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Site-Selective Regulation of Platelet-Derived Growth Factor β Receptor Tyrosine Phosphorylation by T-Cell Protein Tyrosine Phosphatase

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Protein tyrosine phosphatases (PTPs) are natural receptor tyrosine kinase antagonists and serve as regulators of both nonreceptor and receptor tyrosine kinases (28, 29). Recent investigations indicated that each receptor tyrosine kinase associates with and is dephosphorylated by a number of tyrosine phosphatases. The dephosphorylation of the receptor by individual PTPs can be general, thereby terminating receptor signaling. Alternatively, PTPs can site selectively dephosphorylate a subset of tyrosine residues and thereby modulate signaling downstream of the receptor. By regulating the expression and activation of tyrosine phosphatases, the cell consequently might be able to modulate signaling through receptor tyrosine kinases and fine-tune its response.

Platelet-derived growth factors (PDGFs) are a family of growth factors that stimulate cell growth, survival, and motility. PDGF isoforms act by binding to two structurally related protein tyrosine kinases, the PDGF α and β receptors (16). The binding of PDGF to its receptors results in receptor dimerization, promoting phosphorylation in trans between the two receptors in the complex. PDGF-AA forms αα receptor dimers, PDGF-AB forms αα and αβ receptor dimers, and PDGF-BB forms all combinations of receptor dimers. Two more PDGF dimers, PDGF-CC and PDGF-DD, recently were identified (2, 24, 25) and shown to preferentially signal through αα receptor and ββ receptor dimers, respectively, but also may activate both receptor types in cells coexpressing α and β receptors (12, 24).

Phosphorylation of tyrosine 857 (Y857) in the catalytic loop of the PDGF β receptor kinase increases kinase activity (10). In addition, a number of tyrosine residues outside of the catalytic domain are phosphorylated, leading to specific recruitment of signal transduction molecules containing SH2 domains to the activated receptor (16); these molecules include adaptor proteins such as Sca and Grb2 and enzymes such as the Src family tyrosine kinases, phosphatidylinositol 3'kinase (PI 3-kinase), phospholipase Cγ1 (PLCγ1), and tyrosine phosphatase SHP-2. The interactions occur in a specific manner determined by three to six amino acid residues downstream of the phosphorylated tyrosines.

T-cell PTP (TC-PTP) is a ubiquitously expressed phosphatase (8). The TC-PTP transcript is modified by alternative splicing, giving rise to 45- and 48-kDa spliced forms of TC-PTP (27). The 45-kDa spliced form has been reported to be the major gene product in most human and rodent tissues and cell
Regulation of the PDGF β receptor by tyrosine phosphatases is poorly understood. In addition to SHP-2, several phosphatases, including a low-molecular-weight PTP (PTP-1B) and a receptor-like tyrosine phosphatase (DEP-1), interact with and dephosphorylate the PDGF β receptor (4, 13, 18, 22).

Site-selective dephosphorylation of the PDGF β receptor by SHP-2 and PTP-1B has been demonstrated (5, 21). Analyses of DEP-1 dephosphorylation of PDGF receptors showed less efficient dephosphorylation of the autoregulatory site Y857 than of some SH2 binding sites (22, 32). These findings suggest that phosphatases can modulate specific signaling pathways by selectively dephosphorylating specific tyrosine residues on the PDGF β receptor and other receptor tyrosine kinases.

It was recently demonstrated that murine embryonic fibroblasts (MEFs) lacking the TC-PTP displayed a defective PDGF-induced IKK/NF-κB activation pathway, whereas PDGF-induced activation of the PI 3-kinase and Erk signaling pathways was unaffected (17). In this study, we further characterized the effect of TC-PTP depletion on PDGF β receptor signaling. We demonstrate that TC-PTP directly controls PDGF β receptor phosphorylation in a site-selective manner and also negatively regulates the mitogenic response to PDGF.

