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Identification of two C-terminal autophosphorylation sites in the PDGF β-receptor: involvement in the interaction with phospholipase C-γ

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Two novel sites of autophosphorylation were localized to the C-terminal tail of the PDGF β-receptor. To evaluate the importance of these phosphorylation sites, receptor mutants in which Tyr1009, Tyr1021 or both were replaced with phenylalanine residues, were expressed in porcine aortic endothelial (PAE) cells. These mutants were similar to the wild type receptor with regard to protein tyrosine kinase activity and ability to induce mitogenicity in response to PDGF-BB. However, both the Y1009F and Y1021F mutants showed a decreased ability to mediate association with and the tyrosine phosphorylation of phospholipase C-γ (PLC-γ) compared to the wild type PDGF β-receptor; in the case of the Y1009F/Y1021F double mutant, no association or phosphorylation of PLC-γ could be detected. These data show that tyrosine phosphorylation of PLC-γ is dependent on autophosphorylation of the PDGF β-receptor at Tyr1009 and Tyr1021.

Key words: autophosphorylation/PDGF β-receptor/phospholipase C-γ/tyrosine phosphorylation

Introduction

Platelet-derived growth factor (PDGF) is a connective tissue cell mitogen that can occur as homo- and heterodimers of covalently bound A and B polypeptide chains (for reviews, see Heldin and Westermark, 1990; Raines *et al.*, 1990). Two different types of receptors for PDGF exist, the α- and β-receptors, which are structurally similar but have different ligand binding specificities (Yarden *et al.*, 1986; Matsui *et al.*, 1989; Claesson-Welsh *et al.*, 1989a). Each receptor has an extracellular part composed of five immunoglobulin-like domains, a single transmembrane segment and a protein-tyrosine kinase domain with a characteristic insert without homology to kinases.

PDGF binding causes receptor dimerization and induces the intrinsic protein tyrosine kinase activities of the receptors leading to autophosphorylation and to the phosphorylation of target substrates on tyrosine residues (for reviews, see Heldin and Westermark, 1990; Raines *et al.*, 1990). Several lines of evidence indicate that autophosphorylated tyrosine residues form binding sites for potential substrate molecules. In particular, a motif of ~100 amino acid residues, which is known as *src* homology 2 (SH2) domain and is present in several proteins, has been implicated in the interaction with autophosphorylated tyrosine residues (reviewed in Koch *et al.*, 1991; Margolis, 1992). Some of the identified SH2 domain containing proteins can be linked to the early effects of growth factors, including phospholipase C-γ (PLC-γ) (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989), the regulatory subunit (p85) of phosphatidylinositol 3′ kinase (PI-3′-K) (Auger *et al.*, 1989; Coughlin *et al.*, 1989; Kazlauskas and Cooper, 1989), members of the Src family of protein tyrosine kinases (Kypta *et al.*, 1990) and the GTPase activating protein (GAP) of Ras (Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Molloy *et al.*, 1989). These molecules have been found to associate with activated PDGF receptors as well as with other growth factor receptors and to become phosphorylated on tyrosine residues.

Autophosphorylation sites in the PDGF β-receptor were recently mapped to Tyr751 and Tyr857 (Kazlauskas and Cooper, 1989; Kazlauskas *et al.*, 1991) and Tyr740 and Tyr771 (Kashishian *et al.*, 1992). The Tyr857 mutant showed a decreased tyrosine kinase activity compared with the wild type receptor, as well as a reduced level of tyrosine phosphorylation of GAP and PLC-γ (Kazlauskas *et al.*, 1991). The three autophosphorylation sites in the insert have been shown to mediate the binding of PI-3′-K (Tyr740 and Tyr751) and GAP (Tyr771) (Coughlin *et al.*, 1989; Kaplan *et al.*, 1990; Fantl *et al.*, 1992; Kashishian *et al.*, 1992).

We show here that additional autophosphorylation sites exist in the C-terminal tail of the PDGF β-receptor and these sites mediate the interaction with PLC-γ.

Results

Localization of autophosphorylation sites in purified PDGF β-receptor

In order to localize autophosphorylation sites in the PDGF β-receptor, we prepared receptor protein from porcine uterus and subjected it to *in vitro* phosphorylation in the presence of PDGF, followed by trypsin digestion. The tryptic peptides were separated on an HPLC reversed phase column (Figure 1A) and the major ^{32}P radioactivity-containing peak was subjected to amino acid sequence analysis. Two sequences were obtained (Figure 1B) that can be aligned with the known sequence of the human PDGF β-receptor. The sequences correspond to amino acids 1000–1003 and 1010–1023 of the human PDGF β-receptor. Both sequences may continue beyond the identified amino acids. The fragment starting at amino acid 1010 is likely to be generated through a pseudochymotryptic cleavage at Tyr1009. The region from which the isolated peptides were obtained thus contains two potential sites of autophosphorylation, Tyr1009 and Tyr1021 (Figure 1B). The absence of an identifiable amino acid in position 1021 is compatible with a phosphorylated tyrosine in this position, since PTH-phosphotyrosine is not detectable in the sequencing system used. These findings prompted a further investigation of the possibility

