Ligand-stimulation reduces PDGF β-receptor susceptibility to tyrosine dephosphorylation

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Running title:

Ligand-stimulation of the PDGF receptor induces resistance to dephosphorylation

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Summary

Ligand binding to the PDGF β-receptor leads to increased receptor tyrosine phosphorylation as a consequence of dimerization-induced activation of the intrinsic receptor tyrosine kinase activity. In this study we asked if ligand-stimulated PDGF β-receptor tyrosine phosphorylation, to some extent, also involved reduced susceptibility to tyrosine dephosphorylation. To investigate this possibility we compared the sensitivity of ligand-stimulated and nonstimulated forms of tyrosine phosphorylated PDGF β-receptors to dephosphorylation using various preparations containing protein tyrosine phosphatase (PTP) activity. Ligand-stimulated or unstimulated tyrosine phosphorylated receptors were obtained after incubation of cells with pervanadate only, or pervanadate together with PDGF-BB, respectively. Dephosphorylation of receptors immobilized on Wheat Germ Agglutinin-Sepharose, as well as of receptors in intact cell membranes, were investigated under conditions when rephosphorylation did not occur. As compared to unstimulated receptors the ligand-stimulated PDGF β-receptors showed about 10-fold reduced sensitivity to dephosphorylation by cell membranes, a recombinant form of the catalytic domain of density enhanced phosphatase-1 (DEP-1), or recombinant PTP1B. We conclude that ligand-stimulated forms of the PDGF β-receptor display a reduced susceptibility to dephosphorylation. Our findings suggest a novel mechanism whereby ligand-stimulation of PDGF β-receptor, and possibly other tyrosine kinase receptors, leads to a net increase in receptor tyrosine phosphorylation.
Introduction

Receptor tyrosine kinases (RTKs) are critical components of signaling pathways that control cellular processes like proliferation, differentiation, migration and metabolism. RTKs are composed of an extracellular ligand-binding domain and an intracellular portion which contains a tyrosine kinase domain and regulatory sequences. Ligand-binding of RTKs often leads to dimerization and subsequent increase in autophosphorylation of tyrosine residues in the intracellular portion of the receptors (reviewed in 1, 2). Autophosphorylation of intracellular receptor tyrosine residues controls the intrinsic tyrosine kinase activity and creates binding sites to recruit downstream signaling molecules (3, 4). The mechanism whereby ligand-induced dimerization stimulates these phosphorylation events is incompletely understood, but may involve a conformational change of the receptor, or a proximity effect.

RTK net tyrosine phosphorylation is not only controlled by the receptor kinase activity but is also determined by the action of protein tyrosine phosphatases (PTPs). Accumulating evidence suggest that PTPs are regulatory components of RTK signaling pathways. Antisense-studies have demonstrated increased signaling via receptors for insulin, epidermal growth factor (EGF) and hepatocyte growth factor (HGF) after attenuation of expression of the receptor-like PTP LAR (5-7), and disruption of PTP1B in mice results in enhanced insulin sensitivity (8, 9). Furthermore, genetic studies in C. Elegans have identified the receptor-like PTP CLR-1 as a negative regulator of signaling through the fibroblast growth factor (FGF) receptor ortholog EGL-15 (10). Physical association between the insulin receptor and the receptor-like PTP LAR, as well as between PDGF β-receptor and the receptor-like PTP DEP-1, have also been demonstrated (11, 12).

In this study we set out to investigate the possibility that reduced susceptibility to PTP action contributes to ligand-induced increase in net tyrosine phosphorylation of RTKs.
The well-characterized PDGF β-receptor was chosen as a prototype dimerization-activated receptor tyrosine kinase. The PDGF β-receptor is structurally composed of an extracellular domain, a transmembrane region and intracellular tyrosine kinase domain which has a characteristic inserted sequence. The autophosphorylation sites of the PDGF β-receptor have been extensively studied and include a regulatory site, Tyr857, as well as numerous sites which in their phosphorylated form act as binding sites for SH2-domain-containing proteins including c-Src, phospholipase C-γ, (PLC-γ) and phosphatidylinositol-3′-kinase (PI3-kinase) (reviewed in 13).