**Table 1.** Sequences of phosphopeptides used for antibody production

<table>
<thead>
<tr>
<th>Phosphopeptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>pY579</td>
<td>CSSDGHePyYVDPIM</td>
</tr>
<tr>
<td>pY751</td>
<td>CKDESVDpYVPMLDM</td>
</tr>
<tr>
<td>pY771</td>
<td>CADIESSnPyMAPYDNYVPS</td>
</tr>
<tr>
<td>pY1021</td>
<td>CNEGDNpDpYHIPLPD</td>
</tr>
</tbody>
</table>

a Bold type indicates the phosphorylated tyrosine.

Rephrasing with 2 μg of PDGF β receptor antiserum/ml. Bound antibodies were visualized by enhanced chemiluminescence after incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences).

**Cell cultures.** MEF cell lines derived from TC-PTP ko and littermate wt mice were described previously (40). Reexpressing cells were created by transfecting TC-PTP ko MEFs with pcDNA4 vectors containing constructs of either the 45-kDa isoform of mouse wt TC-PTP (17) or a catalytically inactive mutant form of TC-PTP in which the catalytic cysteine is mutated to serine (CS). Two mass cultures stably expressing wt PDGF β (ko/wt1 and ko/wt2) or the CS mutant (ko/CS1 and ko/CS2) were generated. Additionally, wt TC-PTP and the CS mutant were transiently expressed in PDGF ko MEFs using Lipofectamine Plus reagent according to the manufacturer’s protocol (Invitrogen Life Technologies, Carlsbad, Calif.). PTPs ko and wt MEFs (31, PTP-1B ko MEFs (13), and MEFs reconstituted with human wt PTP-1B (PTP-1B wt MEFs) (13) were previously described. All MEFs were grown in Dulbecco minimal essential medium (DMEM) supplemented with 10% fetal calf serum and 1:5,000 plasmocin. Porcine aorta endothelial (PAE) cells stably expressing the PDGF β receptor (6) and PDGF β receptors with tyrosine-to-phenylalanine point mutations (34) were previously described. PAE cells were grown in Ham’s F-12 medium supplemented with 10% fetal calf serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml.

**Peptide synthesis and generation of site-selective phosphotyrosine antibodies.** Peptides corresponding to tyrosine phosphorylation sites of the human PDGF β receptor (Table 1) were synthesized by 9-fluorenylmethoxycarbonyl chemistry with an Applied Biosystems 433A peptide synthesizer and purified by reverse-phase chromatography followed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry to confirm the expected molecular weights. The peptides were conjugated to keyhole limpet hemocyanin by using m-maleimidobenzo-2-nitrosonicotinamide ester (Pierce), and the conjugates were used to immunize rabbits. The antibodies were affinity purified by passing the antisera over three consecutive columns of immobilized nonphosphorylated peptide, phosphotyrosine-agarose, and phosphorylated peptide as previously described (9). The peptides were coupled to SulfoLink (Pierce) and phosphorysorbed to Affigel 10 (Bio-Rad) according to the manufacturers’ instructions. The antibodies were eluted from the phosphopeptide column with 4.6 M MgCl2 and immediately diluted in an equal volume of double-distilled H2O. After dialysis against 20 mM HEPES (pH 7.4); 50 mM NaCl, the antibodies were subjected to ammonium sulfate precipitation. The precipitates were dissolved in phosphate-buffered saline (PBS), and the ammonium sulfate was removed by dialysis against PBS. The antibodies were finally diluted in 50% glycerol and stored at −70°C.

**Cell lysis, receptor precipitation, and immunoblotting analysis.** Cells were starved overnight in medium supplemented with 1 mg of bovine serum albumin (BSA)/ml and were stimulated with 10 ng of PDGF-BB/ml or 50 ng of PDGF-DD/ml for various times. Following stimulation, the cells were rinsed twice in ice-cold PBS and lysed in 20 mM Tris-HCl (pH 7.5); 0.5% Triton X-100; 0.5% deoxycholate; 150 mM NaCl; 10 mM EDTA–0.5 mM Na3VO4; 1% Triton X-100 for 15 min on ice. The lysates were cleared by centrifugation at 16,000 × g for 15 min at 4°C. PDGF β receptors were precipitated with wheat germ agglutinin (WGA)-Sepharose (Pharmacia). The precipitated proteins were washed three times in lysis buffer, separated by SDS-PAGE (7% polyacrylamide gel), and transferred to nitrocellulose membranes, which were incubated with site-selective phosphotyrosine antibodies (3 μg/ml) or PY99 (1 μg/ml). Bound antibodies were visualized by enhanced chemiluminescence after incubation with horseradish peroxi-