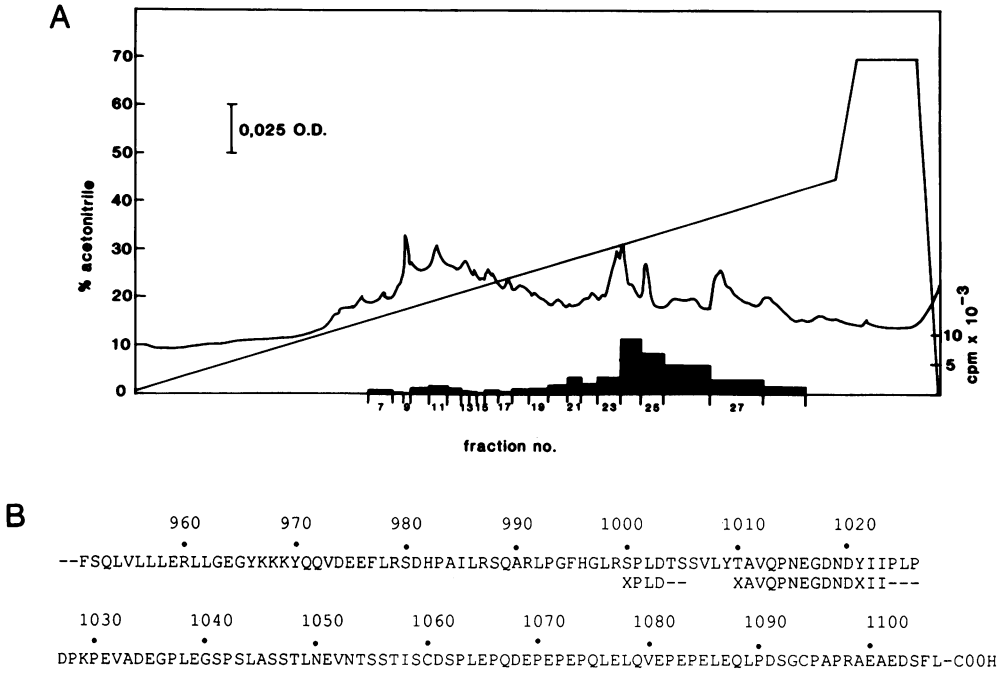

Fig. 1. A. Reversed phase HPLC of tryptic peptides from *in vitro* phosphorylated PDGF β-receptor. Purified porcine PDGF β-receptor was incubated with [γ-^{32}P]ATP in the presence of PDGF-BB and subjected to tryptic digestion. The fragments were separated on a narrow bore Brownlee C4 Aquapore column using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The radioactivity, measured as Cerenkov radiation, is plotted for the individual, manually collected peak fractions. OD was read at 215 nm. Fraction #24 was subjected to amino acid sequencing using an Applied Biosystems Gas Phase Sequencer. **B**. Alignment of the identified sequence of the peptides with the amino acid sequence of the human PDGF β-receptor. Unidentified amino acids are marked with X.

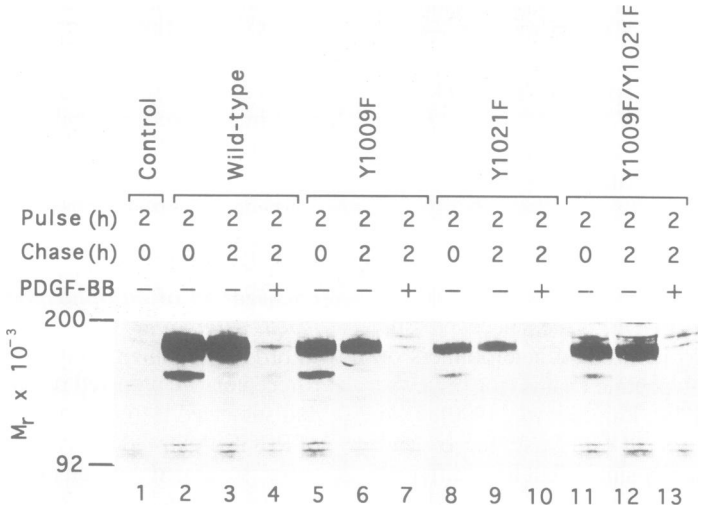

Fig. 2. Immunoprecipitation of wild type and tyrosine residue-mutated PDGF β-receptors. PAE cells expressing the wild type, Y1009F, Y1021F or Y1009F/Y1021F receptors and non-transfected control PAE cells, were pulse-labelled for 2 h at 37°C with [^{35}S]methionine and then chased for 2 h in the presence or absence of 100 ng/ml of PDGF-BB. After incubation, the cells were lysed, immunoprecipitated with PDGFR-3 and analysed by SDS gel electrophoresis and fluorography. The relative migration positions of molecular weight standards (myosin, 200 000; phosphorylase b, 92 500) run in parallel, are indicated.

that Tyr1009 and Tyr1021 of the human PDGF β-receptor are autophosphorylation sites *in vivo*.

Characterization of porcine aortic endothelial (PAE) cell lines expressing Y1009F, Y1021F and Y1009F/Y1021F mutants of the PDGF β-receptor

The Tyr1009 and Tyr1021 codons of the cDNA encoding the wild type β-receptor were changed individually or in combination to phenylalanine codons by site-directed mutagenesis, generating Y1009F, Y1021F and Y1009F/Y1021F receptor mutants, which were stably introduced into PAE cells. Positive clones, identified by binding of ^{125}I-PDGF-BB, were analysed by labelling with [^{35}S]methionine followed by immunoprecipitation with a β-receptor specific antiserum, PDGFR-3. Analysis by SDS–PAGE and fluorography revealed that the wild type and mutant receptor-

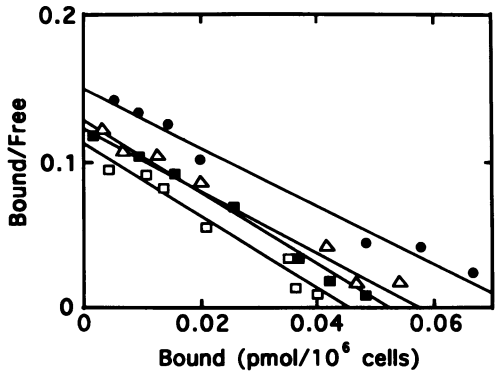

Fig. 3. Scatchard analysis of ^{125}I-PDGF-BB binding to PAE cells expressing wild type or tyrosine residue-mutated PDGF β-receptors. The concentration-dependence of ^{125}I-PDGF-BB binding to cells expressing the wild type (closed circles), Y1009F (closed squares), Y1021F (open squares) or Y1009F/Y1021F (open triangles) receptors were determined. A 90 min incubation at 4°C was used. Binding data were analysed according to Scatchard (1949).

expressing cells contained PDGFR-3 reactive components of 160 and 190 kDa (Figure 2, lanes 2, 5, 8 and 11). These components were not found in non-transfected control PAE cells (Figure 2, lane 1). It was previously shown that the 160 and 190 kDa forms are the precursor and the mature forms of the PDGF β-receptor, respectively (Claesson-Welsh *et al.*, 1988). The intensity of the bands corresponding to the mature forms were substantially reduced when the cells were exposed to PDGF-BB (Figure 2, lanes 4, 7, 10 and 13), due to PDGF-BB-induced internalization and degradation of the receptors (Mori *et al.*, 1991). As judged by Scatchard analysis (Figure 3), PDGF-BB bound with similar affinity to cells expressing the wild-type and the mutant receptors, with dissociation constants of 0.51, 0.41, 0.39 and 0.47 nM for wild type, Y1009F, Y1021F and Y1009F/Y1021F receptor mutants, respectively. The number of binding sites on the cells expressing the wild type, Y1009F, Y1021F or Y1009F/Y1021F mutant receptors were 4.5×10^4, 3.2×10^4, 2.8×10^4 and 3.4×10^4 per cell.