To study the effects of ligand-stimulation on PTP sensitivity, preparations of tyrosine phosphorylated ligand-stimulated and unstimulated PDGF β-receptors were obtained. Using these preparations we demonstrate that ligand-stimulated forms of the PDGF β-receptor display a reduced susceptibility to dephosphorylation, as compared to unstimulated forms.
Experimental procedures

Cell culture and antibodies

Parental PAE cells and PAE cells stably transfected with PDGF β-receptor (14) were cultured in Ham’s F-12 medium (GIBCO), supplemented with 10% fetal calf serum (GIBCO), 100 unit/ml of penicillin and 100 µg/ml streptomycin.

Analysis of receptor dimerization

After overnight incubation in serum-free Ham’s F-12, supplemented with 1 mg/ml BSA, PAE/PDGFβR cells were left unstimulated or treated with 100 ng/ml PDGF-BB for 60 min on ice, or with 100 µM pervanadate for 30 min at 37°C and 60 min on ice, or with pervanadate for 30 min at 37°C and then stimulated with 100 ng/ml PDGF-BB, in the presence of pervanadate, for 60 min on ice. After stimulation, cells were washed with ice-cold PBS. Ligand-receptor complexes were cross-linked by incubation in 1 mM bis(sulfosuccinimidyl)suberate for 1 h on ice. After incubation with 70 mM methylammonium chloride for 10 min, cell lysates were prepared by lysis in 20 mM Tris pH 7.5, 0.5% Triton X-100, 0.15 M NaCl, 0.5% deoxycholic acid, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol (Bayer) and 100 µM Na₃VO₄ for 15 min at 4°C. After elution with 0.3 M N-acetylglucosamine, PDGF β-receptors were immunoprecipitated and analyzed by SDS-PAGE, followed by immunoblotting with the rabbit antiserum PDGFR-3 (14) and the phosphotyrosine antibody PY99 (Santa Cruz Biotechnology).

Immunoprecipitation of PDGF β-receptor and analysis of associating proteins

For detection of PDGF β-receptor associating proteins, cells were lysed as described above and immunoprecipitated with the PDGF receptor antiserum CED raised against a
peptide corresponding to the five carboxy-terminal amino acids of the PDGF β-receptor. Immunoprecipitated PDGF β-receptor was detected with P-20 (Santa Cruz Biotechnology). Co-precipitating tyrosine phosphorylated proteins were detected by immunoblotting with the phosphotyrosine antibody PY99 (Santa Cruz Biotechnology) and co-precipitating p85 was detected by a rabbit antiserum (Santa Cruz Biotechnology).

**Dephosphorylation analysis of immunoprecipitated PDGF β-receptor**

Cells were stimulated and cell lysates were prepared as described above. Lysates were incubated with wheat germ agglutinin (WGA)-Sepharose (EC Diagnostics AB, Uppsala, Sweden) at 4°C overnight, and then incubated in 15 mM iodoacetamide (Sigma) for 30 min at room temperature. The pellets were washed 5 times with lysis buffer without Na₃VO₄ and once in a buffer containing 25 mM imidazol, 0.1 mg/ml BSA and 10 mM dithiothreitol (DTT). The samples were incubated at 37°C with vigorous agitation for 15 min with a recombinant form of DEP-1, composed of an amino-terminal GST-domain, a DEP-1 portion encompassing human DEP-1 amino-acids 997-1137 and a carboxy-terminal HA-tag or with recombinant PTP1B. To stop the dephosphorylation reaction, the samples were washed once with lysis buffer containing 100 μM Na₃VO₄ and then eluted with overnight incubation at 4°C in 0.3 M N-acetylglucosamine (Sigma) in the presence of 3 μM of the PDGF receptor selective tyrosine kinase inhibitor AG1296 (16). After centrifugation, supernatants were incubated with a rabbit antiserum against the PDGF β-receptor for 2 h at 4°C, then with Protein A-Sepharose 6 MB (Amersham Pharmacia Biotech) for 1 h at 4°C. After washing 3 times with lysis buffer and once with 20 mM Tris pH 7.5, the precipitates were heated for 3 min at 95°C in SDS-sample buffer and subjected to SDS-PAGE, followed by immunoblotting with the rabbit antiserum PDGFR-3 or the phosphotyrosine antibody PY99.
Preparation of intact cell membranes and dephosphorylation of PDGF β-receptor in vitro