**Phosphatase assay.** Cells were lysed in the buffer described above but supplemented with 1 mM 1,4-dithiothreitol and lacking Na3VO4. Following immunoprecipitation of TC-PTP, the beads were washed three times in lysis buffer and once in phosphate assay buffer (25 mM imidazole [pH 7.4], 10 mM dithio-
threitol, 0.1 mg of BSA/ml. The precipitates were resuspended in assay buffer, and phosphatase activity was determined by using a 32P-labeled peptide (amino acid sequence AEّEE​YGGF​EAAKKK) as a substrate as previously described (37). Assays were performed in duplicate, and phosphatase activity was expressed as the relative amount of 32P-labeled radioactivity released from the peptide after 7 min of incubation at 30°C.

In vitro PDGF β receptor dephosphorylation. PAE cells stably expressing the PDGF β receptor were stimulated with 100 ng of PDGF-BB/ml for 1 h on ice. The cells were lysed in the buffer described above but with the addition of 15 mM iodoacetic acid and 1 mM benzamidine. The phosphorylated receptors were immunoprecipitated with WGA-Sepharose, followed by four washes in phosphatase assay buffer. The precipitates were resuspended in a total volume of 100 μl of assay buffer or assay buffer containing various amounts of recombinant TC-PTP (New England Biolabs). The samples were incubated at 30°C for 10 min, receptor dephosphorylation was terminated by the addition of 1 ml of ice-cold lysis buffer including phosphatase inhibitors, and the samples were immediately washed. After the completion of washing, phosphorylation of the different tyrosine residues was detected by immunoblotting as described above.

Insoluble phosphate formation. Cells were plated at 10^5 cells per well in 12-well plates and incubated with serum-free M199 medium supplemented with 1 mg of BSA/ml overnight. After being labeled with 4 μCi of myo-[3H]inositol (Amersham) per well for 24 h, the cells were stimulated with various amounts of PDGF-BB in the presence of LiCl at 37°C for 30 min. Isolation and detection of the released insoluble phosphate fraction were performed as described previously (1).

PDGF β receptor-associated PI 3-kinase activity. Cells were starved overnight in medium supplemented with 1 mg of BSA/ml and then stimulated with 50 ng of PDGF-BB/ml at room temperature for 10 min. PDGF β receptors were immunoprecipitated with 2 μg of anti-PDGF β receptor antibody at 4°C for 3 h. Aliquots of the corresponding immunoprecipitates were subjected to analysis of PDGF β receptor levels by immunoblotting. The collected beads were subjected to a kinase reaction in the presence of 20 μCi of [γ-32P]ATP and with 0.2 μg of preconsoned phosphatidylinositol/μl as a substrate as previously described (18). The kinase reaction was performed at 37°C for 30 min and was stopped with 100 μl of chloroform-methanol-HCl (50:100:1 [vol/vol/vol]). Following extraction, the phospholipids were concentrated and applied to a thin-layer chromatography plate (Silica G60; Whatman LKGDF). The plate was developed with 2 M acetic acid–propanol (35:65 [vol/vol]) and exposed to a PhosphorImager.