Demonstration that Tyr1009 and Tyr1021 are in vivo phosphorylation sites in the PDGF β-receptor

To investigate whether Tyr1009 and Tyr1021 are *in vivo* phosphorylation sites and also whether the different mutants are functional as PDGF-stimulative kinases, PAE cells expressing wild type, Y1009F, Y1021F or Y1009F/Y1021F mutant receptors were labelled with [^{32}P]orthophosphate and stimulated with PDGF-BB. Labelled cells were analysed by immunoprecipitation with affinity-purified rabbit anti-phosphotyrosine antibodies, followed by SDS gel electrophoresis. Both wild type and Y1009F/Y1021F mutant receptors responded to PDGF-BB with increased autophosphorylation, as well as increased phosphorylation of a number of cellular substrates (Figure 4).

To analyse the phosphorylation sites of the different mutants, [^{32}P]orthophosphate-labelled wild type, as well as mutant β-receptors were digested with trypsin. Trypsin digestion of the human PDGF β-receptor is expected to create a long C-terminal fragment from amino acid 1000 through the end of the protein, since the tail contains no arginine residues and the only lysine residue (Lys1029) is followed by a proline residue, thus preventing cleavage by trypsin (see Figure 1B). This C-terminal fragment contains

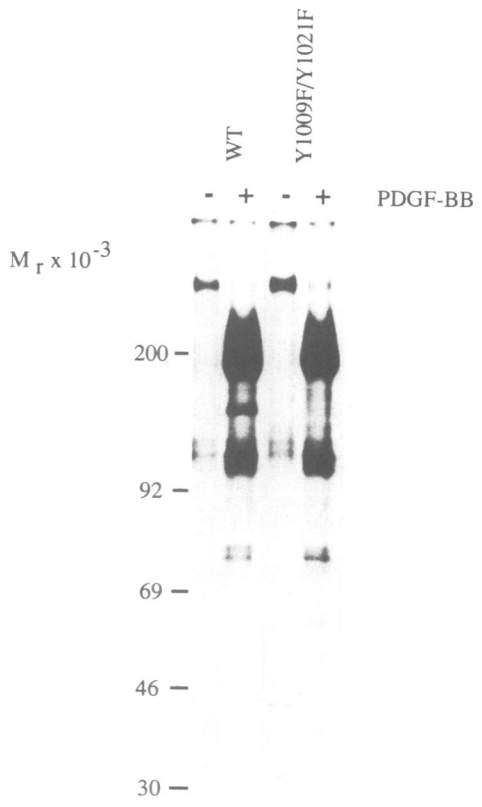

Fig. 4. Orthophosphate labelling of PAE cells expressing wild type or tyrosine residue-mutated PDGF β-receptors. Cells expressing the wild type or Y1009F Y1021F receptors were labelled with [^{32}P]orthophosphate, stimulated with PDGF-BB, lysed and immunoprecipitated with affinity-purified rabbit anti-phosphotyrosine antibodies. Samples were analysed by SDS gel electrophoresis, followed by autoradiography.

only two tyrosine residues, Tyr1009 and Tyr1021. An antiserum, PDGFR-3, raised against a peptide comprising amino acid residues 981–994 of the murine β-receptor sequence (corresponding to amino acid residues 1013–1026 of the human β-receptor), was used to immunoprecipitate the C-terminal fragment. Immunoprecipitation of tryptic digests of [^{32}P]orthophosphate labelled PDGF receptor with the PDGFR-3 antiserum yielded a radiolabelled band. Phosphoamino acid analysis of the precipitated fragment showed the presence of both phosphotyrosine and phosphoserine (data not shown). Therefore, the tryptic digest was further digested with mild acid, which cleaves the C-terminus of the PDGF β-receptor between Asp1027 and Pro1028. This yields one fragment between amino acids 1000 and 1027 that would be expected to react with the PDGFR-3 antiserum. Immunoprecipitation of a tryptic–acid digest from wild-type β-receptor yielded strongly phosphorylated bands (Figure 5). Phosphoamino acid analysis revealed that these peptides contained only phosphotyrosine (data not shown). Thus, the phosphorylated serine residue(s) is located between amino acid 1028 and the C-terminus of the receptor. In contrast, when a tryptic–acid digest from the Y1009F mutant receptor was subjected to immunoprecipitation with the PDGFR-3 antiserum, the intensities of the precipitated bands were only 29% of that of the wild type. Similarly, the Y1021F mutant yielded bands of markedly reduced intensity (40%) compared with the wild type receptor. The tryptic–acid digest from the double mutant Y1009F/Y1021F

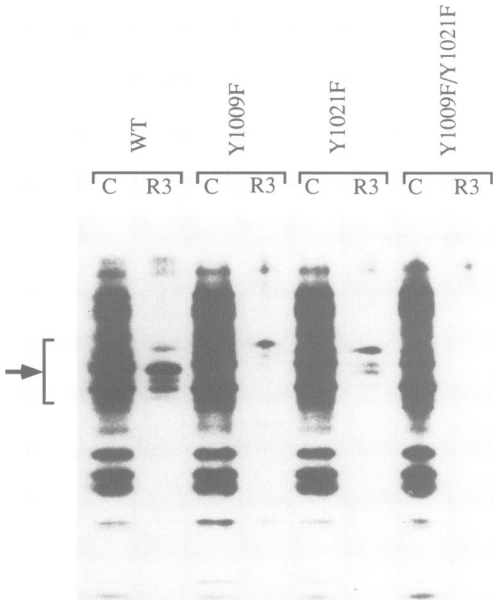

Fig. 5. Immunoprecipitation of tryptic peptides of autophosphorylated wild type or tyrosine residue-mutated PDGF β-receptors. Cells expressing the wild type, or tyrosine residue-mutated PDGF β-receptors were labelled with [^{32}P]orthophosphate, stimulated with PDGF-BB, immunoprecipitated with affinity purified anti-phosphotyrosine antibodies and separated by SDS gel electrophoresis. The band corresponding to the PDGF β-receptor was cut out and extracted from the gel pieces, oxidized and digested extensively with trypsin, followed by mild acid digestion. The resulting digest was either run directly on a gel (C), or immunoprecipitated with the PDGFR-3 antiserum (R3). The digest and the immunoprecipitates were separated on a 40% polyacrylamide gel, dried and autoradiographed. The arrow indicates the fragments precipitated by the R3 antiserum. The radioactivity was visualized by a Phosphorimager, and quantitated using the ImageQuant program.

essentially lacked any ^{32}P-labelled material precipitable with the PDGFR-3 antiserum (2% compared with the wild-type receptor). Furthermore, phosphoamino acid analysis of immunoprecipitated peptides showed the presence of phosphotyrosine only in the Y1009F and Y1021F mutants, but no detectable phosphotyrosine in the immunoprecipitate from the double mutant Y1009F/Y1021F (data not shown).