After overnight incubation in serum-free Ham's F-12, supplemented with 1 mg/ml BSA, PAE/PDGFβR cells were treated with pervanadate for 30 min at 37°C and 60 min on ice, or with pervanadate for 30 min at 37°C and then stimulated with 100 ng/ml PDGF-BB, in the presence of pervanadate, for 60 min on ice and washed with ice-cold PBS and collected in 5 ml of ice-cold PBS. The cells were pelleted and incubated in hypotonic buffer (20 mM Tris pH 7.5, 10 mM NaCl, 1% Trasylol) for 30 min at 4°C. The cells were homogenized with 80 strokes in a Dounce homogenizator and the nuclei were pelleted at 3000 g for 5 min. The supernatants were then centrifuged at 100 000 g for 25 min. The membranous sediments were resolved in hypotonic buffer containing 10 mM DTT and 3 µM AG1296 using a syringe with a 0.6 x 26 mm needle and incubated with 15 mM iodoacetamide for 30 min at room temperature. Preparations were kept at -80°C until use. To obtain PTP activity containing membranes to be used in dephosphorylation reactions, membranous sediments from unstimulated parental PAE cells were prepared by hypotonic lysis and centrifugation, as described above. After centrifugation, membranes were resuspended in a buffer containing 25 mM imidazol, 0.1 mg/ml BSA and 10 mM DTT. For the dephosphorylation assay, PAE/PDGFβR membranes containing 10 mg of protein were mixed with membranes from parental PAE cells for 10 min at 37°C during vigorous agitation or incubated with recombinant DEP-1 for 15 min at 4°C. The dephosphorylation was stopped by addition of 100 µM Na3VO4 and an equal volume 2 x lysis buffer for 10 min at 4°C. After centrifugation at 13000 rpm for 15 min, the PDGF β-receptor were immunoprecipitated with PDGFR-3 antiserum, followed by immunoblotting with the PDGF β-receptor antibody P-20 or the phosphotyrosine antibody PY99.
Orthophosphate labeling and phosphopeptide mapping

Orthophosphate labeling and phosphopeptide mapping was performed essentially as described (17). Briefly, PAE/PDGFR cells were labeled by incubation in phosphate-free Ham’s F-12 medium supplemented with 2 mCi/ml [32P]orthophosphate for 3 h at 37°C. Cells were left unstimulated or were stimulated with 100 ng/ml PDGF-BB for 60 min on ice, or with 100 µM pervanadate for 30 min at 37°C and 60 min on ice, or with pervanadate for 30 min at 37°C and then stimulated with 100 ng/ml PDGF-BB, in the presence of pervanadate, for 60 min on ice. The cells were lysed in 20 mM Tris pH 7.5, 0.5% Triton X-100, 0.15 M NaCl, 0.5% sodium deoxycholate, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol and 100 µM Na3VO4. Immunoprecipitation was performed using PDGFR-3 antiserum; immunoprecipitates were then subjected to SDS-PAGE and thereafter transferred onto nitrocellulose membrane. The bands corresponding to radioactively labeled PDGF β-receptor were cut out and incubated with 0.5% polyvinylpyrrolidone-40, 0.6% acetic acid for 30 min at 37°C. Then, the filter pieces were rinsed 3 times with water and subjected to in situ tryptic digestion by incubation with 200 µl of 50 mM ammonium bicarbonate containing 1 µg of modified sequencing grade trypsin (Promega Corporation) for 12 h at 37°C. Peptides released from nitrocellulose were then lyophilized, oxidized in performic acid for 1 h on ice, diluted with 500 µl water, frozen and lyophilized again. The samples were then dissolved in 50 µl of 50 mM ammonium bicarbonate, sonicated and incubated with 1 µg of trypsin for another 12 h at 37°C. The samples were again lyophilized, dissolved in electrophoresis buffer consisting of 88% formic acid-glacial acetic acid-H2O (50: 156:1794), pH 1.9, and analyzed by two-dimensional phosphopeptide mapping on thin layer cellulose plates, using electrophoretic separation at pH 1.9 in the first dimension, followed by ascending chromatography (isobutyric acid: pyridine: glacial acetic acid: H2O: n-butanol, 1250: 96: 58: 558: 38) in the second dimension. After two-dimensional electrophoresis plates were analyzed by autoradiography.
Results

Preparation and characterization of tyrosine phosphorylated ligand-stimulated and unstimulated forms of PDGF β-receptors