Cell migration assay. Cell migration was determined by using a 96-well Chemo-TX (Neuroprobe) cell migration microplate with a pore size of 3.2 μm. The filters were coated with 50 μg of fibronectin (BD Biochemicals)/ml in PBS for 1 h at room temperature, rinsed twice in distilled water, and air dried. Serum-starved cells were trypsinized to yield single cells, and trypsinization was terminated by the addition of 150 μl of Trysol/ml of cell suspension. The cells were pelleted and diluted to a final concentration of 2.5 × 10⁶ cells/ml in DMEM supplemented with 1 mg of BSA/ml (DMEM/BSA). The wells of the Chemotx microplate were filled with DMEM/BSA or with DMEM/BSA supplemented with either 10% fetal bovine serum or various concentrations of PDGF-BB. The filters were placed on the wells to allow contact with the medium, and 50,000 cells were added on top of each filter. The chamber was incubated for 4 h at 37°C in 5% CO₂. Cells adhering to the top of the filter were removed, and cells adhering to the bottom of the filter were fixed by 3 min of incubation in 96% ethanol. The filters were washed three times in distilled water, and adherent cells were stained with 0.04% (wt/vol) crystal violet in 4% (vol/vol) ethanol and detected spectro-photometrically by using a BioRad100 automated laboratory workstation (Beckman) with a 600-nm filter. All experiments were performed in quadruplicate.

RESULTS

PDGF β receptor hyperphosphorylation in vivo following TC-PTP deletion. The effect of TC-PTP deletion on in vivo phosphorylation of the PDGF β receptor was determined with TC-PTP−/− (wt) and TC-PTP−/− (TC-PTP ko) E14.5 mouse embryos. Immunoprecipitation of PDGF β receptors revealed hyperphosphorylation of the receptors obtained from TC-PTP ko embryos compared to receptors obtained from wt embryos (Fig. 1), indicating that TC-PTP regulates PDGF β receptor tyrosine phosphorylation in vivo.

TC-PTP ko MEFs display increased PDGF β receptor phosphorylation. The role of TC-PTP in PDGF β receptor phosphorylation and signal transduction was further investigated by using MEF cell lines obtained from TC-PTP ko and littermate wt mice (17). Deletion of TC-PTP did not induce detectable tyrosine phosphorylation of the PDGF β receptor in serum-starved, nonstimulated cells (Fig. 2A, left panel). In both cell lines, 7 min of stimulation with PDGF-BB induced tyrosine phosphorylation of the PDGF β receptor (Fig. 2A, left panel). Densitometric analysis revealed that receptor phosphorylation in stimulated TC-PTP ko cells was threefold higher than that in wt cells (Fig. 2A, left panel).

To confirm that the increased tyrosine phosphorylation in TC-PTP ko cells was caused by TC-PTP depletion, TC-PTP ko MEFs were transfected with wt TC-PTP or the catalytically inactive CS mutant form of TC-PTP. Two independent mass cultures from each transfection were established. Analysis of PDGF expression in cells reconstituted with wt TC-PTP by phosphatase assays indicated expression levels corresponding to 10 and 16% those seen in wt MEFs (Fig. 2B, left panel). We were unable to establish cell lines with higher levels of TC-PTP expression, indicating selection against cells expressing high levels of TC-PTP.

In agreement with a direct effect of TC-PTP on PDGF β receptor phosphorylation, reconstitution of TC-PTP ko MEFs with wt TC-PTP led to a decrease in PDGF β receptor phosphorylation (Fig. 2A, left panel). In contrast, receptor phosphorylation in TC-PTP ko cells transfected with the CS mutant was similar to that in parental ko cells (Fig. 2A, left panel). To verify the effect of TC-PTP reconstitution, we transiently expressed wt TC-PTP and the CS mutant in TC-PTP ko MEFs. Analysis of wt TC-PTP expression indicated that the expression of TC-PTP activity corresponding to 27% that seen in wt MEFs (Fig. 2B, right panel) was sufficient to restore PDGF β receptor phosphorylation to the levels detected in wt MEFs (Fig. 2A, right panel). Expression of the CS mutant led to a slight increase in PDGF β receptor phosphorylation, presumably due to its ability to act as a substrate trap (Fig. 2A, right panel). Together, the results in Fig. 2 indicate that the PDGF β receptor is directly dephosphorylated by TC-PTP.
Generation of a panel of site-specific PDGF β receptor antibodies. To study the phosphorylation of the individual tyrosine residues of the PDGF β receptor, we generated antibodies against phosphopeptides derived from the human PDGF β receptor, corresponding to four phosphorylation sites (Table 1). The designations of the antibodies are based on the sequence of the human receptor. To test the specificity of affinity-purified antibodies, PAE cells stably expressing the PDGF β receptor were used. As a control for site selectivity, we used a series of PAE cell lines each expressing PDGF β receptor mutants containing tyrosine-to-phenylalanine point mutations of one or two of the tyrosine residues corresponding to the receptor phosphorylation sites. To obtain maximal receptor tyrosine phosphorylation, the cells were stimulated with 100 ng of PDGF-BB/ml for 1 h on ice. None of the antibodies bound to the unphosphorylated PDGF β receptor in an im-