To verify further that Tyr1009 and Tyr1021 are phosphorylation sites, two-dimensional tryptic–thermolytic phosphopeptide maps were run on the *in vivo* labelled wild type PDGF β-receptor and the mutants Y1009F, Y1021F and the double mutant Y1009F/Y1021F. These maps showed the absence of one phosphorylated spot in the digest from the Y1009F and the Y1009F/Y1021F mutants, compared with the wild type receptor (Figure 6). Phosphoamino acid analysis of this spot showed that it contained phosphotyrosine (data not shown). However, despite the fact that the results shown in Figure 5 indicate that Tyr1021 also is an autophosphorylation site, the pattern obtained by the Y1021F mutant was indistinguishable from the pattern of the wild type receptor. It is possible that the peptide containing Tyr1021 co-migrates with other peptides in the two-dimensional system or that the peptide is lost in the preparation for two-dimensional analysis.

Taken together, these data indicate that both Tyr1009 and Tyr1021 are *in vivo* phosphorylation sites of the PDGF β-receptor; furthermore, the data indicate that the phosphorylation of these sites is not a prerequisite for the phosphorylation of other sites in the receptor.

Phosphorylation of PLC-γ is dependent on autophosphorylated Tyr1009 and Tyr1021 residues in the PDGF β-receptor

Autophosphorylation sites in the C-terminal tails of the EGF receptor (Margolis *et al.*, 1990) and the FGF receptor (Mohammadi *et al.*, 1991) mediate the interaction with

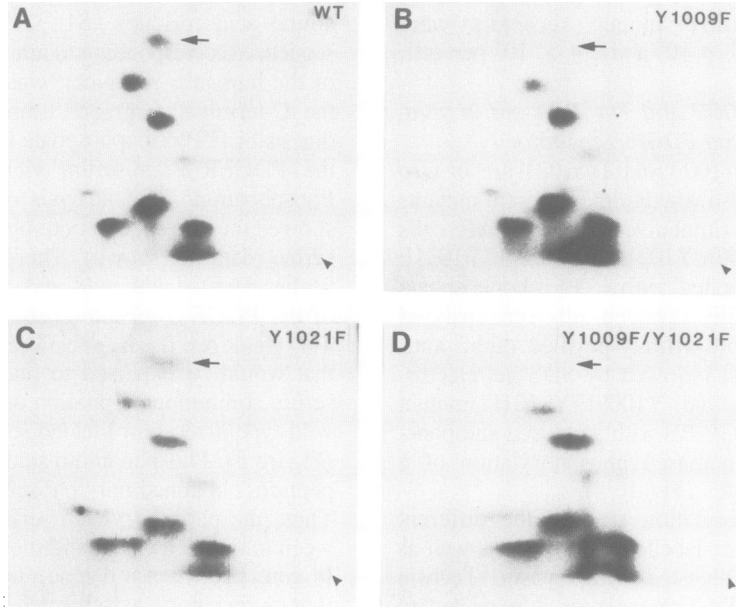

Fig. 6. Two-dimensional tryptic–thermolytic phosphopeptide maps of the wild type or tyrosine residue-mutated PDGF β-receptors. *In vivo* labelled PDGF β-receptors were prepared according to the legend to Figure 5, extensively digested with trypsin, followed by digestion with thermolysin. The resulting digests were then separated electrophoretically at pH 8.9, followed by ascending chromatography (isobutyric acid:pyridine:glacial acetic acid:H_2O:butanol, 65:5:3:2:29). Radiolabelled phosphopeptides were visualized by autoradiography. **A**, wild type PDGF β-receptor, **B**, Y1009F mutant; **C**, Y1021F mutant; **D**, Y1009F/Y1021F mutant. In each case the origin is marked with an arrowhead. Arrows indicate the position of the phosphopeptide containing Tyr1009.

PLC-γ. When subjected to *in vitro* kinase assay the wild type β-receptor phosphorylates a 145 kDa component, i.e. the expected size of PLC-γ (data not shown). In contrast, a mutant PDGF β-receptor with a C-terminal deletion of 98 amino acids, thus lacking Tyr1009 and Tyr1021 (Mori *et al.*, 1991), did not show phosphorylation of this component when subjected to an *in vitro* kinase assay or when assayed using immunoblotting with phosphotyrosine antibodies on extracts from PDGF-stimulated cells (data not shown). We therefore explored the possibility that phosphorylation of PLC-γ was dependent on autophosphorylation of Tyr1009 and/or Tyr1021 of the PDGF β-receptor. Cells expressing wild type or mutant receptors were exposed to PDGF-BB, lysed and immunoprecipitated with a receptor antiserum (PDGFR-HL2; Figure 7A) or with an anti-PLC-γ antiserum (Figure 7B). The immunoprecipitates were separated by SDS−PAGE and transferred onto a nitrocellulose membrane. The blots were then probed with an anti-phosphotyrosine monoclonal antibody. As shown in Figure 7, PDGF-BB stimulation of the wild type receptor expressing cells led to autophosphorylation of the receptor (Figure 7A) as well as to the phosphorylation of PLC-γ (Figure 7B). In Figure 7B, in addition to the phosphorylated PLC-γ band, a band was also detected which most likely represents the tyrosine-phosphorylated wild type receptor co-immunoprecipitated with PLC-γ. PDGF-BB-stimulated phosphorylation of PLC-γ and its association with the phosphorylated receptor was also observed in cells expressing the Y1009F or Y1021F mutant receptors, although to a lesser extent compared to the wild type receptor-expressing cells (Figure 7B). In contrast, Y1009F/Y1021F receptor-expressing cells completely failed to phosphorylate PLC-γ in response to PDGF-BB (Figure 7B). Reprobing of the same filter with the anti-PLC-γ antiserum demonstrated that the difference in phosphorylation of PLC-γ was not due to differences in the amount of PLC-γ immunoprecipitated from the various cell lines (data not shown). In order to estimate the efficiency of PLC-γ phosphorylation by the receptors, we compared the intensity of the PLC-γ band with the receptor band for each cell type by densitometric scanning of the fluorogram (Figure 7). The apparent efficiencies of Y1009F, Y1021F and Y1009F/Y1021F mutant receptors to phosphorylate PLC-γ were found to be 64, 6 and 0%, respectively, compared with the wild-type receptor.

