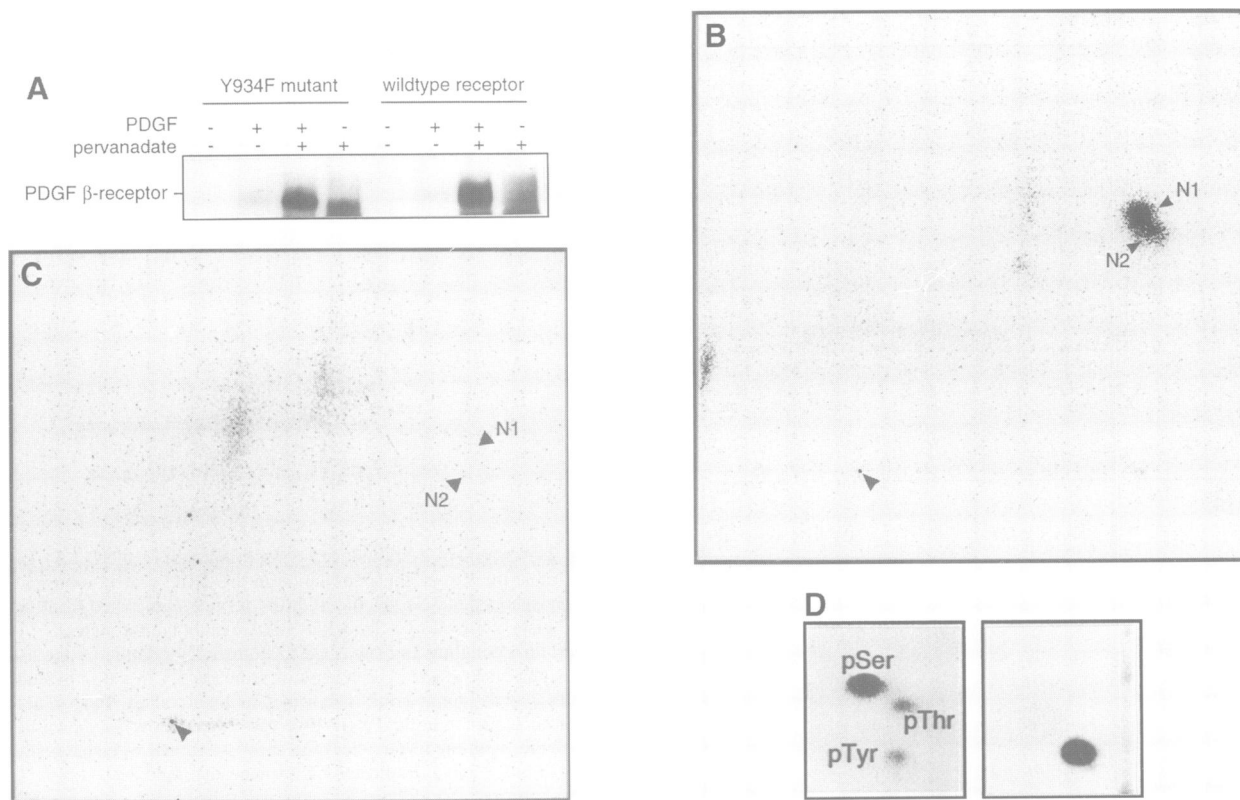
	Amino acid residues
VIESVSSDGHE <b>Y</b> IYDPMQLPYDSTWELPR	568-597
ATLINESPVLS <b>Y</b> MDLVGFSYQVANGMEFLASK	789-821
WMAPE <b>S</b> IFNSL <b>Y</b> TTLSDVWSFGILLWEIFTLGGTPYPELPMNEQFYNAIK	869-918
MAQPAHASDEI <b>Y</b> EIMQK	923-939

Arrows indicate possible cleavage sites for endoproteinase Glu-C, which was employed for secondary cleavage of phosphopeptides N1 and N2.

To distinguish between these possibilities, phosphopeptide N1 was cleaved further by endoproteinase Glu-C (*Staphylococcal* V8 protease), which cleaves C-terminal of glutamic acid residues. Two phosphopeptides with novel migrations were obtained, N1A and N1B (Figure 2B). The only one of the four putative peptides (Table I) which contains glutamic acid residues is the one corresponding to amino acid residues 923-939; this peptide is thus likely to correspond to phosphopeptide N1. Edman degradation of N1A and N1B yielded radioactivity in

cycles 2 (Figure 2C) and 12 (data not shown), respectively. This finding is consistent with the interpretation that peptide N1A represents peptide N1 cleaved C-terminal of Glu932, and possibly also after Glu935; peptide N1B may represent a peptide cleaved only after Glu935.

Edman degradation of phosphopeptide N2 also led to release of radioactivity in cycle 12. Furthermore, this peptide was also sensitive to degradation with endoprotease Glu-C, indicating that N2 also contained phosphorylated Tyr934. Taken together, these results indicate that the



**Fig. 3.** *In vivo* [ $^{32}\text{P}$ ]orthophosphate labeling of wild-type and Y934F mutant PDGF  $\beta$ -receptors. (A) PAE cells expressing wild-type or Y934F mutant PDGF  $\beta$ -receptors were labeled *in vivo* with [ $^{32}\text{P}$ ]orthophosphate. Cells were left unstimulated or were stimulated with PDGF-BB (100 ng/ml) in the absence or presence of the phosphotyrosine phosphatase inhibitor pervanadate, as indicated. After stimulation, cells were washed, lysed and processed for immunoprecipitation with anti-phosphotyrosine antibodies (PY20). The immunoprecipitates were analyzed by SDS-PAGE and electrotransferred to a nitrocellulose membrane; an X-ray film exposure of the membrane is shown. The amounts of radioactivity incorporated into wild-type and mutant receptors (determined as Cerenkov radiation) in the individual samples were: Y934F mutant receptor (clone A)—unstimulated control, 264 c.p.m.; PDGF-BB, 17 663 c.p.m.; PDGF-BB and pervanadate, 92 414 c.p.m.; pervanadate, 48 207 c.p.m.; wild-type  $\beta$ -receptor (ZnR5)—unstimulated control, 535 c.p.m.; PDGF-BB, 10 176 c.p.m.; PDGF-BB and pervanadate, 142 405 c.p.m.; pervanadate, 64 824 c.p.m. (B) and (C) The  $^{32}\text{P}$ -labeled wild-type  $\beta$ -receptor stimulated with PDGF-BB in the presence of pervanadate was subjected to tryptic cleavage and immunoprecipitation with  $\beta\text{YEI}$  antiserum (B) or pre-immune serum (C), followed by separation of phosphopeptides on TLC plates. The sample application points as well as the positions of the N1 and N2 phosphopeptides are marked by arrowheads. (D) Phosphopeptides N1 and N2, immunoprecipitated by  $\beta\text{YEI}$  antibodies from cells with wild-type PDGF  $\beta$ -receptors stimulated with PDGF-BB in the presence of pervanadate, were subjected to phosphoamino acid analysis. The left hand figure shows phosphoamino acid standards (revealed by ninhydrin spraying), while the right hand figure shows the result of the exposure on a Bio-Imager screen. Only phosphotyrosine was detected.

major site in  $\beta\text{RIC}$  that is phosphorylated by c-Src is Tyr934, which is located in the second part of the kinase domain.

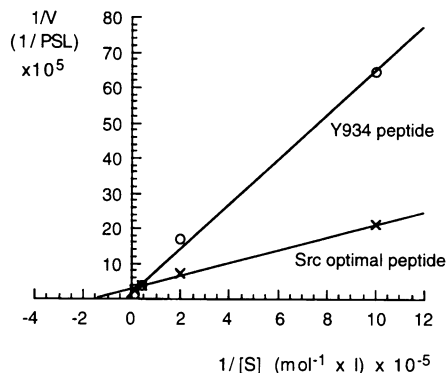
#### ***In vivo* phosphorylation of the PDGF $\beta$ -receptor on Tyr934**

A PDGF  $\beta$ -receptor mutant in which Tyr934 was replaced with a phenylalanine residue was established by site-directed mutagenesis. The mutant receptor was stably expressed in porcine aortic endothelial (PAE) cells, which do not contain endogenous PDGF receptors. Scatchard analysis of three different mutant clones (A, B and C) revealed a  $K_d$  for binding of PDGF-BB of 0.5 nM and ~45 000, 68 000 and 84 000 receptors per cell, respectively (data not shown). The wild-type receptor-expressing cell clone used in the present study,  $\beta\text{1.3}$ , has 53 000 receptors per cell with a  $K_d$  for binding of PDGF-BB of 0.5 nM (data not shown).

To investigate whether Tyr934 is phosphorylated *in vivo*, PAE cells expressing the wild-type PDGF  $\beta$ -receptor or the mutant Y934F receptor were incubated for 3 h with [ $^{32}\text{P}$ ]orthophosphate, then with or without PDGF-BB (100 ng/ml), in the presence or absence of the tyrosine

phosphatase inhibitor pervanadate (100  $\mu\text{M}$ ), for 7 min at 37°C. Cells were then lysed in a Nonidet P-40 buffer and processed for immunoprecipitation with anti-phosphotyrosine antibodies (PY20). The immunoprecipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The amount of  $^{32}\text{P}$  radioactivity incorporated in the ligand-stimulated wild-type PDGF  $\beta$ -receptor was >10-fold higher in the presence of pervanadate (Figure 3A). Even in the absence of PDGF-BB, pervanadate induced phosphorylation of the PDGF receptor. Two-dimensional tryptic maps revealed a minor spot at the expected position of phosphopeptide N1 in the tryptic map of the PDGF-stimulated wild-type receptor but not in that of the Y934F mutant receptor (data not shown).