![Graph](image_url)
In the investigation of PDGF/H925 receptor phosphorylation, it was observed that increases in Y579 phosphorylation were similar to the increase in total tyrosine phosphorylation. In contrast, the phosphorylation of Y751 changed less than the increase in total tyrosine phosphorylation. An increase in Y771 phosphorylation greatly exceeded the increase seen in total tyrosine phosphorylation. It was also noted that Y1021 displayed an increase in phosphorylation which was higher than the increase in total tyrosine phosphorylation.

Mutations of individual phosphorylation sites of the PDGF receptor were used to achieve selective activation of the PDGF receptor. As shown in Fig. 4B, stimulation with PDGF-BB led to increased tyrosine phosphorylation at all four sites investigated. Densitometric quantitation revealed that Y579/581F, Y751F, Y771F, and Y1021F phosphorylation differed between different sites.

The increases in receptor tyrosine phosphorylation in TC-PTP ko MEFs were uniformly distributed over the sites investigated. The increased phosphorylation induced by PDGF-DD in TC-PTP ko MEFs displayed a larger increase in phosphorylation than Y579 and Y751 in TC-PTP ko MEFs, indicating that these two sites are preferred substrates for TC-PTP. Further investigation showed that TC-PTP displayed site selectivity against the different phosphorylation sites in the PDGF β receptor, which was expected because TC-PTP displayed site selectivity against PDGF receptor phosphorylation sites, Y1021 and Y771. These results were consistent with the in vivo data, suggesting that the rate of dephosphorylation was not affected by binding of SH2 domain-containing proteins to the receptor.

Deletion of TC-PTP causes a site-selective increase in PDGF β receptor phosphorylation. Using the site-selective antibodies, we next investigated whether deletion of TC-PTP differentially affected individual autophosphorylation sites. To achieve selective activation of the PDGF β receptor, MEFs were stimulated with 50 ng of PDGF-DD/ml (2). Stimulation with PDGF-DD induced fivefold more PDGF β receptor phosphorylation in TC-PTP ko MEFs than in wt MEFs (Fig. 4A). To investigate whether the increases in receptor tyrosine phosphorylation in TC-PTP ko cells were uniformly distributed between the PDGF β receptor phosphorylation sites, we used the site-specific phosphotyrosine antibodies directed against the PDGF β receptor. As shown in Fig. 4B, stimulation with PDGF-DD led to increased tyrosine phosphorylation at all four sites investigated. Densitometric quantitation revealed that Y1021 displayed an increase in phosphorylation which greatly exceeded the increase seen in total tyrosine phosphorylation (Fig. 4C). The total increase in Y771 phosphorylation was higher than the increase in total tyrosine phosphorylation. In contrast, the phosphorylation of Y751 changed less than the total tyrosine phosphorylation of the receptor, whereas the increase in Y579 phosphorylation was similar to the increase in total tyrosine phosphorylation (Fig. 4C). Thus, the increases in phosphorylation differed between different sites.