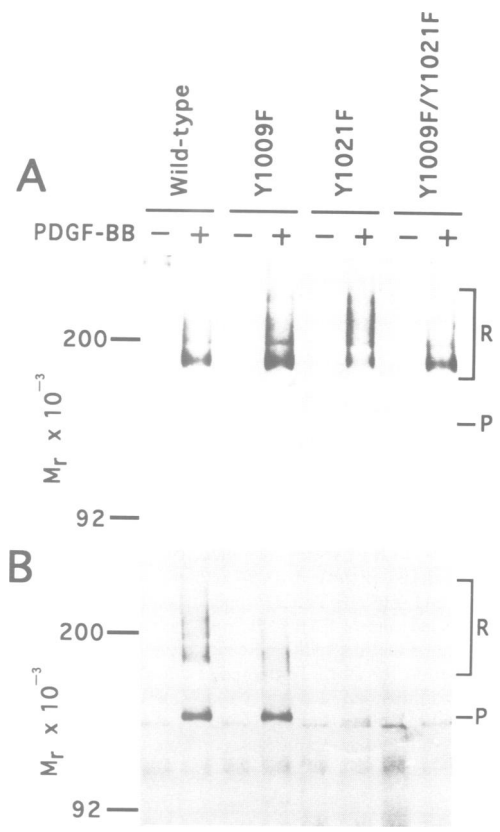

Fig. 7. Tyrosine phosphorylation of PLC-γ after PDGF-BB stimulation of PAE cells expressing wild type or tyrosine residue-mutated PDGF β-receptors. Cells expressing the wild type, Y1009F, Y1021F or Y1009F Y1021F mutant receptors were incubated with or without 100 ng/ml of PDGF-BB for 1 h at 4°C. After incubation, the cells were lysed and immunoprecipitated with the receptor antiserum PDGFR-HL2 (**A**) or with the anti-PLC-γ antiserum (**B**). The immunoprecipitates were separated by SDS gel electrophoresis and transferred onto nitrocellulose membranes. The blots were probed with the anti-phosphotyrosine monoclonal antibody and sites of antibody binding were visualized using the ECL Western blotting detection system (Amersham). The migration positions of the receptor (R) and PLC-γ (P) are indicated. Ubiquitination of the PDGF β-receptor accounts for the smeary appearance of the receptor band (Mori *et al.*, 1992). For a list of molecular weight standards, see the legend to Figure 2.

PDGF-BB stimulated [^{3}H]thymidine incorporation in cells expressing wild type or tyrosine residue-mutated PDGF β-receptors

The abilities of the wild type and mutant receptors to transduce mitogenic signals were examined by a [^{3}H]thymidine incorporation assay (Figure 8). Except for non-transfected control PAE cells, all the cells examined were found to respond to stimulation by PDGF-BB with a similar dose-dependency. Thus, the inability of the mutant receptors to phosphorylate PLC-γ did not perturb their capacity to mediate mitogenic signals.

Discussion

We show in the present work that Tyr1009 and Tyr1021 in the C-terminus of the PDGF β-receptor can serve as autophosphorylation sites. Furthermore, these autophosphorylation sites mediate, in a cooperative manner, the interaction with PLC-γ. A receptor mutant in which both these tyrosine residues were changed to phenylalanine residues underwent autophosphorylation on other tyrosine residues, but was unable to phosphorylate PLC-γ.

The results of the present study, together with other observations, show that there is selectivity in the binding between substrates and different autophosphorylation sites in the PDGF β-receptor. Thus, Tyr740 and Tyr751 in the kinase insert domain of the β-receptor have been implicated in the binding of PI-3′-K, and Tyr771 in the binding of GAP; mutation of these tyrosine residues perturbed the interaction with the respective substrates (Kashishian *et al.*, 1992; Kazlauskas *et al.*, 1992) and phosphorylated peptides containing these residues specifically inhibited the interaction with them (Escobedo *et al.*, 1991; Fantl *et al.*, 1992). Moreover, Tyr857 in the second part of the kinase domain has also been identified as an autophosphorylation site (Kazlauskas and Cooper, 1989). Mutation of Tyr857 has been found to perturb the interaction with members of the Src family (Courtneidge *et al.*, 1991); however, this could

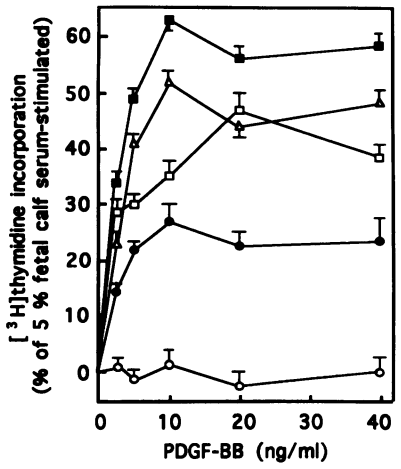

Fig. 8. Stimulation of [^{3}H]thymidine-incorporation by PDGF-BB in PAE cells expressing wild type or tyrosine residue-mutated PDGF β-receptors. After 48 h of serum starvation, cells expressing the wild type receptors (closed circles), Y1009F (closed squares), Y1021F (open squares) and Y1009F Y1021F (open triangles) mutant receptors and non-transfected control PAE cells (open circles), were incubated with [^{3}H]thymidine and the indicated concentrations of PDGF-BB or 5% fetal calf serum for 24 h. Trichloroacetic acid-precipitable radioactivity was measured as described in Materials and methods. Data were calculated relative to the values for the cells stimulated by 5% fetal calf serum and are expressed as means ± standard error of triplicate determinations.

be due to a generally lowered autophosphorylation of the mutant (Kazlauskas *et al.*, 1991). Thus, the six candidate autophosphorylation sites so far identified in the PDGF β-receptor differ in their abilities to interact with the known substrates (Fantl *et al.*, 1992; Kashishian *et al.*, 1992; Kazlauskas *et al.*, 1992; Figure 9). All of these sites, except Tyr1009, are conserved in the α-receptor. Mutations of Tyr731 and Tyr742 in the α-receptor (corresponding to Tyr740 and Tyr751 in the β-receptor) impaired the association with PI-3′-K (Yu *et al.*, 1991), but the sites involved in the interaction with the other substrates have not been identified.