To obtain tyrosine-phosphorylated forms of ligand-stimulated and unstimulated PDGF β-receptors, porcine aortic endothelial (PAE) cells stably transfected with the human PDGF β-receptor (PAE/PDGFβR cells) were treated with pervanadate with or without PDGF-BB. To investigate if receptors occurred as monomers or dimers after stimulation with PDGF-BB alone, pervanadate alone, or pervanadate together with PDGF-BB, cross-linking of cell-surface proteins was performed and WGA-Sepharose fractions isolated. After elution with N-acetyl-glucosamine, PDGF β-receptors were immunoprecipitated and analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 1, lower panel, dimeric receptors were recovered from cells stimulated with PDGF-BB, or pervanadate and PDGF-BB, but not from cells stimulated with pervanadate only. Furthermore, treatment with pervanadate only and pervanadate combined with PDGF-BB yielded receptors that were tyrosine phosphorylated to the same extent, as determined by phospho-tyrosine immunoblotting (Fig. 1, upper panel).

To determine the pattern of tyrosine-phosphorylation of the ligand-stimulated and unstimulated receptor preparations, in vivo 32P-labeled receptors were isolated by immunoprecipitations and subjected to two-dimensional phospho-peptide mapping (Fig. 2 A). No major differences were seen in the autophosphorylation patterns after the different stimulations.

To characterize the pattern of association of SH2-domain-containing signaling molecules with the PDGF β-receptor after stimulation with pervanadate alone, or pervanadate and PDGF-BB, PDGF β-receptor were immunoprecipitated and subjected to immunoblotting with phosphotyrosine antibody, PDGF β-receptor antiserum and p85
antiserum (Fig. 2B). With the exception of a component of a 120 kDa, found exclusively co-precipitating with the ligand-stimulated receptor, both stimulations induced a similar pattern of PDGF β-receptor-coprecipitating tyrosine phosphorylated proteins (Fig. 2B, top panel). The tyrosine phosphorylated component with an apparent molecular mass of about 200 kDa was recognized by an antibody to the PDGF β-receptor (Fig. 2B, second panel from the top). Furthermore, equal amount p85 subunit of PI3-kinase coprecipitated with the PDGF β-receptor after incubation with pervanadate alone, and pervanadate and PDGF-BB (Fig. 2B, lower two panels).

In conclusion, stimulation with pervanadate only, or pervanadate and PDGF-BB, yield PDGF receptors that display similar patterns of tyrosine phosphorylation and substrate association, but differ with regard to occurrence as receptor dimers.

**In vitro dephosphorylation of unstimulated and ligand-stimulated PDGF β-receptors immobilized on WGA-Sepharose**

To compare the susceptibility of unstimulated and ligand-stimulated PDGF β-receptors to dephosphorylation, WGA-Sepharose fractions from cells stimulated with pervanadate alone, or pervanadate together with PDGF-BB, were isolated. To avoid dephosphorylation mediated by PTPs present in the WGA-Sepharose fractions, these were inactivated by incubation with the alkylating agent iodoacetamide. After washes in vanadate-free buffers, the WGA-Sepharose fractions were subsequently mixed with either recombinant catalytic domain of DEP-1, a receptor-like PTP (Fig. 3A), or recombinant PTP1B (Fig. 3C). The dephosphorylation reaction was stopped by addition of vanadate. After elution of receptors with N-acetyl-glucosamine, receptors were immunoprecipitated and subjected to phospho-tyrosine immunoblotting. As shown in Fig. 3, incubation of WGA-Sepharose-immobilized PDGF β-receptors with either PTP1B or DEP-1, led to a dose-dependent dephosphorylation. However, both when PTP1B and when DEP-1 were used as source of PTP activity, 10-100 fold larger
amounts were required for dephosphorylation of ligand-stimulated receptors, as compared to unstimulated receptors. It is unlikely that the more abundant phosphorylation of ligand-stimulated receptors is due to rephosphorylation in trans by the kinases in the ligand-induced receptor dimer, since the analysis was done in the absence of ATP. Moreover, the same difference in susceptibility to dephosphorylation of ligand-stimulated receptors as compared to monomers was seen when the dephosphorylation was performed in the presence of the PDGF receptor kinase inhibitor AG1296 (data not shown).

Together, these experiments demonstrate that ligand-stimulated forms of PDGF β-receptors, when immobilized on WGA-Sepharose, display a reduced susceptibility to tyrosine dephosphorylation as compared to unstimulated forms.