In order to increase the sensitivity of the phosphorylation analysis, we used a peptide antiserum ( $\beta\text{YEI}$ ), which was raised against the region in the PDGF  $\beta$ -receptor covering amino acid residues 923–939. Immunoprecipitation of a total tryptic digest of the wild-type PDGF  $\beta$ -receptor from  $^{32}\text{P}$ -labeled cells using the  $\beta\text{YEI}$  antiserum revealed that a pair of peptides recognized by the antibody was phosphorylated *in vivo* after ligand stimulation of the receptor

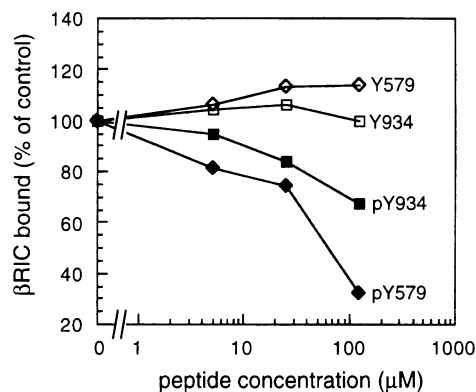


**Fig. 4.** Kinetics of phosphorylation of the Y934 and the c-Src optimal peptides by c-Src. Different concentrations of the Y934 peptide or the c-Src optimal peptide and a fixed amount of purified c-Src were subjected to *in vitro* kinase assay for 2 min at 20°C in the presence of [ $^{32}$ P]ATP. The kinase reactions were stopped by quenching with EDTA. After the reaction, Src was precipitated in 10% (w/v) TCA in the presence of BSA as a carrier. Five  $\mu$ l of the supernatant was spotted on phosphocellulose paper. After washing, the radioactivity incorporated into the peptides was estimated by exposure on a Bio-Imager screen and quantified using a Fuji Bio-Imager. The results were expressed as PSL values and plotted according to Lineweaver-Burk.

in the presence of pervanadate (Figure 3B and C). These phosphopeptides migrated similarly to N1 and N2, and were not observed after immunoprecipitation with the pre-immune serum (Figure 3C). Phosphoamino acid analysis of the immunoprecipitated phosphopeptide eluted from the TLC plate in Figure 3B showed phosphorylation only on tyrosine (Figure 3D). The fact that the phosphorylation was only observed in the presence of the tyrosine phosphatase inhibitor pervanadate indicates that this PDGF-induced phosphorylation is tightly regulated by dephosphorylation by a protein tyrosine phosphatase. No corresponding phosphorylated peptide was observed in immunoprecipitations with  $\beta$ YE1 antibodies of a tryptic digest of PDGF receptors from the cell line expressing the Y934F mutant receptor (data not shown). These results show that Tyr934 in the second part of the kinase domain of the PDGF  $\beta$ -receptor is phosphorylated *in vivo* in response to PDGF-BB.

#### **The Y934 peptide is an efficient substrate for c-Src**

Using a peptide library approach, Songyang *et al.* (1995) recently deduced that the peptide AEEIYGEFEAKKKK is an optimal peptide substrate for c-Src *in vitro*. We therefore compared this Src optimal substrate peptide with the Y934 peptide in an *in vitro* kinase assay with purified Src. Phosphorylation reactions were performed with varying concentrations of peptide in the presence of a fixed amount of Src and [ $^{32}$ P]ATP. The kinase reactions were stopped after 2 min of incubation at 20°C by addition of EDTA. The Src protein was precipitated in 10% trichloroacetic acid (TCA) and part of the supernatant was spotted onto phosphocellulose paper. After careful washing in 75 mM phosphoric acid, the phosphocellulose paper was exposed on a Bio-Imager screen. After scanning and quantification using a Fuji Bio-Imager, the data were plotted according to Lineweaver-Burk (Figure 4). From the intersections with the abscissa, we calculated the  $K_m$  values for the Y934 peptide and the Src optimal peptide to be 31 and 9  $\mu$ M, respectively, for phosphorylation by



**Fig. 5.** Peptide competition for binding of the autophosphorylated  $\beta$ IRIC kinase to the c-Src SH2 domain.  $\beta$ IRIC autophosphorylated in the presence of [ $^{32}$ P]ATP was incubated with a fusion protein of GST and the SH2 domain of c-Src, in the absence or presence of different concentrations of phosphorylated (closed symbols) or non-phosphorylated (open symbols) Y934 peptide (squares) or Y579 peptide (diamonds). Samples were incubated for 2 h before the GST-SH2 fusion protein was adsorbed to glutathione-Sepharose beads by incubation for another 30 min. After washing, the bound  $\beta$ IRIC protein was eluted and subjected to SDS-PAGE. The gel was dried and exposed on a Bio-Imager screen, and the radioactive  $\beta$ IRIC protein recovered in each sample was quantitated.

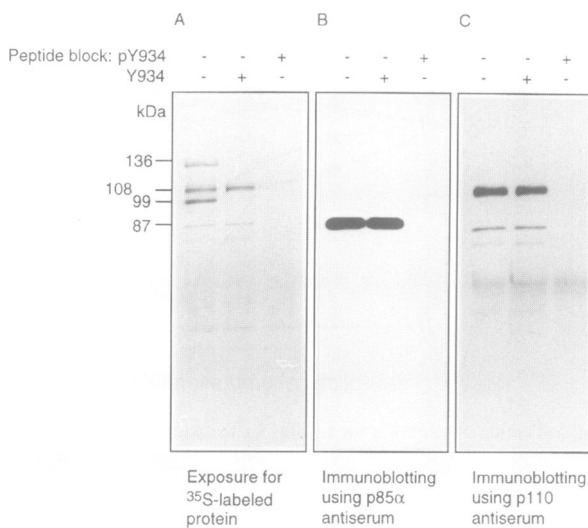
Src. Thus, the Y934 peptide is an efficient substrate for the Src kinase.

#### **The phosphorylated Y934 peptide interacts with the Src SH2 domain with low affinity**

It has been suggested that the SH2 domain of Src tends to bind to phosphorylated tyrosine residues in sequences which can be phosphorylated by Src itself (Songyang *et al.*, 1995). To investigate whether this was the case for the Tyr934 site in the PDGF  $\beta$ -receptor, we incubated  $\beta$ IRIC kinase phosphorylated in the presence of [ $^{32}$ P]ATP with a GST fusion protein containing the SH2 domain of Src in the presence of varying concentrations of phosphorylated or non-phosphorylated Y934 peptide. As a positive control, we included in parallel a phosphorylated peptide representing the juxtamembrane sequence of the PDGF  $\beta$ -receptor containing Tyr579 (Y579 peptide) which has been shown to interact with c-Src (Mori *et al.*, 1993). After incubating the GST-SH2 fusion protein with  $\beta$ IRIC and peptides for 1 h, glutathione-Sepharose was added and the incubation prolonged for 30 min. After extensive washing, Laemmli SDS sample buffer was added to the samples and proteins separated by SDS-PAGE. The dried gel was exposed on a Bio-Imager screen for quantification. The Y579 phosphorylated peptide interfered with the binding of  $\beta$ IRIC to the SH2 domain of Src with half-maximum inhibition at 125  $\mu$ M, while the Y934 phosphorylated peptide was less efficient with half-maximum inhibition at a peptide concentration of >1 mM; none of the unphosphorylated peptides competed (Figure 5). Based on these data, we conclude that the Src phosphorylation site Tyr934 in the PDGF  $\beta$ -receptor has, after phosphorylation, only low affinity for the c-Src SH2 domain.

#### **Phosphopeptide affinity chromatography employing the phosphorylated Y934 peptide**

In order to investigate whether phosphorylated Tyr934 could mediate binding to specific signal transduction



**Fig. 6.** Affinity purification of [<sup>35</sup>S]methionine- and [<sup>35</sup>S]cysteine-labeled cellular proteins using immobilized phosphorylated Y934 peptide. (A) Three mg of protein from a [<sup>35</sup>S]methionine- and [<sup>35</sup>S]cysteine-labeled lysate of HeLa cells was pre-cleared and applied to phosphorylated Y934 peptide coupled to Affigel-10. As controls for specificity, aliquots of the sample were blocked with 200 μM of non-phosphorylated Y934 peptide or 200 μM of phosphorylated Y934 peptide. After 2 h of incubation, the samples were washed extensively. The eluted proteins were separated by SDS-PAGE and electrotransferred to a PVDF membrane; the membrane was exposed for radioactivity on an X-ray film. The calculated sizes of the protein bands, as determined from the migration of <sup>14</sup>C-labeled standards, are indicated. (B) The PVDF membrane exposed for <sup>35</sup>S-radioactivity in (A) was blocked with 5% BSA in Tris-buffered saline before immunoblotting with antibodies against the p85α regulatory subunit of PI3'-kinase. The blot was developed by chemiluminescence and exposed on X-ray film. (C) After blocking the membrane again in 5% BSA, without removing the p85α antibody, the membrane was re-probed with polyclonal antibodies against the p110 catalytic subunit of the PI3'-kinase and developed by chemiluminescence.