PLC-γ has also been found to interact with autophosphorylation sites in the C-terminus of two other receptors, the EGF receptor (Margolis *et al.*, 1990) and the FGF receptor (Mohammadi *et al.*, 1991). Tyr992 and its flanking sequences has been shown to be responsible for the high affinity binding of PLC-γ to the EGF receptor (Rotin *et al.*, 1992). In the *flg*-encoded FGF receptor, Tyr766 was found to interact with PLC-γ (Mohammadi *et al.*, 1991). PI-3′-K has been shown to interact with autophosphorylation sites surrounded by specific amino acid residues (YM/VXM; Cantley *et al.*, 1991; Fantl *et al.*, 1992). There is a slight similarity in the sequences around the PLC-γ-binding autophosphorylation sites in the EGF and FGF receptors and that around Tyr1021 of the PDGF β-receptor (V/LXX-XXEYL/I; Rotin *et al.*, 1992); however, Tyr1009 has another flanking sequence with no apparent similarity to the proposed motif.

The PDGF and EGF receptors phosphorylates PLC-γ on tyrosine residues 472, 771, 783 and 1254 (Kim *et al.*, 1990, 1991; Wahl *et al.*, 1990), which leads to an increase in the catalytic activity of the enzyme (Nishibe *et al.*, 1990). A requirement of physical association between EGF receptor and phosphorylated PLC-γ for enzyme activation *in vivo* has been demonstrated (Vega *et al.*, 1992). PLC-γ catalyses the degradation of phosphatidylinositol bisphosphate to diacylglycerol and inositol trisphosphate, two second messengers implicated in the activation of protein kinase C and in the mobilization of intracellular Ca^{2+}, respectively (reviewed in Rhee *et al.*, 1989; Wahl and Carpenter, 1991). Apparently, association of PLC-γ with the PDGF receptor or direct phosphorylation by the PDGF receptor, is not a prerequisite for PDGF-induced mitogenicity (Figure 8). This observation is consistent with previous findings by others (Hill *et al.*, 1990). Thus, the activation of this metabolic pathway may not be necessary for PDGF-induced mitogenic signalling. Alternatively, the degradation of phosphatidylinositol bisphosphate may be performed by other isoenzymes, e.g. PLC-βs or PLC-δs, which may be activated via other signal pathways (Rhee *et al.*, 1989). Whether the activation of PLC-γ is of significance for other effects of growth factors on cells, e.g. the stimulation of chemotaxis, remains to be elucidated.

Our data show that PLC-γ interacts with two autophosphorylation sites in the PDGF β-receptor. Mutation of either one of these tyrosine residues led to partial loss of the phosphorylation of PLC-γ and association with the PDGF β-receptor, whereas mutation of both residues led to a complete loss of PLC-γ phosphorylation and receptor association. PLC-γ contains two SH2 domains and thus it is possible that these domains bind to one autophosphorylation site each and both interactions are necessary for high affinity binding. A similar situation may prevail for PI-3′-K, which also has two SH2 domains that interact with two autophosphorylation sites (Tyr740 and Tyr751) (Fantl *et al.*, 1992; Kashishian *et al.*, 1992) located at approximately the same distance from each other as Tyr1009 and Tyr1021. Further studies are required to determine the individual binding affinities of the two SH2 domains of PLC-γ to the respective C-terminal autophosphorylation sites in the PDGF β-receptor and whether both SH2 domains can bind simultaneously to the same receptor molecule.

Materials and methods

Preparation of tryptic peptides from in vitro phosphorylated PDGF β-receptor

PDGF receptor was purified from porcine uterus essentially according to Rönnstrand *et al.* (1987). MonoQ fractions containing PDGF receptor (~100 μg of receptor) were phosphorylated overnight in the presence of 1 μg/ml of PDGF-BB, 50 μM ATP and 10×10^6 c.p.m./ml of [γ-^{32}P]ATP on ice. The sample was passed over a 1 ml anti-phosphotyrosine Sepharose column (5 mg IgG/ml) previously equilibrated with 0.2% Triton X-100, 10% glycerol, 0.15 M NaCl, 1 mM EGTA, 1 mM dithiothreitol and 20 mM HEPES, pH 7.4 at a flow rate of 10 ml/h. The column was washed with 10 ml of equilibration buffer, followed by 10 ml of 0.05% Triton X-100 and 50 mM NH_4HCO_3. Bound material was eluted with 0.05% Triton X-100, 50 mM NH_4HCO_3, and 40 mM phenyl phosphate. Fractions of 1 ml were collected and counted for Cerenkov radiation. Fractions containing radioactivity were pooled and evaporated in a Speedivac. For reduction, the dry material was dissolved in 6 M guanidinium–HCl, 0.25 M Tris–HCl, pH 8.5 and 0.5 mg/ml dithiothreitol, and incubated at room temperature for 3 h, followed by alkylation with 4-vinylpyridine for 2 h at room temperature.

Detergent was separated from protein essentially according to Downward *et al.* (1984). In short, a Superose 6 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) was equilibrated with 6 M guanidinium–HCl and 50 mM NH_4HCO_3, pH 8.0. Reduced and alkylated receptor was applied to the column and eluted at 0.5 ml/min. Fractions were collected and counted for Cerenkov radiation. Fractions containing radioactivity were pooled and extensively dialysed against 1% ammonia–10% propanol, followed by evaporation in a Speedivac. The dried material was dissolved in 2 M urea, 50 mM NH_4HCO_3 and digested with 0.2 μg of TPCK-treated trypsin (Worthington, Freehold, NJ) for 6 h at 37°C, followed by 0.2 μg of trypsin for further 6 h. Tryptic peptides were applied on a narrow bore Brownlee