**Dephosphorylation of unstimulated and ligand-stimulated PDGF β-receptors in intact cell membranes**

To investigate the sensitivity of unstimulated and ligand-stimulated PDGF β-receptors to the action of PTPs in a more physiological setting, their dephosphorylation in intact cell membranes were investigated. After stimulation of PAE/PDGFβR cells with pervanadate only, or pervanadate together with PDGF-BB, membrane fractions were isolated and incubated with iodoacetamide to irreversibly block PTP activity in the membrane preparations. Membranes containing unstimulated and ligand-stimulated tyrosine phosphorylated PDGF β-receptors were then incubated with either membrane preparations from untransfected PAE cells or with recombinant DEP-1 (Fig. 4). Dephosphorylation was stopped by addition of vanadate and the tyrosine phosphorylation status of PDGF β-receptors was determined by phosphotyrosine immunoblotting of receptors immunoprecipitated from membrane lysates (Fig. 4A and B). Ligand-stimulated PDGF β-receptors displayed a reduced susceptibility to dephosphorylation as compared to the unstimulated form. Whereas 100 µg of PAE
membranes was required for 50% dephosphorylation of ligand-stimulated receptors, 10 µg of PAE membranes led to more than 50% dephosphorylation of unstimulated receptors (Fig. 4A). A similar tendency was observed when recombinant DEP-1 was used as source of PTP-activity (Fig. 4B).

We thus conclude that also in intact cell membranes ligand-stimulated PDGF β-receptors are more resistant to PTP-mediated dephosphorylation, as compared to unstimulated PDGF β-receptors.
Discussion

Our results clearly demonstrate that ligand-stimulated forms of the PDGF β-receptor display a reduced susceptibility to dephosphorylation by PTPs in vitro. This was observed both when WGA-Sepharose-immobilized receptors and receptors in non-solubilized cell membranes were analyzed. Furthermore, the effect of ligand-stimulation on susceptibility to dephosphorylation could be detected when either purified recombinant PTPs or PTPs present in non-solubilized cell membranes were used as source of PTP activity. Our results suggest a novel mechanism contributing to the increased receptor tyrosine phosphorylation occurring after ligand-stimulation.

The mechanism(s) underlying the difference between ligand-stimulated and unstimulated receptors with regard to dephosphorylation susceptibility remains to be clarified. The characterization of the receptors with regard to tyrosine phosphorylation pattern did not reveal any major differences (Fig. 4A). Furthermore, analysis by two-dimensional phospho-peptide mapping of receptors after dephosphorylation indicated that dephosphorylation occurred of most sites (data not shown). This argues against the possibility of the difference being due to the presence of particular ligand-induced phosphatase resistant sites in ligand-stimulated receptors. In contrast, a clear difference between the two receptor preparations was detected when they were analyzed with regard to occurrence as crosslinkable receptor dimers (Fig. 1). This suggests that dimerization might underlie differences in dephosphorylation sensitivity.

At least two principally different mechanisms whereby dimerization can affect sensitivity to PTPs can be envisioned; dimeric forms of the receptors can be intrinsically poorer substrates for PTPs, or alternatively, the difference between monomeric and dimeric forms could be caused by modulation of PTP activity. The latter possibility could involve an association with dimeric receptors of a PTP inhibitor, or an association with monomeric receptors of a PTP activator. Earlier studies have demonstrated H$_2$O$_2$
production, and subsequent inhibition of PTP activity, following stimulation with PDGF or EGF (18, 19). The PDGF-stimulated \( \text{H}_2\text{O}_2 \) production was recently shown to be dependent upon PI3-kinase activation (20). Although similar amounts of PI3-kinase was found associated with the monomeric and dimeric forms of the receptor in our study, the possibility that the difference in dephosphorylation between monomeric and dimeric receptors is caused by trans-inhibition of PTPs should be further studied. In this context, identification of the 120 kDa component, which was exclusively detected in immunoprecipitates of ligand-stimulated receptors (Fig. 2B), appears as an important goal in future studies.