molecules, affinity chromatography using immobilized phosphorylated Y934 peptide was used. HeLa cells grown for 3 h in the presence of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine were lysed, cleared by centrifugation and incubated with phosphorylated Y934 peptide coupled to Affigel-10. To determine the specificities in the interactions, samples in which 200 μM of either non-phosphorylated or phosphorylated Y934 peptide were added to the lysates were included as well. After incubation for 2 h, samples were washed and bound proteins eluted, separated by SDS-PAGE and transferred to a PVDF membrane by electroblotting. Exposure of the membrane revealed four different <sup>35</sup>S-labeled protein components which bound directly or indirectly to the phosphorylated Y934 peptide (Figure 6A). The interaction with two of these components, i.e. components of 136 and 99 kDa, was blocked by the non-phosphorylated as well as the phosphorylated Y934 peptide, indicating that at least one of these proteins binds to this region in the PDGF β-receptor, but independently of phosphorylation of Tyr934. In contrast, the phosphorylated, but not the unphosphorylated, Y934 peptide blocked the binding of two protein components of molecular masses 87 and 108 kDa. Since the sequence C-terminal of the Tyr934 in the PDGF β-receptor is a consensus sequence for the binding of the SH2 domains of the PI3'-kinase, YXXM (Songyang *et al.*, 1993), we suspected that the 87 and 108 kDa components represented the regulatory

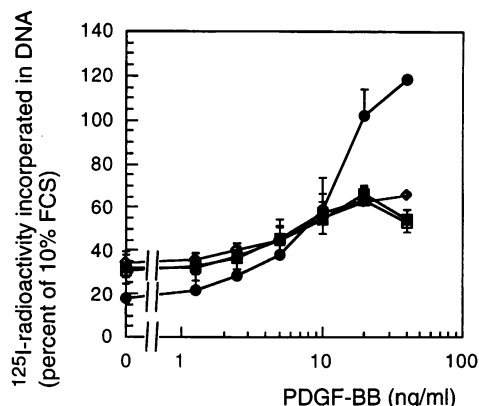
and catalytic subunits of PI3'-kinase, respectively. Probing of the PVDF membrane shown in Figure 6A with antibodies against the p85α subunit revealed that the radioactive 87 kDa component co-migrated exactly with the regulatory subunit of the PI3'-kinase (Figure 6B). In order to exclude the possibility that another protein with the size of 87 kDa co-purified with the regulatory subunit of PI3'-kinase, a larger amount of the 87 kDa protein was purified using the Y934 phosphopeptide column, employing HeLa cells grown in suspension culture as starting material, and was subjected to microsequencing. Three different tryptic peptides from the 87 kDa protein all showed 100% identity to the human p85α subunit (data not shown). We therefore conclude that the p85α regulatory subunit of PI3'-kinase binds to the phosphorylated Y934 peptide. That the <sup>35</sup>S-labeled 108 kDa protein (Figure 6A) corresponded to the catalytic subunit (p110) of the PI3'-kinase, was confirmed by re-probing the PVDF membrane with antibodies against the p110 protein (Figure 6C). The PVDF membrane was not stripped for p85α antibodies before the re-probing with p110 antibodies, and therefore the p85α protein band is also seen in Figure 6C. Furthermore, binding experiments with p85α GST-SH2 fusion proteins showed that both the N- and the C-terminal SH2 domain of p85α can bind to the phosphorylated Y934 peptide (data not shown). In conclusion, Tyr934 in the PDGF β-receptor, phosphorylated by c-Src, binds the SH2 domains of p85α of PI3'-kinase. Re-probing the membrane shown in Figure 6 with anti-Src antibodies did not reveal any significant c-Src binding to the Y934 phosphopeptide-coupled beads (data not shown).

#### **Y934F mutant PDGF receptor-expressing cells show a reduced mitogenic response to PDGF-BB**

Three different cell clones expressing the Y934F mutant receptor were compared with wild-type PDGF β-receptor cells in their ability to respond mitogenically to PDGF-BB. Cells at confluency were starved for 24 h in Ham's F12 medium containing 0.3% fetal calf serum (FCS). After incubation with PDGF-BB at different concentrations for 16 h, the medium was removed and replaced with fresh medium containing [5-<sup>125</sup>I]iododeoxyuridine, whereafter the incubation was prolonged for 4 h. All three different cell lines with Y934F mutant receptors responded to PDGF-BB with a reduced incorporation of [5-<sup>125</sup>I]-iododeoxyuridine compared with the wild-type PDGF β-receptor cells (Figure 7).

#### **Y934F mutant PDGF receptor-expressing cells show an increased chemotactic response to PDGF-BB**

PAE cells expressing the Y934F mutant receptor were analyzed further for their ability to migrate against a gradient of PDGF-BB, employing the leading front assay in a modified Boyden chamber. Cells at a density of 2 × 10<sup>5</sup>/ml were resuspended after trypsinization and centrifugation in EMEM medium containing 10% FCS and seeded in the upper part of the modified Boyden chamber, while media containing 10% FCS and PDGF-BB at different concentrations were added below the 150 μm thick micropore filter. The migration of cells under conditions where medium containing 10% FCS without PDGF was added below the micropore filter was used as a



**Fig. 7.** Mitogenic response to PDGF-BB of Y934F mutant cells. Cells expressing Y934F mutant or wild-type PDGF  $\beta$ -receptors were grown to confluency and serum starved for 24 h before FCS or PDGF-BB were added at different concentrations. After incubation for 16 h, the cells were labeled with [5- $^{125}$ I]iododeoxyuridine for 4 h. The incorporation of radioactivity into DNA in response to different concentrations of PDGF-BB was expressed in relation to the radioactivity incorporated into DNA in the presence of 10% FCS (set as 100%). The wild-type clone employed was  $\beta$ 1.3 (filled circles) and the mutant receptor cell clones were Y934F clones A, B and C.

measure of random migration, also referred to as chemokinesis. Compared with wild-type PDGF  $\beta$ -receptor cells, three different clones of Y934F cells all showed increased chemotaxis with initial responses at much lower doses of PDGF-BB (Figure 8A).

#### **The chemotactic response to PDGF is influenced by PI3'-kinase-dependent as well as -independent pathways**

It has been shown previously that activation of the PI3'-kinase is an important downstream event of PDGF  $\beta$ -receptor-induced cell migration (Wennström *et al.*, 1994). Therefore, we tested whether the PI3'-kinase inhibitor LY294002 was able to block the increased chemotactic response of the Y934F mutant.

Wild-type PDGF  $\beta$ -receptor-expressing cells or Y934F mutant receptor cells were pre-treated with LY294002 at the indicated concentrations for 10 min at 37°C before cells were subjected to the chemotaxis assay using the modified Boyden chamber. The concentration of PDGF-BB was kept constant at 10 ng/ml throughout the assay, i.e. the concentration giving maximal chemotactic response in both wild-type and Y934F mutant receptor cells (Figure 8A), while the concentration of the PI3'-kinase inhibitor LY294002 in the medium was varied (Figure 8C). For the wild-type  $\beta$ -receptor-expressing cells, there was a clear dose-dependent inhibition of cell migration by the PI3'-kinase inhibitor, while the migratory response of the Y934F mutant cells was unaffected by inhibition of PI3'-kinase. To investigate whether the independency of PI3'-kinase activity for the migratory response in the Y934F mutant was dependent on the dose of PDGF, we incubated cells at a fixed concentration of LY294002 (1.4  $\mu$ M) and measured the chemotactic response at increasing concentrations of PDGF (Figure 8B). The wild-type PDGF  $\beta$ -receptor-expressing cells were dependent on the activity of PI3'-kinase, with a clear inhibitory effect of LY294002 over the whole concentration range of PDGF-BB giving stimulation of chemotaxis. In contrast, there was no