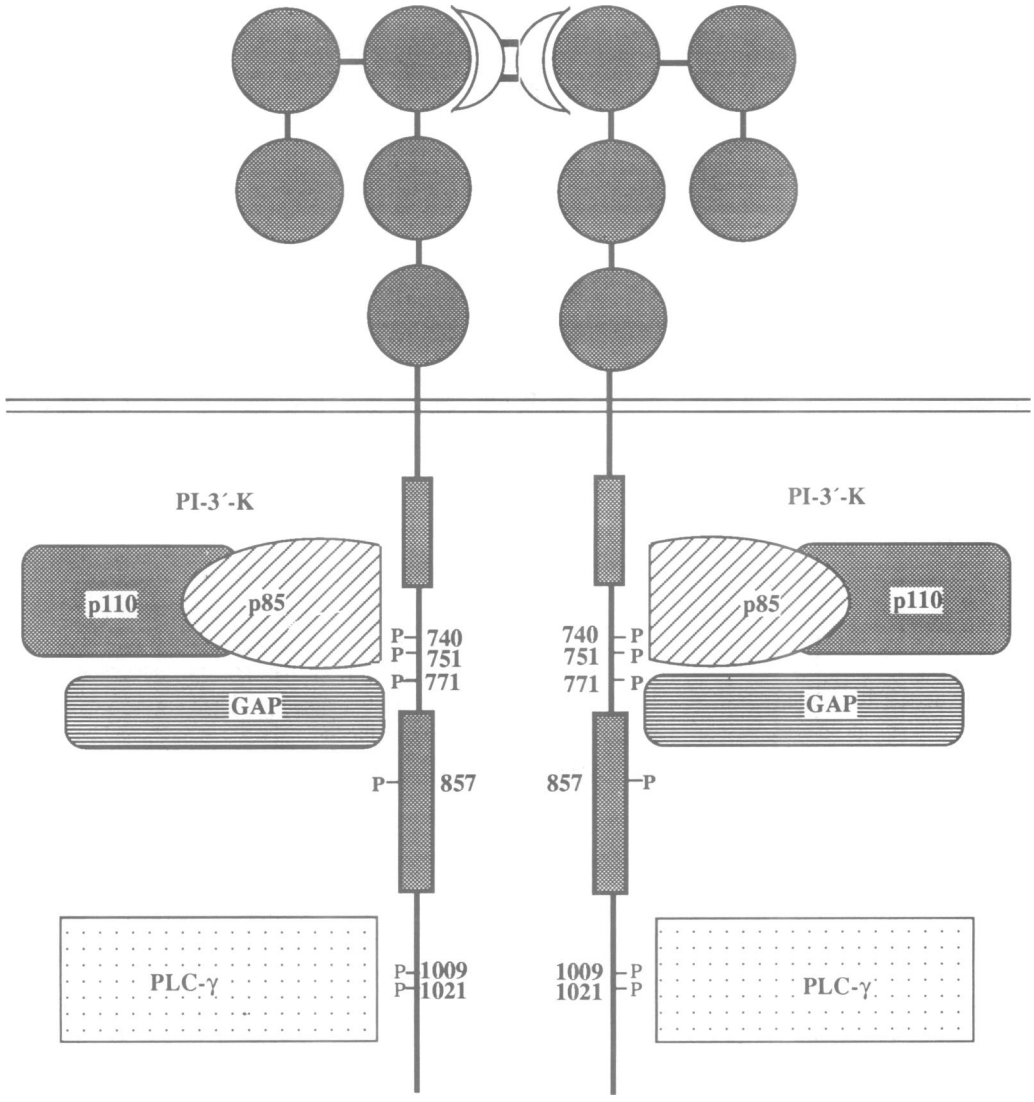

Fig. 9. Schematic illustration of the specificity in the interaction between different substrates and the different activated and autophosphorylation sites in the activated and dimerized PDGF β-receptor. Note that it is not known whether more than one substrate molecule can bind to the receptors simultaneously (not drawn to scale).

C4 Aquapore 2.1 × 30 mm column and eluted by a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Peak fractions were manually collected and counted for Cerenkov radiation. Fractions containing the majority of the radioactivity were subjected to amino acid sequencing on an Applied Biosystems (Foster City, CA) Gas Phase Sequencer Model 470A, equipped with an on-line PTH analyzer, model 120A.

Plasmids

Site-directed mutagenesis was performed using the Altered Sites™ Mutagenesis System (Promega Corporation). A cDNA encoding the full-length human PDGF β-receptor (Claesson-Welsh *et al.*, 1988), which was designated wild type receptor in this study, was subcloned into pSELECT™-1 vector using 5′ *Eco*RI and 3′ *Hin*dIII (at nucleotide 3629 according to Claesson-Welsh *et al.*, 1988) sites of the insert. Point mutations, which change tyrosine residues (Tyr1009 and Tyr1021) to phenylalanine residues, were then introduced into the insert with the oligonucleotides 5′-TCCGTCCTCTTTACTGCCGTG-3′ (Y1009F) and 5′-GACAACGA-CTTTATCATCCCC-3′ (Y1021F). All mutations were confirmed by sequencing. The mutated cDNA was cut out from the vector and cloned into the cytomegalovirus expression vector pcDNAI/NEO (Invitrogen), as described by Mori *et al.* (1991). The wild type receptor cDNA was cloned into the retroviral expression vector pZipNeo (Cepko *et al.*, 1984), as described by Westermark *et al.* (1990).

Cell culture and transfection

The PAE cell line (Miyazono *et al.*, 1987), which lacks endogenous PDGF α- and β-receptors, was cultured in Ham's F12 medium (Gibco) supplemented with 10% fetal calf serum (GIBCO) and 100 units/ml of penicillin. The circular DNA was introduced into PAE cells using electroporation, as described (Claesson-Welsh *et al.*, 1988). Selection of transfected cells was initiated after 48 h by adding Geneticin (G418 sulfate; Gibco) at 0.5 mg/ml to the culture medium. Clones were picked after 2 weeks.

^{125}I-PDGF-BB binding experiments

PDGF-BB was a recombinant form expressed in the yeast strain *Saccharomyces cerevisiae* (Östman *et al.*, 1989). PDGF-BB was labelled with ^{125}I to a specific activity of ~50 000 c.p.m./ng as described by Bolton and Hunter (1973). Binding of ^{125}I-PDGF-BB to transfected cells was evaluated by Scatchard analysis (Scatchard, 1949), as described by Mori *et al.* (1991). Briefly, the cells were incubated on ice for 90 min with ^{125}I-PDGF-BB (≈50 000 c.p.m./well) and unlabelled PDGF-BB at concentrations ranging from 0–400 ng/ml. The cells were then washed, lysed and radioactivity in the lysate was determined in a gamma counter. The value for radioactivity bound in the presence of 1 μg/ml of unlabelled ligand was subtracted as nonspecific binding.

Antisera

The rabbit peptide antiserum PDGFR-3 was generated using a synthetic peptide corresponding to the murine PDGF β-receptor amino acids 981–994 (Yarden *et al.*, 1986) corresponding to amino acids 1013–1026 in the human receptor, this antiserum reacts in a specific manner with the human PDGF β-receptor (Claesson-Welsh *et al.*, 1989b). The rabbit peptide antiserum PDGFR-HL2 was kindly provided by Dr S.M.Weima (Weima *et al.*, 1990);

this antiserum was generated using a synthetic peptide corresponding to the murine PDGF β-receptor amino acids 701–732 (Yarden *et al.*, 1986), which corresponds to amino acids 733–764 of the human β-receptor. The rabbit anti-PLC-γ antiserum was a kind gift from Drs A.Sorkin and G.Carpenter (Arteaga *et al.*, 1991). The mouse anti-phosphotyrosine monoclonal antibody (PY20) was from ICN Biomedicals, Inc. (Costa Messa, CA, USA). Affinity-purified rabbit anti-phosphotyrosine antibodies were prepared according to Rönnstrand *et al.* (1987). Peroxidase-conjugated swine anti-rabbit immunoglobulins was from DAKOPATTS (Copenhagen, Denmark). Peroxidase-conjugated sheep anti-mouse immunoglobulins were from Amersham.