TC-PTP displays site-selective dephosphorylation of the PDGF β receptor in an in vitro assay. The fact that two of the investigated PDGF β receptor sites, Y1021 and Y771, displayed a larger increase in phosphorylation than Y579 and Y751 in TC-PTP ko MEFs was expected because TC-PTP was able to dephosphorylate a WGA-immobilized PDGF β receptor. The phosphorylation status of two of the sites, Y579 and Y1021, was determined by immunoblotting after incubation with TC-PTP. After incubation with TC-PTP, 40% of the total receptor phosphorysine signal was lost, compared to the results obtained with receptor precipitates incubated with assay buffer only (Fig. 5). Dephosphorylation of Y579 occurred to the same extent as total receptor dephosphorylation (Fig. 5). Consistent with the in vivo data, a larger decrease in Y1021 phosphorylation than in total tyrosine phosphorylation was observed (Fig. 5). To ensure that the rate of dephosphorylation was not affected by binding of SH2 domain-containing proteins to the receptor, the experiment was repeated with PDGF β receptors phosphorylated in an in vitro kinase assay. The rates of dephosphorylation of the different sites were the same as those observed with PDGF β receptors phosphorylated by stimulation with PDGF-BB (data not shown).

Deletion of PTP-1B but not of PTPε is associated with site-selective effects on PDGF β receptor phosphorylation. To investigate the effects of losses of other PTPs on the phosphorylation of selected sites in the PDGF β receptor, we used MEFs from PTP-1B and PTPε ko mice. MEFs from PTP-1B ko mice displayed greater phosphorylation of the PDGF β receptor following stimulation with PDGF-DD than did MEFs reconstituted with human PTP-1B (Fig. 6A), in agreement with published results (13). In contrast, PTPε ko MEFs did not differ in receptor phosphorylation from the corresponding MEFs from wt mice (Fig. 6A). The phosphorylation of the four different sites of the PDGF β receptor was examined by using these cell lines. The increased phosphorylation induced by a loss of PTP-1B was not evenly distributed over the sites investigated. In contrast to the results for TC-PTP ko MEFs (data

**FIG. 3.** Specificity of site-selective phosphotyrosine antibodies in immunoblotting. PAE cells stably expressing the wt PDGF β receptor or PDGF β receptors carrying tyrosine-to-phenylalanine point mutations at the indicated tyrosine residues were left untreated or were stimulated with 100 ng of PDGF-BB/ml for 1 h on ice. Receptors were precipitated with WGA-Sepharose, and the precipitated proteins were separated by SDS-PAGE. Total receptor phosphorylation (top panel) was detected by immunoblotting (IB) with antiphosphotyrosine antibody PY99, and phosphorylation of the indicated individual phosphorylation sites (lower panel) was detected by immunoblotting with the indicated site-selective phosphotyrosine antibodies.
FIG. 4. Site-selective increase in tyrosine phosphorylation of the PDGF β receptor in TC-PTP ko cells. (A) Cells were stimulated with 50 ng of PDGF-DD/ml and lysed. Receptors were precipitated with WGA-Sepharose, and the precipitated proteins were separated by SDS-PAGE. Receptor phosphorylation was detected by immunoblotting with PY99 and PDGF β receptor (β-rec) antibodies. (B) Cells were stimulated with 50 ng of PDGF-DD/ml and lysed. Receptors were precipitated with WGA-Sepharose, and the precipitated proteins were separated by SDS-PAGE. Phosphorylation of individual phosphorylation sites was determined by immunoblotting (IB) with site-selective phosphotyrosine antibodies. Aliquots of the immunoprecipitates were separated by SDS-PAGE, and the amounts of PDGF β receptors immunoprecipitated were determined. (C) Densitometric analysis of changes in site-selective PDGF β receptor phosphorylation in TC-PTP ko MEFs compared to wt MEFs. Data are given as the increase (mean and standard error of the mean; n = 5) in phosphorylation/receptor in TC-PTP ko cells compared to wt cells. The total phosphorylation/receptor and the phosphorylation of each site/receptor in wt cells were set to 1.
The investigated (Fig. 6B).