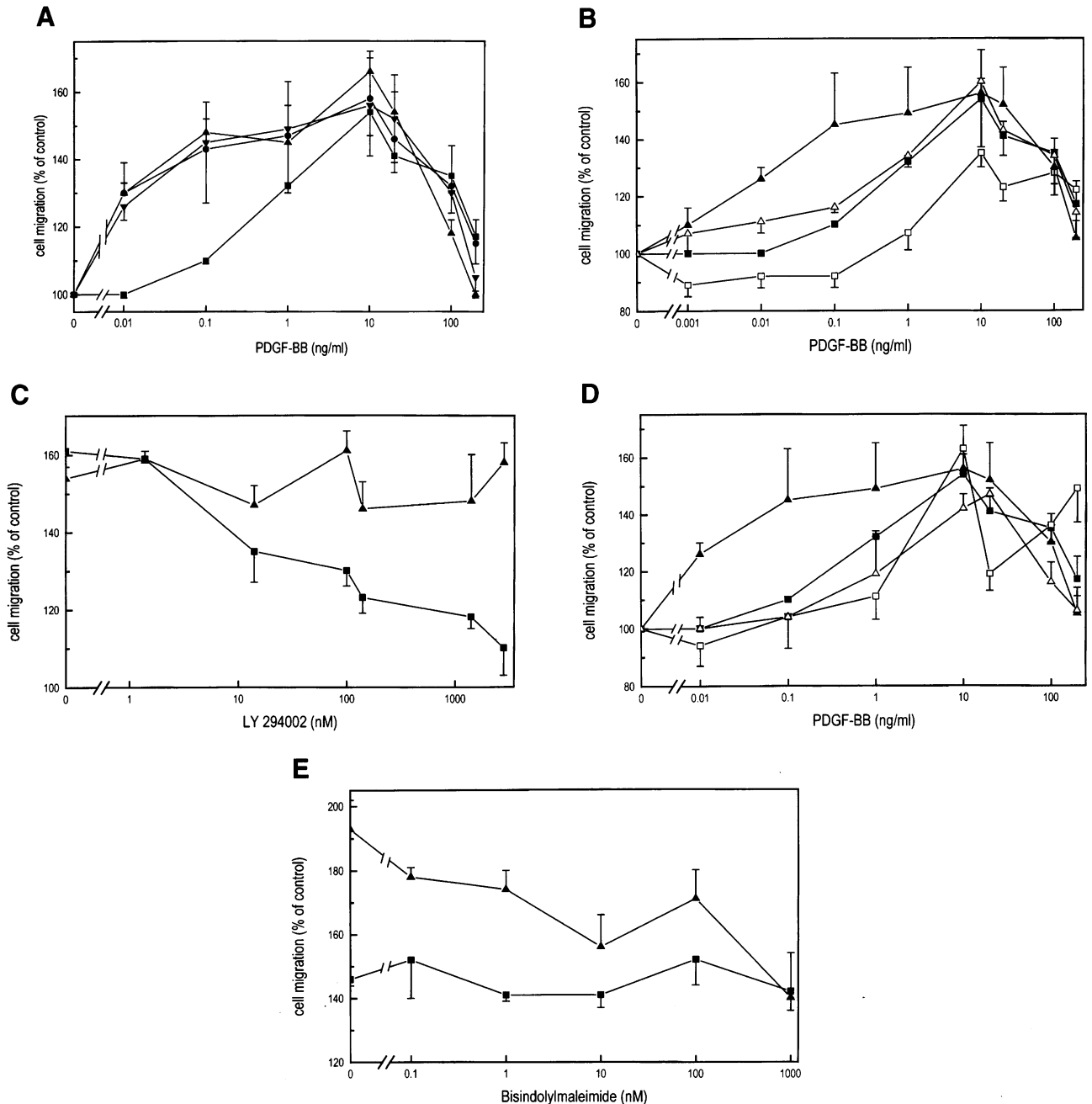
inhibition of cell migration in the Y934F mutant cell lines at the optimal concentration of PDGF-BB (10 ng/ml) and only a small inhibition at lower doses of PDGF (Figure 8B). In conclusion, the results indicate that both PI3'-kinase-dependent and -independent pathways exist for the regulation of chemotaxis.

#### **Increased PDGF-BB-induced actin reorganization in Y934F mutant receptor-expressing cells**

PDGF-BB stimulation of PAE cells expressing the wild-type PDGF  $\beta$ -receptor induces reorganization of actin filaments seen as membrane ruffling, a response possibly correlated to cell movement (Mellström *et al.*, 1983). In order to analyze actin reorganization in response to PDGF-BB in Y934F mutant receptor cells, these cells, as well as cells expressing wild-type receptor, were seeded on chamber slides and incubated in the absence or presence of PDGF-BB for 20 min at 37°C, before fixation and incubation with TRITC-conjugated phalloidin. Analysis by fluorescence microscopy revealed that membrane ruffling was much more pronounced in the cells expressing the Y934F mutant receptor as compared with wild-type PDGF  $\beta$ -receptor cells, especially at low doses of PDGF-BB (compare Figure 9B and F). Furthermore, the membrane ruffling response in cells expressing the Y934F mutant receptor could not be blocked by the PI3'-kinase inhibitor LY294002 at a concentration of 1.4  $\mu$ M (Figure 9C and D), whereas the membrane ruffling response in the wild-type receptor-expressing cells was shown to be sensitive to inhibition by LY294002 (Figure 9G and H). Thus, cells expressing the Y934F mutant receptor showed increased motility responses, including actin reorganization as well as chemotaxis, compared with cells expressing the wild-type receptor. Furthermore, both the increased membrane ruffling and the increased chemotaxis were independent of the activity of PI3'-kinase.

#### **Increased tyrosine phosphorylation of PLC- $\gamma$ in cells expressing the Y934F mutant receptor**

It has been suggested previously that the PLC- $\gamma$  pathway, at least in some cell types, is important for cell migratory responses (Kundra *et al.*, 1994a,b). In order to investigate PLC- $\gamma$  tyrosine phosphorylation in response to PDGF-BB, which is a measure of PLC- $\gamma$  activation (Lee and Rhee, 1995), cells expressing wild-type or Y934F mutant receptors were labeled with [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine before stimulation with various concentrations of PDGF-BB. After 10 min of incubation, the cells were lysed and PLC- $\gamma$  was immunoprecipitated, subjected to separation by SDS-PAGE, and transferred to a PVDF membrane by electroblotting. After exposure for  $^{35}$ S-labeled protein on a Bio-Imager screen (Figure 10A), the membrane was processed for immunoblotting with anti-phosphotyrosine antibodies and  $^{125}$ I-labeled protein A (Figure 10B). By comparing the signals from exposure of  $^{35}$ S-labeled PLC- $\gamma$  with that from the  $^{125}$ I exposure, the extent of tyrosine phosphorylation of PLC- $\gamma$  was determined; a significantly increased tyrosine phosphorylation of PLC- $\gamma$  was seen in cells expressing Y934F mutant receptor compared with those expressing the PDGF wild-type  $\beta$ -receptor (Figure 10C).



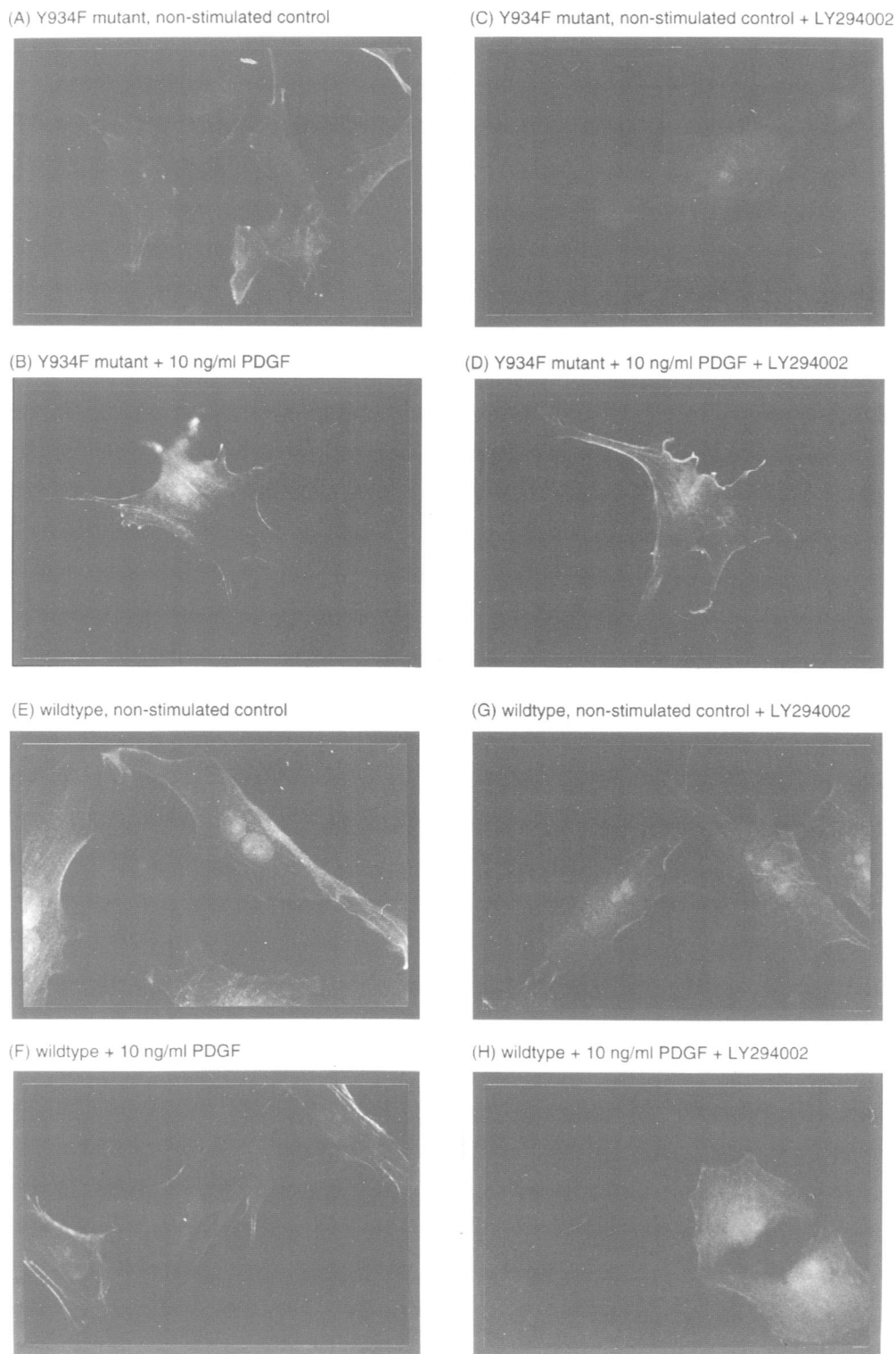
**Fig. 8.** PDGF-BB-induced chemotaxis of PAE cells expressing Y934F and wild-type receptors. (A) The migration of PAE cells expressing the PDGF wild-type  $\beta$ -receptor ( $\beta$ 1.3) (■) or three different cell clones of the Y934F mutant receptor [clones A (▲), B (▼) and C (●)] in response to different concentrations of PDGF-BB, was measured by the leading front assay using a modified Boyden chamber. The migration of cells under conditions where no PDGF-BB was added to the medium below the micropore filter was used as a measure of random migration (chemokinesis) and set to 100%. The result shown is based on three independent experiments (mean  $\pm$  SEM). (B and C) The migration of PAE cells expressing wild-type receptor ( $\beta$ 1.3; squares) or clone A of the Y934F mutant receptor (triangles), in response to different concentrations of PDGF-BB in the presence (open symbols) or absence (filled symbols) of 1.4  $\mu$ M of the PI3'-kinase inhibitor LY294002 (B), or in response to 10 ng/ml of PDGF-BB and different concentrations of LY294002 (C). The result shown is based on three independent experiments (mean  $\pm$  SEM). Similar data were obtained when assaying clones B and C of mutant Y934F for chemotaxis. (D and E) Migration of PAE cells expressing wild-type  $\beta$ -receptors ( $\beta$ 1.3; squares) or clone A of the Y934F mutant receptor (triangles) in response to different concentrations of PDGF-BB in the presence (open symbols) or absence (filled symbols) of 10 nM of the PKC inhibitor bisindolylmaleimide (D), or in response to 10 ng/ml PDGF-BB in the presence of different concentrations of bisindolylmaleimide (E) (results are mean  $\pm$  SEM of at least three independent experiments). Similar results were obtained with clones B and C of the Y934F mutant.