Accumulating evidence suggest that PTPs act as negative regulators of ligand-activated tyrosine kinase receptors. The best evidence is derived from studies using anti-sense-mediated suppression of PTP expression and from studies on mice deficient in expression of specific PTPs. For example, increased insulin receptor signaling has been demonstrated in cell lines with reduced LAR expression, as well as in PTP1B knockout mice (5, 8, 9). There are also some indications suggesting that modulation of PTP activity is involved in ligand-independent activation of tyrosine kinase receptors. For example, UV-induced increase in EGF receptor tyrosine phosphorylation is caused by reduced receptor-directed PTP activity, rather than increased tyrosine kinase activity (21). In agreement with this notion, a direct inhibitory effect by UV light on the specific activities of PTP-\( \alpha \), PTP-\( \sigma \), DEP-1 and SHP-1 have recently been demonstrated (22). These observations, together with the findings of the present paper thus suggest important functions for PTPs in controlling activation, as well as inactivation, of both unoccupied and ligand-occupied tyrosine kinase receptors.
Acknowledgements

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References


Footnotes

The abbreviations used are: DEP-1, density enhanced phosphatase-1; DTT, dithiothreitol; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; PAE cells, porcine aortic endothelial cells; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; PI3-kinase, phosphatidylinositol-3'-kinase; PTP, protein tyrosine phosphatase; RTK, receptor tyrosine kinase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SH2 domain, Src homology 2 domain; WGA, wheat germ agglutinin.
Figure Legends

**Fig. 1. Treatment with PDGF-BB, but not pervanadate, induces formation of PDGF β-receptor dimers.** PAE/PDGFβR cells were stimulated with PDGF-BB, pervanadate, or pervanadate and PDGF-BB. After treatment without (-) or with (+) the cross-linker bis(sulfosuccinimidyl)suberate, cell lysates were subjected to immunoprecipitation with PDGF β-receptor antiserum followed by SDS-PAGE in 5% gels. After transfer to nitrocellulose filters, samples were analyzed by immunoblotting (IB) with a phosphotyrosine antibody (upper panel) and PDGF β-receptor antiserum (lower panel). The positions of dimeric and monomeric receptors are indicated to the left, and the position of a 200 kDa marker protein is shown to the right.

**Fig. 2. Treatment with pervanadate only or pervanadate and PDGF-BB, induces similar pattern of receptor tyrosine phosphorylation and substrate association.** A, PDGF β-receptor expressing PAE cells were labeled *in vivo* with $[^{32}P]$orthophosphate. Cells were left unstimulated or were stimulated with pervanadate, PDGF-BB or pervanadate and PDGF-BB. Immunoprecipitated PDGF β-receptor were subjected to tryptic cleavage and analyzed by cellulose thin-layer chromatography followed by ascending chromatography and exposure to film. Thick and thin arrows indicate the position of peptides containing phosphorylated Tyr857 and Tyr751 of PDGF β-receptor, respectively. B, After stimulation with pervanadate, or pervanadate and PDGF, cell lysates were immunoprecipitated with PDGF β-receptor antibody. The immunoprecipitates were analyzed by SDS-PAGE, and immunoblotted with phosphotyrosine antibody (top panel), PDGF β-receptor antiserum (second panel from the top) or antibodies recognizing the regulatory p85 subunit of PI3-kinase (lower panel). The positions of molecular size markers are indicated on the left of the upper panel.
Fig. 3. WGA-Sepharose immobilized ligand-stimulated receptors display a reduced susceptibility to tyrosine dephosphorylation as compared to unstimulated forms. PDGF β-receptor expressing PAE cells were treated with PDGF-BB only, pervanadate only or pervanadate and PDGF-BB, and WGA-Sepharose fractions were isolated. After inactivation of tyrosine phosphatases in the WGA-Sepharose fractions, samples were incubated with the catalytic domain of DEP-1 (A) or PTP 1B (C). After dephosphorylation, PDGF β-receptors were eluted, immunoprecipitated with PDGF β-receptor antiserum and subjected to phospho-tyrosine immunoblotting. The amount of remaining tyrosine phosphorylated receptor, relative to the untreated control, is indicated below each of the phosphotyrosine immunoblot panels. (B) To confirm that equal amounts of PDGF β-receptor were used for the DEP-1 dephosphorylation experiment, an aliquot of the WGA-Sepharose fractions from cells stimulated with PDGF-BB (lane 2), pervanadate (lane 3) or PDGF-BB and pervanadate (lane 4) were immunoblotted with PDGF β-receptor antibodies. In the PTP1B dephosphorylation experiment, reprobing of the filter with PDGF β-receptor antiserum confirmed that equal amounts of receptors was used in the dephosphorylation experiment (C). The position of PDGF β-receptor is indicated to the left.