Metabolic labelling, immunoprecipitation and SDS–PAGE

Cells were labelled in methionine-free MCDB 104 medium (McKeehan *et al.*, 1976) supplemented with 100 μCi/ml of [^{35}S]methionine (specific activity >800 Ci/mmol; Amersham) for 2 h at 37°C. Thereafter, the cells were incubated for 2 h at 37°C in the presence or absence of 100 ng/ml of PDGF-BB in Ham's F12 medium containing a 10-fold molar excess of unlabelled methionine and 1 mg/ml bovine serum albumin. After incubation, the cells were washed, lysed and immunoprecipitated, as described by Claesson-Welsh *et al.* (1989b), using the PDGFR-3 antiserum, followed by adsorption to protein A–Sepharose CL-4B (Pharmacia). SDS–PAGE was carried out in gradient slab gels of 5–10% polyacrylamide, according to Blobel and Dobberstein (1975). Gels were prepared for fluorography by soaking in Amplify (Amersham), then dried and exposed to Hyperfilm MP (Amersham).

Immunoblotting

Immunoblotting was performed as described by Mori *et al.* (1992). Cells were incubated with or without 100 ng/ml of PDGF-BB at 4°C for 1 h. After incubation, the cells were washed and lysed in a lysis buffer consisting of 20 mM HEPES, pH 7.5, 1 mM $MnCl_2$, 100 mM NaCl, 0.1% Triton X-100, 500 μM Na_3VO_4, 10% glycerol, 1% Trasylol (Bayer, Leverkusen, Germany) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma). The lysates were centrifuged to remove insoluble materials and the supernatants were incubated with the PDGFR-HL2 antiserum or the anti-PLC-γ antiserum for 2 h at 4°C. Protein A–Sepharose CL-4B was used to precipitate the immune complexes. The immunoprecipitates were separated by SDS gel electrophoresis and the proteins of the gel were electrophoretically transferred onto nitrocellulose membranes (Hybond-C extra, Amersham). Blots were blocked and incubated with the anti-PLC-γ antiserum (1:400 dilution) or with the anti-phosphotyrosine monoclonal antibody (1:1000 dilution). The blots were washed and then incubated with the peroxidase-conjugated swine anti-rabbit or sheep anti-mouse immunoglobulins (1:1000 dilution). After washing, sites of antibody binding were visualized using the ECL Western blotting detection system (Amersham). The blots were reprobed after removal of the first probe, as described by Mori *et al.* (1992).

Thymidine incorporation assay

The thymidine incorporation assay was performed as described previously by Mori *et al.* (1991). Briefly, cells were seeded sparsely in 12-well plates. Two days later, while the cell layer was still subconfluent, the medium was changed to serum-free medium and after an additional 48 h, 0.5 μCi [^{3}H]thymidine/ml (Amersham) and PDGF-BB (0–40 ng/ml) or 5% fetal calf serum, were added. After incubation for 24 h, the cells were washed and high molecular mass ^{3}H radioactivity was precipitated with 5% trichloroacetic acid. The precipitate was washed, solubilized and ^{3}H radioactivity was measured in a liquid scintillation counter.

[^{32}P]orthophosphate labelling of cells for peptide mapping

Confluent 75 cm^2 flasks of PAE cells expressing either wild type or mutant PDGF β-type receptors were washed twice with phosphate-free F 12 medium containing 0.1% dialysed fetal calf serum, 10 mM HEPES, pH 7.4. Fifteen millilitres of phosphate-free medium were added and the cells were incubated at 37°C for 30 min. After this time the medium was aspirated and 3 ml of fresh medium were added together with 6 mCi of [^{32}P]orthophosphate (Amersham) and 50 μM Na_3VO_4. Cells were incubated at 37°C for 2.5 h, after which time they were transferred onto ice. PDGF-BB was added at 100 ng/ml and incubation was prolonged on ice for an additional 1 h. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with 1 ml of 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.15 M NaCl, 20 mM Tris–HCl, pH 7.4, 30 mM $Na_4P_2O_7$, 250 μM Na_3VO_4, 10 mM EDTA, 1 mM PMSF and 1% Trasylol, on ice for 10 min. The lysates were centrifuged at 12 000 *g* for 10 min and the supernatants were incubated for 1 h at 4°C with affinity-purified anti-phosphotyrosine antibodies. Precipitates were collected using protein A–Sepharose CL4B and washed five times using lysis buffer, four times with 1% Triton X-100, 0.5 M NaCl and 20 mM Tris–HCl, pH 7.4, and finally once with 20 mM Tris, pH 7.4. Samples were analysed by SDS gel electrophoresis on a 5–10% gradient polyacrylamide gel (Blobel and Dobberstein, 1975), followed by autoradiography.

Phosphopeptide mapping

The band corresponding to the PDGF β-receptor was cut out from non-fixed, wet polyacrylamide gels and extracted and digested essentially according to Kazlauskas and Cooper (1989). The extracted and performic acid-oxidized proteins were suspended in 50 μl of 50 mM NH_4HCO_3 and 1 μg of modified sequencing grade trypsin (Promega) was added, followed by incubation for 12 h at 37°C, after which time 1 μg of trypsin was added and incubation was prolonged for another 12 h at 37°C. The tryptic digest was then further digested with 70% formic acid at 37°C for 24 h. After removal of formic acid by evaporation in a Speedivac, the tryptic–acid digests were immunoprecipitated essentially according to Laudano and Buchanan (1986), with the exception that immobilized α_2-macroglobulin (Boehringer Mannheim) was used to remove trypsin from the samples. Immunoprecipitated peptides were eluted from the beads by incubation with 8 M urea, 0.125 M Tris–HCl, pH 6.8 and 50 mM dithiothreitol to 37°C for 10 min and then separated on 40% polyacrylamide gels according to Pantazis and Bonner (1981). The radioactivity was visualized using a Phosphorimager (Molecular Dynamics) and the intensity of the bands quantified by use of the ImageQuant Program (Molecular Dynamics).

Alternatively, the PDGF β-receptor was digested with trypsin as above, followed by digestion with thermolysin and analysis by two-dimensional phosphopeptide mapping, essentially as described by Kashishian *et al.* (1992).

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