#### Decreased PDGF-BB-induced chemotaxis of cells by inhibition of PKC

PLC- $\gamma$  catalyzes the hydrolysis of phosphatidylinositol bisphosphate (PIP<sub>2</sub>) leading to the formation of diacylglycerol (DAG) and inositol tris-phosphate (IP<sub>3</sub>). IP<sub>3</sub> mobilizes

Ca<sup>2+</sup> from internal stores, which together with DAG activates certain isoforms of protein kinase C (PKC). In view of the increased tyrosine phosphorylation of PLC- $\gamma$  in the Y934F mutant receptor cells, we tested whether the PKC inhibitor bisindolylmaleimide (GF109203X) had any

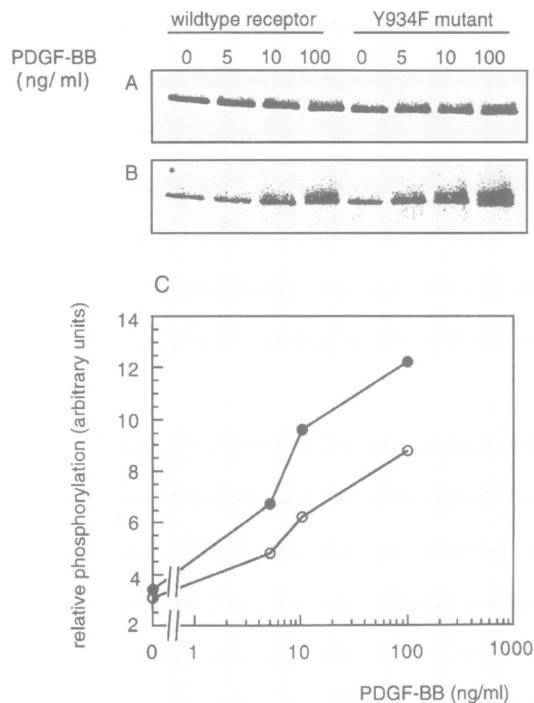




**Fig. 9.** Actin reorganization induced by PDGF-BB in PAE cells expressing wild-type or Y934F mutant receptors. PAE cells expressing Y934F mutant (A–D) or wild-type (E–H) PDGF  $\beta$ -receptors were seeded sparsely on chamber slides and incubated for 24 h in the presence of 10% FCS. Cells were then incubated in the absence (A and E) or presence (B and F) of 10 ng/ml PDGF-BB for 20 min, before slides were washed briefly in PBS and fixed in formaldehyde (5% w/v). Certain cell cultures were treated with the PI3'-kinase inhibitor LY294002, at a concentration of 1.4  $\mu$ M in the absence (C and G) or presence of 10 ng/ml PDGF-BB (D and H). LY294002 was added 10 min before stimulation with PDGF-BB and was also present during the stimulation with PDGF-BB. Actin filaments were visualized by incubation with TRITC-conjugated phalloidin and photographed with Kodak T-MAX 400 film using an Olympus microscope.

effect on the PDGF-BB-induced chemotactic response in cells expressing the mutant or the wild-type PDGF  $\beta$ -receptor. At a concentration of bisindolylmaleimide

giving half-maximal inhibition of PKC (10 nM), the chemotactic response to low doses of PDGF-BB in the Y934F mutant was completely inhibited, while at higher



**Fig. 10.** Tyrosine phosphorylation of PLC- $\gamma$  in response to PDGF. Semi-confluent and serum-starved cells were labeled in medium containing [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine for 3 h. Sodium orthovanadate (100  $\mu$ M) was added during the last 30 min of the labeling period, before the cells were stimulated with PDGF for 10 min at the indicated concentrations, or left unstimulated. Cells were lysed in RIPA lysis buffer and processed for immunoprecipitation with PLC- $\gamma$  antibodies. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to a PVDF membrane.  $^{35}$ S-Labeled PLC- $\gamma$  was revealed by exposure on a Bio-Imager screen as shown in (A). The membrane was then processed for immunoblotting with anti-phosphotyrosine antibodies (anti-pTyr6) and [ $^{125}$ I]protein A. The membrane was exposed for  $^{125}$ I on a Bio-Imager screen (B) and quantitated. The relative tyrosine phosphorylation of PLC- $\gamma$  in response to different concentrations of PDGF-BB for wild-type PDGF  $\beta$ -receptor-expressing cells (○) or the Y934F mutant PDGF  $\beta$ -receptor-expressing cells (clone A; ●) is shown in (C).

doses of PDGF-BB the inhibition was less pronounced (Figure 8D). For the wild-type PDGF  $\beta$ -receptor-expressing cells, there was some inhibition of the chemotactic response by bisindolylmaleimide at concentrations of PDGF-BB below 10 ng/ml (Figure 8D). When cells were stimulated with a fixed concentration of PDGF-BB (10 ng/ml) and exposed to increasing concentrations of bisindolylmaleimide, the chemotactic response of cells expressing the Y934F mutant receptor was blocked in a dose-dependent manner, while the wild-type PDGF  $\beta$ -receptor cells were only slightly inhibited (Figure 8E). These results, together with the results obtained by employing the PI3'-kinase inhibitor LY294002, show that the chemotactic response induced by PDGF-BB in the mutant Y934F receptor cells is less dependent on the PI3'-kinase pathway compared with the wild-type receptor-expressing cells, and that a pathway which may involve the activation of PLC- $\gamma$  dominates.

## Discussion

Here we show that c-Src phosphorylates Tyr934 in the second part of the kinase domain of the PDGF  $\beta$ -receptor.

A receptor mutant in which Tyr934 was replaced with a phenylalanine residue mediated a decreased mitogenic signal, but increased signals for chemotaxis and membrane ruffling, compared with the wild-type receptor. The results suggest that Src via phosphorylation of Tyr934 potentiates mitogenic signaling from the PDGF  $\beta$ -receptor, but negatively controls the signals leading to motility responses via this receptor.

Our *in vitro* kinase experiments show that when Src is added in excess to autophosphorylated and kinase-inactivated  $\beta$ RIC, additional phosphorylation of a number of sites occurs, including most, maybe all, of the known autophosphorylation sites (Figure 1). However, when Src was allowed first to bind to autophosphorylated  $\beta$ RIC and the unbound Src was removed by washing before the phosphorylation reaction, a specific phosphorylation of Tyr934 by Src was observed. Thus, the specificity of Src for tyrosine residues in the PDGF receptor was not very pronounced when added in excess in relation to  $\beta$ RIC. However, after binding through its SH2 domain to certain phosphorylated tyrosine residues in the receptor, preferentially to Tyr579 (Mori *et al.*, 1993), the specificity of c-Src became more restricted and was directed primarily to Tyr934. This tyrosine residue is conserved in the PDGF  $\alpha$ -receptor; however, no phosphorylation of this residue by Src was observed in a similarly designed experiment (S.Ekman, K.Hansen, C.-H.Heldin and L.Rönstrand, unpublished observation).

Phosphorylated Tyr934 was shown to bind PI3'-kinase (Figure 6), and the p85 $\alpha$  subunit of the PI3'-kinase could also be purified from a lysate of HeLa cells using an immobilized peptide containing phosphorylated Tyr934 (data not shown). However, it is unclear how important this interaction is compared with the well characterized binding of PI3'-kinase to phosphorylated Tyr740 and Tyr751 in the kinase insert of the PDGF  $\beta$ -receptor (Fantl *et al.*, 1992; Kazlauskas *et al.*, 1992). Determination of receptor-associated PI3'-kinase activity after stimulation of cells with PDGF revealed no difference between the wild-type receptor and the Y934F mutant receptor (data not shown), suggesting that, at least under these conditions, the contribution to binding and activation of PI3'-kinase by Tyr934 is minor. It should be noted though, that phosphorylation of Tyr934 *in vivo* was seen only in the presence of the protein tyrosine phosphatase inhibitor pervanadate. It is thus possible that the phosphorylation of Tyr934 is tightly controlled by phosphatases and that, under conditions when such phosphatases are modulated, the phosphorylation of Tyr934 might be quantitatively more important.