Fig. 4. Ligand-stimulated PDGF β-receptors in intact cell membranes display a reduced susceptibility to tyrosine dephosphorylation as compared to unstimulated forms. After stimulation of PAE/PDGFβR cells with pervanadate or pervanadate and PDGF-BB, membrane fractions were isolated after hypotonic lysis. Membranes containing unstimulated and ligand-stimulated tyrosine-phosphorylated PDGF β-receptors were incubated with either membrane preparations from parental PAE cells (A) or recombinant DEP-1 (B). After dephosphorylation, the tyrosine phosphorylation status of PDGF β-receptors was determined by immunoprecipitation of receptors from membrane lysates, followed by SDS-PAGE and phosphotyrosine immunoblotting. After stripping, membranes were reprobed with PDGF β-receptor antibody. The position of PDGF β-receptor is indicated to the left. The amount of phosphorylated receptor
remaining, relative to the untreated control, is given below the phosphotyrosine immunoblotting panels.
Fig. 1, Shimizu et al.
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Fig. 3, Shimizu et al
Fig. 4, Shimizu et al.

A

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PDGF R

IB: P-Tyr

IB: PDGF R

B

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PDGF R

IB: P-Tyr

IB: PDGF R
PAE/PDGF-B cells were treated with pervanadate only or with pervanadate and PDGF-BB. Cell lysates were incubated with WGA-sepharose and bound material was eluted with 0.3 M N-acetyl-glucoseamine. The WGA eluates were applied to a Superose-6 column and size exclusion chromatography was performed in 0.2 % Triton X-100, 0.15 M NaCl, 10 % glycerol, 1 mM DTT, 20 mM Hepes pH 7.5. Fractions of 300 μl were collected and subjected to immunoprecipitation with the rabbit antiserum CED recognizing the PDGF-B. The immunoprecipitated material was analyzed, after SDS-PAGE, by immunoblotting with the phosphotyrosine antibody PY99.

As shown, elution of receptors from PDGF BB stimulated cells started in fraction 19 (10.1 ml) whereas elution of unstimulated receptors was not detected before fraction 21 (11.9 ml). It should be noted that under the experimental conditions, including a long gel chromatography, a major part of receptor dimers are likely to dissociate to monomers (with elution positions overlapping with the receptors from non-ligand-stimulated cells). The differences in elution position are consistent with previous studies of monomeric and dimeric forms of the PDGF-B receptor (Heldin et al. 1989) J. Biol. Chem. 264, 8905-8912). Hence these results suggest that the preparation from PDGF BB stimulated cells contains dimeric receptors and the preparation from unstimulated cells contains monomeric receptors.
WGA-eluates from pervanadate or pervanadate and PDGF-BB-treated PAE/PDGFR cells were prepared. One half of the eluates were cross-linked by incubation with 0.2 mM DSS for 30 min at room temperature. The reaction was quenched by addition of 50 mM metylammonium chloride and PDGF-R were immunoprecipitated using CED antiserum. The samples were analyzed, after SDS-PAGE, by immunoblotting with the phosphotyrosine antibody PY99.

Treatment with cross-linker of WGA eluates from PDGF-BB stimulated cells led to the appearance of easily detectable high molecular weight forms, most likely corresponding to receptor dimers. Smaller amounts of the same components were also detected in eluates from unstimulated cells, most likely representing unspecifically cross-linked components or ligand-independent receptor dimers.
Appendix 3

PAE/PDGF R cells were labeled with $^{32}$P orthophosphate in vivo followed by stimulation with pervanadate only or pervanadate and PDGF-BB. Intact cell membranes were prepared and incubated with membranes containing PTP activity obtained from unstimulated parental PAE cells. The PDGF R receptors were subsequently solubilized in Triton X-100, immunoprecipitated with the CED rabbit antiserum. The receptor band was cut out and subjected to trypsin degradation and peptides were then analyzed by two-dimensional phosphopeptide mapping.

The two-dimensional phosphopeptide maps reveal that the different phosphotyrosines were dephosphorylated to similar extent and that the phosphorylation pattern, prior to dephosphorylation, was similar in unstimulated and ligand-stimulated cells. Four phosphopeptides are indicated with arrows.
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