Ligand Stimulation Reduces Platelet-derived Growth Factor β-Receptor Susceptibility to Tyrosine Dephosphorylation*

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Ligand binding to the platelet-derived growth factor (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 whether 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 non-stimulated forms of tyrosine-phosphorylated PDGF β-receptors to dephosphorylation using various preparations containing protein-tyrosine phosphatase 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, was investigated under conditions when rephosphorylation did not occur. As compared with 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, or recombinant protein-tyrosine phosphatase 1B. 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.

Receptor tyrosine kinases (RTKs)† are critical components of signaling pathways that control cellular processes like proliferation, differentiation, migration, and metabolism. Ligand binding of RTKs often leads to dimerization and subsequent increases in autophosphorylation of tyrosine residues in the intracellular portion of the receptors (reviewed in Refs. 1 and 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 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 Caenorhabditis elegans have identified the receptor-like PTP CLR-1 as a negative regulator of signaling through the fibroblast growth factor 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 increases in net tyrosine phosphorylation of RTKs. The well characterized PDGF β-receptor was chosen as a prototype dimerization-activated receptor tyrosine kinase. 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, and phosphatidylinositol 3'-kinase (PI3-kinase) (reviewed in Ref. 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 with 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 (Life Technologies, Inc.), supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 unit/ml 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/ PDGF-BβR cells were left unstimulated or treated with 100 ng/ml PDGF-BB for 60 min on ice, with 100 μM pervanadate or with 30 μM pervanadate and 10 μM calyculin A. Ligand-receptor complexes were cross-linked by incubation with 100 μM WGA at 0 °C for 30 min.

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tion in 1 m M bis(sulfosuccinimidyl)suberate for 1 h on ice. After incubation with 70 m M methylammonium chloride for 10 min, cell lysates were prepared by lysis in 20 m M Tris, pH 7.5, 0.5 % Triton X-100, 0.15 m NaCl, 0.5 % deoxycholic acid, 10 m M EDTA, 1 m M phenylmethylsulfonyl fluoride, 1 % Trasylol (Bayer), and 100 m M Na3VO4 for 15 min at 4 °C. After elution with 0.3 m 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 carboxyl-terminal amino acids of the PDGF β-receptor. Immunoprecipitated PDGF β-receptor was detected with P-20 (Santa Cruz Biotechnology). Coprecipitating tyrosine-phosphorylated proteins were detected by immunoblotting with the phosphotyrosine antibody PY99 (Santa Cruz Biotechnology), and coprecipitating p85 was detected by a rabbit antiserum (Upstate 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 m M iodoacetamide (Sigma) for 30 min at room temperature. The pellets were washed five times with lysis buffer without Na3VO4 and once in a buffer containing 25 m M imidazol, 0.1 m M mg/ml BSA, and 10 m M 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 glutathione S-transferase domain, a DEP-1 portion encompassing human DEP-1 amino acids 997–1337, and a carboxyl-terminal hemagglutinin tag or with recombinant PTP1B. To stop the dephosphorylation reaction, the samples were washed once with lysis buffer containing 100 m M Na3VO4, and then eluted with overnight incubation at 4 °C in 0.3 m M N-acetylglucosamine (Sigma) in the presence of 3 m M 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 and then with protein A-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C. After washing three times with lysis buffer and once with 20 m M Tris, pH 7.5, the precipitates were heat treated for 5 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 m M mg/ml BSA, PAE/PDGF βR 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 m M PDGF-BB, in the presence of pervanadate, for 60 min on ice and washed with ice-cold PBS and collected in 5 m l of ice-cold PBS. The cells were pelleted and incubated in hypotonic buffer (20 m M Tris, pH 7.5, 10 m M NaCl, 1 % Trasylol) for 30 min at 4 °C. The cells were homogenized with 80 strokes in a Dounce homogenizer, and the nuclei were pelleted at 3000 X g for 5 min. The supernatants were then centrifuged at 100,000 X g for 25 min. The membranous sediments were resolved in hypotonic buffer containing 10 m M DT and 3 m M AG1296 using a syringe with a 0.6 X 26-mm needle and incubated with 15 m M iodoacetamide at 37 °C for 10 min at 37 °C, 0.6 X 26-mm needle and incubated with 15 m M iodoacetamide at 37 °C for 10 min. 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 m M imidazol, 0.1 m M mg/ml BSA, and 10 m M DT. For the dephosphorylation assay, PAE/PDGF βR membranes containing 10 m g of protein were mixed with membranes from parental PAE for 10 min at 37 °C during tyrosine phosphorylation or incubated with recombinant DEP-1 for 15 min at 4 °C. The dephosphorylation was stopped by addition of 100 m M Na3VO4 and an equal volume of 2 X lysis buffer for 10 min at 4 °C. After centrifugation at 13,000 rpm for 15 min, the PDGF β-receptor was immunoprecipitated with PDGFR-3 antiserum, followed by immunoblotting with the PDGF β-receptor antibody P-20 or the phosphotyrosine antibody PY99.

[32P]Orthophosphate Labeling and Phosphopeptide Mapping—[32P]Orthophosphate peptide mapping was performed essentially as described (17).

RESULTS AND DISCUSSION

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, PAE cells stably transfected with the human PDGF β-receptor (PAE/PDGF βR cells) were treated with pervanadate with or without PDGF-BB. To investigate whether 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 were isolated. After elution with N-acetylglucosamine, 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 phosphotyrosine 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 immunoprecipitation and subjected to two-dimensional phosphopeptide mapping (Fig. 2A). 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 was immunoprecipitated and subjected to immunoblotting with phosphotyrosine antibody, PDGF β-receptor antiserum, and p85 antiserum (Fig. 2B). With the exception of a component of 120 kDa, found exclusively coprecipitating 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
Ligand Stimulation of PDGFβR Protects from Dephosphorylation

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 and PDGF-BB, were isolated. To avoid dephosphorylation mediated by PTPs present in the WGA-Sepharose fractions, these were inactivated by incubation with the alkylation agent iodoacetamide. After washing in vanadate-free buffers, the WGA-Sepharose fractions were subsequently mixed with the 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-acetylglucosamine, receptors were immunoprecipitated and subjected to phosphotyrosine 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 the source of PTP activity, 10–100-fold larger amounts were required for dephosphorylation of ligand-stimulated receptors as compared with unstimulated receptors. It is unlikely that the more abundant phosphorylation of ligand-stimulated receptors is because of rephosphorylation in trans by the kinases in the ligand-induced receptor dimer, because the analysis was done in the absence of ATP. Moreover, the same difference in susceptibility to dephosphorylation of ligand-stimulated receptors as compared with 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 with unstimulated forms.
Ligand-stimulated PDGFβR immunoprecipitated from membrane lysates (Fig. 4), to dephosphorylation by PTPs demonstrated H2O2 production and subsequent inhibition of monomeric receptors of a PTP activator. Earlier studies have demonstrated that PTPs act as negative regulators of ligand-activated tyrosine kinase receptors (15). 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-α, PTP-η, 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.

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