Apart from PI3'-kinase, components of 99 and 136 kDa were found to bind to an immobilized phosphorylated peptide containing Tyr934. However, in these cases, the binding could be competed with the phosphorylated as well as the non-phosphorylated Tyr934 peptide. Since these interactions are phosphorylation independent, they are not likely to be of primary importance in signaling mechanisms.

The Y934F mutant PDGF  $\beta$ -receptor mediated an increased phosphorylation of PLC- $\gamma$ , compared with the wild-type receptor. There is still no clear picture regarding the involvement of PLC- $\gamma$  in mitogenic signaling. Valius and Kazlauskas (1993) showed that when a PDGF

$\beta$ -receptor mutant deprived of interactions with several signal transduction molecules was given back the ability to interact with PLC- $\gamma$ , it regained some mitogenic signaling. On the other hand, overexpression of PLC- $\gamma$  in NIH3T3 cells (Cuadrado and Molloy, 1990) did not lead to an increased mitogenic signal in response to PDGF or basic fibroblast growth factor, and mutation of the PLC- $\gamma$  binding site (Tyr1021) in the PDGF  $\beta$ -receptor (expressed in porcine aortic endothelial cells; Rönstrand *et al.*, 1992) in fact resulted in an increased mitogenic response to PDGF (Mori *et al.*, 1993). In a recent study, Obermeier *et al.* (1996) showed that exchanging the nerve growth factor receptor/Trk and EGF receptor PLC- $\gamma$  binding sites resulted in the transfer of their distinct affinities for PLC- $\gamma$ . Relative to the wild-type EGF receptor, the increase in PLC- $\gamma$  affinity of the mutant EGF receptor led to an increase in its IP<sub>3</sub> and calcium signals. Furthermore, the mutant EGF receptor showed a significantly decreased mitogenic and transforming potential in NIH3T3 cells. These data suggest a negative role for PLC- $\gamma$  in the mitogenic signals transmitted through the EGF receptor. These observations are consistent with the observation in the present study that the Y934F mutant PDGF receptor, which shows an increased ability to phosphorylate and activate PLC- $\gamma$ , has an decreased ability to mediate a mitogenic response (Figure 7).

An important question that arises from the present study is how the increased activation of PLC- $\gamma$  in the Y934F mutant PDGF  $\beta$ -receptor is related to the increased PDGF-induced chemotaxis of cells expressing this mutant receptor. Kundra and co-workers (1994a,b), found that PLC- $\gamma$  is important for PDGF-induced chemotaxis. In contrast, mutating the association sites for PLC- $\gamma$  on the PDGF  $\beta$ -receptor (Tyr1009 and Tyr1021) to phenylalanine residues did not block the motility response towards the ligand (Wennström *et al.*, 1994). It has been shown that c-Src is able to associate with and phosphorylate PLC- $\gamma$  (Liao *et al.*, 1993; Nakanishi *et al.*, 1993b), suggesting an additional pathway for activation of PLC- $\gamma$ . We have observed tyrosine phosphorylation of PLC- $\gamma$  in the Y1009F/Y1021F mutant in response to PDGF (data not shown) which, although much less pronounced compared with that in wild-type PDGF  $\beta$ -receptor expressing cells, could be sufficient to elicit a response. Moreover, activation of PLC- $\beta$  has been implicated in the action of chemotactic peptides on lymphocytes (for review, see Bokoch, 1995). On the other hand, preventing the association of the p85 $\alpha$  subunit of PI3'-kinase with the PDGF  $\beta$ -receptor, by mutating Tyr740 and Tyr751 to phenylalanine residues, blocked the PDGF-induced chemotactic response (Wennström *et al.*, 1994). Together with the finding that PDGF-induced chemotaxis is inhibited by wortmannin (S. Wennström, L. Claesson-Welsh, R. Hooshmand-Rad, K. Yokote, C.H. Heldin and A. Siegbahn, unpublished data), these observations indicate a role for PI3'-kinase in the induction of chemotaxis, possibly via activation of Rac (Hawkins *et al.*, 1995). Taken together, the available data support the notion that different pathways, involving PI3'-kinase and PLC, respectively, are involved in chemotactic signaling.

The mechanism by which PLC- $\gamma$  affects chemotaxis is not known. IP<sub>3</sub>, generated through the action of PLC- $\gamma$ , induces the release of Ca<sup>2+</sup> to the cytoplasm, which has

been shown to be necessary for cell migration (Nomoto *et al.*, 1988). The other product of PLC- $\gamma$  action, DAG, activates the classical PKCs ( $\alpha$ ,  $\beta$ I,  $\beta$ II and  $\gamma$ ; Dekker and Parker, 1994) and is involved in cell flattening and formation of lamellipodia (Gilbert *et al.*, 1994). Several investigators have reported the ability of DAG to induce a chemotactic response in lymphocytes (Wright *et al.*, 1988; Boonen *et al.*, 1993), suggesting a possible role for PKC in chemotaxis. However, another possibility is that DAG is metabolized further to yield eicosanoids that might function as mediators of chemotaxis; by treatment of neutrophils with cyclooxygenase inhibitors, several groups have shown that leukocyte chemotaxis is prostaglandin dependent (Hagihara *et al.*, 1991; Hyers *et al.*, 1992). PLC- $\gamma$  has also been shown to be important for the PDGF-induced activation of phospholipase D (PLD), an enzyme that hydrolyzes phosphatidylcholine to yield choline and phosphatidic acid which can be metabolized further to DAG by dephosphorylation (Lee *et al.*, 1994; Yeo *et al.*, 1994). Thus, PLD constitutes an alternative pathway for generation of DAG, further enhancing the levels of DAG in cells in response to PDGF. In support of the possibility that stimulation of PKC by DAG is involved in PLC- $\gamma$ -induced stimulation of chemotaxis, we found that the increased chemotactic response in the mutant cell line Y934F was blocked by the PKC inhibitor bisindolylmaleimide.

The results of the present study, together with previous studies, show that chemotaxis in PDGF-stimulated cells is affected by different pathways involving PI3'-kinase as well as PLC- $\gamma$ . Which is then the downstream target upon which the PLC- $\gamma$  and PI3'-kinase pathways converge? Possible candidates are members of the PKC family. Recent data show that PLC- $\gamma$  and PI3'-kinase independently can activate PKC- $\epsilon$  (Moriya *et al.*, 1996) and that activation of PKC- $\lambda$  is dependent on the activity of PI3'-kinase (Akimoto *et al.*, 1996). Another candidate could be PKC- $\zeta$  that, under certain conditions has been shown to be activated by PIP<sub>3</sub>, the product of PI3'-kinase (Nakanishi *et al.*, 1993a; Toker *et al.*, 1994). The possibility that PKCs are common downstream targets for PLC- $\gamma$  and PI3'-kinase is complicated by our finding that the PKC inhibitor bisindolylmaleimide blocks the chemotactic response induced by PDGF in the Y934F mutant, while leaving the wild-type receptor-expressing cells unaffected (Figure 4). However, it is possible that a bisindolylmaleimide-insensitive PKC isoform acts downstream of PI3'-kinase; this putative PKC isoform may be able to transmit a downstream signal similar to PKCs acting downstream of PLC- $\gamma$ . Future experiments, aimed at understanding the role of different pathways in the enhanced chemotactic response seen in the Y934F receptor mutant cell line, will provide us with an enhanced knowledge about the mechanisms involved in chemotaxis in normal cells.

## Materials and methods

### Materials

Pre-coated cellulose TLC plates, 20×20 cm, were purchased from Merck (Darmstadt, Germany), modified sequencing grade trypsin from Promega (Madison, WI) and endoproteinase Glu-C from Boehringer Mannheim (sequencing grade; Mannheim, Germany). Hunter thin layer peptide mapping system was from C.B.S. Scientific Co. (Del Mar, CA). Polyvinylpyrrolidone was from Aldrich (Steinheim, Germany) and soybean

trypsin inhibitor-agarose (STI-agarose) from Pierce (Rockford, IL). c-Src kinase, expressed and purified from insect cells, was from Upstate Biotechnology Incorporated (Lake Placid, NY). [<sup>35</sup>S]Methionine and [<sup>35</sup>S]cysteine *in vivo* cell labeling promix as well as Redivue [ $\gamma$ -<sup>32</sup>P]ATP and [<sup>32</sup>P]orthophosphate (PBS43) were purchased from Amersham (Buckinghamshire, UK). *N*-Ethylmaleimide was from Sigma (St Louis, MO). LY294002 was from Biomol (Plymouth Meeting, PA) and bisindolylmaleimide (GF109203X) was purchased from Calbiochem (San Diego, CA). The intracellular part of the PDGF  $\beta$ -receptor ( $\beta$ RIC) was expressed in insect cells by use of the baculovirus system and purified as will be described elsewhere.

#### Antisera

Rabbit antiserum PDGFR3 was raised against a synthetic peptide corresponding to amino acid residues 1013–1028 of the human PDGF  $\beta$ -receptor (Claesson-Welsh *et al.*, 1989), and antiserum  $\beta$ YEI was raised against the peptide MAQPAHASDEIYEIMQK KKK covering amino acid residues 923–939 of the  $\beta$ -receptor extended with three C-terminal lysine residues.

#### Mutagenesis and transfection

Site-directed mutagenesis was performed on a cDNA encoding the full-length PDGF  $\beta$ -receptor (Claesson-Welsh *et al.*, 1988) using the Altered sites *in vitro* Mutagenesis System (Promega Corp.). The following oligonucleotide was used for the mutagenesis: 5'-CTG GGA GAT CTT CAC CTT GGG-3' (Y934F). The mutation was confirmed by DNA sequencing. The mutated cDNA was inserted into the eukaryotic expression vector pcDNA3 (Invitrogen). The constructs were transfected into PAE cells by electroporation and subsequently selected for G418 resistance as described previously (Claesson-Welsh *et al.*, 1988).

#### Peptide synthesis and purification

Peptides were synthesized in an Applied Biosystems peptide synthesizer (model ABI430A) by Fmoc [*N*-(9-fluorenyl)methoxycarbonyl] chemistry and phosphorylated according to Andrews *et al.* (1991), employing di-*t*-butyl-*N,N*-diisopropylphosphoramidite, as described (Mori *et al.*, 1993). The peptides were purified by reversed phase chromatography, and peak fractions were analyzed by plasma desorption mass spectrometry using an Applied Biosystems Bio Ion 20 instrument. Peptide fractions were freeze-dried and stored under dry conditions. Peptide stock solutions of 1 mM were kept in 20 mM HEPES, pH 7.4, 1 mM dithiothreitol (DTT) at –20°C. The following tyrosine phosphorylated peptides were synthesized: MAQPAHASDEIY(p)EIMQK KKK (pY934 peptide), VSS-DGHEY(p)IYVDPMLPY (pY579 peptide). The corresponding non-phosphorylated peptides (Y934 and Y579 peptides) were also synthesized, as well as AEEIYGEFEAK KKK (c-Src optimal peptide; Songyang *et al.*, 1995).

#### In vitro phosphorylation of the $\beta$ RIC protein by c-Src

Dephosphorylated purified  $\beta$ RIC kinase (~1  $\mu$ g) was immunoprecipitated by PDGFR3 antibodies covalently coupled to protein A-Sepharose by the dimethyl pimelidate method (Schneider *et al.*, 1982), and washed in 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, 1% (w/v) NP-40, 1% (v/v) Trasylol, 5  $\mu$ g/ml leupeptin, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were then either pre-phosphorylated for 20 min at 30°C in the presence of 1 mM ATP, in kinase assay buffer (20 mM HEPES, pH 7.4, 1 mM DTT, 20 mM MnCl<sub>2</sub>), or incubated in the same buffer without ATP. After phosphorylation, the samples were washed in the Tris buffer (see above) followed by several washes in the same buffer supplemented with NaCl to 500 mM before washing in 20 mM HEPES, pH 7.4, 150 mM NaCl. Samples were then incubated with 2 mM *N*-ethylmaleimide for 2 h, end-over-end at 4°C to inactivate the  $\beta$ RIC kinase. The kinase-inactivated  $\beta$ RIC protein was then washed extensively in Tris buffer containing 500 mM NaCl, to remove *N*-ethylmaleimide. Samples were then incubated for 1 h at 4°C end-over-end in the absence or presence of c-Src, then washed with the Tris buffer twice, with or without 500 mM NaCl, and finally washed in kinase assay buffer. Phosphorylation was then performed in kinase assay buffer containing 50  $\mu$ Ci of [<sup>32</sup>P]ATP for 15 min at 30°C. One of the samples, which was pre-phosphorylated in the presence of 1 mM non-labeled ATP, was incubated with an excess of c-Src during the final phosphorylation reaction in the presence of [<sup>32</sup>P]ATP.

#### In vitro kinase reactions using peptides

The kinase reactions were performed in 20 mM HEPES, pH 7.4, 1 mM DTT, 10 mM MnCl<sub>2</sub>, 40  $\mu$ M sodium orthovanadate and [<sup>32</sup>P]ATP (from 10 to 50  $\mu$ Ci per reaction; sp. act. 3000 Ci/mmol). In kinetic studies, non-

labeled ATP was added together with [<sup>32</sup>P]ATP to 100  $\mu$ M final concentration.

#### Coupling of peptides to Affigel-10

Peptides were coupled to Affigel-10 through primary amino groups according to the standard protocol from Bio-Rad. Two and a half milligrams of peptide was suspended in 2 ml of 50 mM HEPES, pH 7.2 (pH was adjusted with 5 M NaOH). One ml of Affigel-10, washed with double-distilled water (ddH<sub>2</sub>O), was added to the suspended peptide and incubated end-over-end for 1 h at 20°C. The efficiency of coupling was determined by measuring the OD<sub>280</sub> (or OD<sub>268</sub> for phosphorylated peptides) of the peptide solution before and after coupling. The coupling efficiencies of the four different peptides used in this study were: Y934 peptide, 56%; pY934 peptide, 48%; Y579 peptide, 29%; pY579 peptide, 37%. In experiments with [<sup>35</sup>S]methionine- and [<sup>35</sup>S]cysteine-labeled cell extracts, pre-cleaning of the lysates was performed using rabbit pre-immune serum coupled to Affigel-10. The coupled peptides and pre-immune serum were stored at 4°C in 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT and 0.2% sodium azide.

#### In situ trypsin digestion, two-dimensional phosphopeptide mapping and phosphoamino acid analysis

Phosphorylated proteins were separated by SDS-PAGE, using 8 or 10% polyacrylamide gels and transferred to a nitrocellulose membrane (Hybond-C extra, Amersham) by electrotransfer. Samples were processed for tryptic digestion, two-dimensional phosphopeptide mapping and phosphoamino acid analysis, as described by Blume-Jensen *et al.* (1995).

#### Immunoprecipitation after tryptic cleavage

Phosphoproteins digested with trypsin, as described above, were incubated end-over-end for 1 h at 4°C with 25  $\mu$ l of STI-agarose (Pierce) in a final volume of 300  $\mu$ l of 50 mM NH<sub>4</sub>HCO<sub>3</sub>. The supernatants were then transferred to tubes containing antibodies covalently coupled to protein A-Sepharose (Schneider *et al.*, 1982) and incubated for 1.5 h end-over-end, at 4°C. Immunoprecipitates were then washed three times in 50 mM NH<sub>4</sub>HCO<sub>3</sub>, 0.05% (w/v) NP-40 and once with ddH<sub>2</sub>O, before eluting with pH 1.9 buffer or 1% diethylamine, pH 11.5. After elution, the samples were evaporated and resuspended in 7  $\mu$ l of pH 1.9 buffer, for electrophoresis.

#### Elution of phosphopeptides from TLC plates for secondary cleavage with endoproteinase Glu-C or automated Edman degradation

After exposure of the TLC plate on the Fuji Bio-Imager, the phosphopeptides of interest were scraped off the plate and then eluted with 200  $\mu$ l of isobutyric chromatography buffer or pH 1.9 buffer, depending on the character of the phosphopeptide; this was followed by another elution with 200  $\mu$ l of buffer. The supernatants were then pooled in a new tube and evaporated. Eluted phosphopeptides were resuspended in 50  $\mu$ l of 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.6, and endoproteinase Glu-C (*Staphylococcus* protease V8) was added at a concentration of 4  $\mu$ g/ml; samples were incubated for 2 h at 37°C. After 2 h, fresh protease was added and the digestion continued. The addition of endoproteinase Glu-C was repeated four times, at intervals of 2 h, in order to obtain an efficient cleavage of the peptide. After the last incubation, the sample was evaporated and resuspended in pH 1.9 buffer for electrophoresis. Precipitates were removed by centrifugation before sample application.

#### Automated Edman degradation

Eluted phosphopeptides were coupled to Sequelon™-AA membrane (Millipore), by use of carbodiimide coupling, according to standard procedures as described by the manufacturer, and Edman degradation was performed using an Applied Biosystems gas phase sequencer (Model 477A) as described by Blume-Jensen *et al.* (1995).

#### Metabolic labeling with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine and affinity purification of labeled proteins on immobilized phosphopeptides

Semi-confluent PAE cells (75 cm<sup>2</sup> cell culture flask), 24 h after passaging, were washed once with methionine- and cysteine-free medium (MCDB 104 with 20 mM HEPES, pH 7.2); 4 ml of the same medium containing 250  $\mu$ Ci/ml [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine Promix (Amersham) was then added to the cells, followed by incubation at 37°C for 2 h. Cells were then washed three times in ice-cold 20 mM Tris, pH 7.4, 150 mM NaCl, and lysed in 2.5 ml ice-cold RIPA buffer [20 mM Tris, pH 7.4, 1% (w/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 5  $\mu$ g/ml leupeptin, 1% (v/v) Trasylol, 5 mM EDTA, 200  $\mu$ M

sodium orthovanadate, 150 mM NaCl]. After incubation on ice for 5 min, the cell lysates were transferred to microfuge tubes and spun at 14 000 g for 10 min at 4°C. Before adding the  $^{35}\text{S}$ -labeled cell lysate to the Affigel-bound peptides, the whole lysate was pre-cleared with 400  $\mu\text{l}$  of pre-immune serum coupled to Affigel-10 (30 min end-over-end, at 4°C). Affigel-10-coupled peptides, in the presence or absence of blocking peptide, were then added to the pre-cleared lysate and tubes were incubated end-over-end for 1 h at 4°C. Thereafter, the samples were washed twice with RIPA buffer and three times with high salt RIPA buffer containing 500 mM NaCl, and finally once again in RIPA buffer before bound proteins were eluted with concentrated Laemmli SDS sample buffer [125 mM Tris, pH 6.8, 4% (w/v) SDS, 10% 2-mercaptoethanol (v/v), 17% glycerol and 0.2% (w/v) bromophenol blue].

#### Immunoprecipitation and Western blotting

Immunoprecipitations and Western blotting was performed essentially according to Blume-Jensen *et al.* (1993).

#### Large-scale purification of proteins on peptide columns

In order to obtain enough protein material for peptide sequencing, extracts of HeLa cells grown in suspension culture were lysed in RIPA buffer, as described above, and mixed with [ $^{35}\text{S}$ ]methionine- and [ $^{35}\text{S}$ ]cysteine-labeled cell lysate (prepared as described above). After lysis of the HeLa cells ( $1 \times 10^{10}$  cells) on ice in a final volume of 200 ml, the lysate was cleared by high speed centrifugation (14 000 g) for 30 min, followed by ultracentrifugation at 100 000 g for another 30 min. The lysate was cleared further by incubation for 1 h at 4°C with rabbit pre-immune serum coupled to Affigel-10, followed by incubation with a mixture of equal parts of gelatin-Affigel-10 and DNase I-Affigel-10 for 1 h. Glycerol was then added to the cleared lysate, to a final concentration of 8%, and lysates were frozen at -70°C. Next day, the lysate was thawed and cleared by ultracentrifugation (100 000 g, 30 min), followed by incubation with pre-immune serum coupled to Affigel-10. The cleared lysate was incubated with pY934 peptide coupled to Affigel-10, end-over-end for 2–3 h, at 4°C. The beads were then washed twice with RIPA buffer followed by four washes in RIPA buffer containing 0.5 M NaCl and finally once with RIPA buffer, before elution of the bound proteins by addition of 200  $\mu\text{M}$  of pY934 peptide followed by incubation for 3 h on ice with occasional vortexing. Concentrated Laemmli SDS sample buffer was then added to the eluate which, after heating at 95°C for reduction, were separated by SDS-PAGE using a 10% acrylamide gel. To make sure that all proteins were eluted with competing peptide, the beads were eluted further directly with Laemmli SDS sample buffer and this eluate was applied on the same gel. After electrophoresis, the gel was fixed and stained with Coomassie Blue R-250.

#### In vivo [ $^{32}\text{P}$ ]orthophosphate labeling of cells

Subconfluent cells in monolayer cultures were starved overnight in 0.3% (v/v) FCS. Cells were washed with phosphate-free medium (Ham's F12) containing 0.2% (v/v) dialyzed FCS, and 20 mM HEPES, pH 7.2, before addition of [ $^{32}\text{P}$ ]orthophosphate (3 mCi/ml) in phosphate-free medium. Cells were then incubated for 3 h at 37°C before stimulation with PDGF-BB (100 ng/ml) and/or sodium pervanadate (100  $\mu\text{M}$ ) for 7 min at 37°C. After stimulation, the cells were washed three times in ice-cold Tris-buffered saline, pH 7.4, before lysis in 20 mM Tris, pH 7.4, 150 mM NaCl, 1% (w/v) NP-40, 1% Trasyolol, 5  $\mu\text{g}/\text{ml}$  leupeptin, 5 mM EDTA, 1 mM PMSF, 200  $\mu\text{M}$  sodium orthovanadate. Non-soluble material was removed by centrifugation at 14 000 g for 10 min at 4°C. The cleared lysates were then incubated with anti-phosphotyrosine antibodies (PY20, Transduction Laboratories, Lexington, KY) at a concentration of 1  $\mu\text{g}$  antibody per mg of cell lysate protein. After 2 h of incubation at 4°C, protein A-Sepharose was added and incubation was prolonged, end-over-end, for an additional 45 min. Immunoprecipitates were washed extensively in lysis buffer followed by lysis buffer supplemented with 500 mM NaCl, before elution with concentrated Laemmli SDS sample buffer. Samples were heated to 95°C and subjected to SDS-PAGE using a 10% acrylamide gel. The separated proteins were then transferred to a nitrocellulose membrane (Hybond C-extra), as described above. After electrotransfer, the membrane was washed in ddH<sub>2</sub>O before exposure on a Bio-Imager screen. The phosphorylated bands corresponding to the PDGF  $\beta$ -receptor were cut out and processed for tryptic cleavage as described above.

#### Cell motility assay

The chemotactic response of PAE cells expressing wild-type PDGF receptor and receptor mutants was assayed by means of the leading front

technique in a modified Boyden chamber as described previously (Siegbahn *et al.*, 1990). The two compartments in the Boyden chamber were separated by a 150  $\mu\text{m}$  thick micropore filter (Millipore), which was pre-coated with type-1 collagen (Vitrogen 100; Collagen Corporation). Exponentially growing cells were detached from the culture flask by trypsin treatment, washed in Hank's balanced salt solution and suspended at a final concentration of  $3 \times 10^5$  cells per ml in Ham's F12 medium supplemented with 10% FCS and antibiotics. One hundred  $\mu\text{l}$  of the cell suspension was added above the filter in the Boyden chamber, while the same medium containing PDGF-BB was added below the filter. In experiments where the effect of enzyme inhibitors on the motility response were tested, the detached cells were in all cases pre-incubated for 10 min at 37°C with the inhibitor at the indicated concentrations, before the cells were seeded out in the upper compartment of the Boyden chamber. The inhibitors were added to the medium below the filter in the Boyden chamber at the same concentration as employed for pre-incubation. The control in each experiment was either medium without ligand and inhibitor or medium without ligand but including inhibitor. The motility response was estimated after 6 h incubation at 37°C. For each set of experiments, the motility response of each cell clone in response to medium containing 10% FCS without ligand and inhibitor served as the control and was referred to as 100% (stimulated random migration or chemokinesis). In experiments with a fixed concentration of inhibitor and a varying concentration of PDGF-BB, the inhibitor was included in the control as well. In all experiments, the samples were analyzed in duplicate, and all experiments were repeated at least three times. In experiments with the Y934F mutant receptor, at least two different clones were employed. The wild-type clone  $\beta 1.3$  used in the present study has been used previously in chemotaxis assays and shown to respond similarly to other wild-type clones (Wennström *et al.*, 1994, and our unpublished results).

#### Examination of actin reorganization employing TRITC-conjugated phalloidin

Cells were seeded out sparsely in chamber slides (Nunc) and grown overnight in Ham's F12 medium containing 10% FCS. In experiments in which the PI3'-kinase inhibitor LY294002 was employed, cells were pre-treated for 10 min with 1.4  $\mu\text{M}$  of the inhibitor ( $\text{IC}_{50} = 1.4 \mu\text{M}$ ) before PDGF-BB was added to the cells at different concentrations. After 20 min at 37°C in the presence or absence of PDGF-BB, the medium was aspirated and the cells washed briefly in phosphate-buffered saline (PBS). Cells were then fixed in 5% (w/v) formaldehyde for 30 min at 4°C. After fixation, the cells were permeabilized by incubation in acetone for 30 min at 4°C, and then washed in PBS followed by incubation in TRITC-conjugated phalloidin (62.5 ng/ml) for 3 h at 20°C. Cells were then washed several times in PBS before adding fluoromount (Southern Biotechnology Associates, Inc, Birmingham, AL) and a coverglass. The specimens were analyzed in an Olympus photomicroscope equipped for epifluorescence, and photographs were taken using Kodak T-MAX 400 film.

#### $\beta\text{RIC}$ binding to the Src SH2 domain in the presence of competing peptides

Purified and dephosphorylated PDGF  $\beta$ -receptor intracellular domain expressed in insect cells by use of the baculovirus system ( $\beta\text{RIC}$ ) was phosphorylated in kinase buffer containing [ $^{32}\text{P}$ ]ATP (20 mM HEPES, pH 7.2, 10 mM  $\text{MnCl}_2$ , 1 mM DTT, 10  $\mu\text{Ci}$  [ $^{32}\text{P}$ ]ATP) for 10 min at 30°C, followed by phosphorylation in the presence of 1 mM non-labeled ATP for 30 min at 30°C. The phosphorylated  $\beta\text{RIC}$  protein was separated from ATP by gel chromatography using a PD-10 column equilibrated in 20 mM Tris, pH 7.4, 150 mM NaCl and 1% (w/v) NP-40. The  $^{32}\text{P}$ -labeled  $\beta\text{RIC}$  protein (100 ng) was then incubated with Src SH2 domain as a GST fusion protein (1  $\mu\text{g}$ ) in the absence or presence of different concentrations of phosphorylated or non-phosphorylated peptides in binding buffer [20 mM Tris, pH 7.4, 150 mM NaCl, 1% (w/v) NP-40] for 2 h before addition of glutathione-Sepharose. After incubation for another 45 min, the precipitated complexes were washed with binding buffer followed by binding buffer adjusted to 500 mM NaCl; the  $\beta\text{RIC}$  protein was then eluted with Laemmli sample buffer, separated by SDS-PAGE in a 10% gel, and quantified by exposure of the gel on a Bio-Imager screen.

#### Mitogenicity assay

PAE cells expressing PDGF receptors were grown to confluency in 12-well plates and then starved for 24 h in 0.3% FCS. PDGF-BB was added to the cells at concentrations up to 40 ng/ml. Cells stimulated with 10% FCS were analyzed in parallel as a positive control. After incubation for

16 h, the cells were given 1  $\mu\text{Ci/ml}$  of  $[5\text{-}^{125}\text{I}]\text{jiododeoxyuridine}$  and incubation was prolonged for 4 h before the labeling medium was aspirated and macromolecules precipitated with 10% (w/v) TCA. After 15 min at 20°C, the TCA was aspirated, the samples were dissolved in 1 M NaOH and radioactivity incorporated in DNA was determined in a  $\gamma$ -counter.

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