The p85 subunit of PI 3-kinase associates with Y751 of the receptor. Stimulation of wt cells with PDGF-BB dose-dependently activated PLC1, measured as the production of inositol phosphate (Fig. 7A). Consistent with the observed hyperphosphorylation of Y1021 in TC-PTP ko cells, ligand-induced PLC1 activation was increased in these cells (Fig. 7A). PLC1 expression levels were not affected by deletion of TC-PTP (Fig. 7A, inset).

The p85 subunit of PI 3-kinase associates with Y751 of the activated PDGF β receptor, the site least affected by deletion of TC-PTP. As shown in Fig. 7B, stimulation of both TC-PTP wt and ko cells led to the association of similar amounts of PI 3-kinase activity with the receptor. In accordance with this finding, no differences in PDGF-BB-induced phosphorylation of the serine/threonine kinase Akt, which is downstream of PI 3-kinase, were detected in the two cell lines (Fig. 7C). A low level of PI 3-kinase activity was associated with the receptor in unstimulated TC-PTP ko cells (Fig. 7B). However, Akt was not phosphorylated in unstimulated cells (Fig. 7C). Furthermore, no differences in PDGF-BB-induced phosphorylation of Erk1/2 were detected in TC-PTP wt and ko cells (data not shown), in accordance with previous reports (17).

Thus, the large increase in the phosphorylation of Y1021 observed in TC-PTP ko MEFs correlated with an increase in PLC1 activation, whereas the more modest increase in the phosphorylation of Y751 had no observable effect on downstream signaling.

Loss of TC-PTP but not of PTP-1B increases PDGF-induced cell migration. Overactivation of PLC1 has been linked to an increased chemotactic response following PDGF stimulation (35). Therefore, we investigated the effect of a loss of TC-PTP on cell migration in response to PDGF-BB. TC-PTP ko MEFs displayed a 1.5-fold-higher level of random migration than did wt MEFs. Both cell types displayed an increased migration rate in response to PDGF-BB (Fig. 8A), but the increase was larger for TC-PTP ko MEFs than for wt MEFs (nine- and sixfold increases, respectively, in response to 10 ng of PDGF-BB/ml). This effect was partially reversed in TC-PTP ko cells stably transfected with wt TC-PTP (data not shown). When serum was used as a stimulus, the TC-PTP wt MEFs responded with a greater increase in migration than did the TC-PTP ko MEFs (15- and 4-fold increases, respectively), indicating that the increase seen following PDGF stimulation was not due to increased general cell motility following the loss of TC-PTP (Fig. 8A).

Since a loss of PTP-1B also increased PDGF β receptor phosphorylation after ligand binding, we investigated whether the loss of this PTP affected cell migration. As shown in Fig. 8B, unstimulated MEFs lacking PTP-1B expression showed a lower level of background migration than did MEFs expressing human PTP-1B. Notably, both cell types migrated at similar rates toward both PDGF-BB and serum, consistent with the fact that in these cells, Y1021 is not selectively overphosphorylated. Moreover, PDGF and serum were equally efficient in stimulating migration.

DISCUSSION

In this report, we present findings, derived from in vivo analyses and tissue culture studies, which identify TC-PTP as a previously unrecognized negative regulator of PDGF β receptor phosphorylation (Fig. 1 and 2). Detailed characterization of the consequence of TC-PTP deletion indicated site-selective effects of TC-PTP, with the most pronounced hyperphosphorylation of Y1021 of the PDGF β receptor (Fig. 3 to 6). Importantly, the increase in tyrosine phosphorylation of the PDGF β receptor following the deletion of PTP-1B occurred mainly on Y579 (Fig. 6). These results demonstrate that these two tyrosine phosphatases regulate the phosphorylation of distinct tyrosine residues. Also, the increased PDGF β receptor phosphorylation in cells from TC-PTP ko mice was associated with increased PDGF-induced activation of PLC1 and increased cell migration in response to PDGF (Fig. 7 and 8).
PTPs that were previously implicated in the control of PDGF receptor phosphorylation include the classical PTPs SHP-1, SHP-2, PTP-1B, PTP-PEST, DEP-1, and LMW-PTP (4, 14, 20, 22, 26, 41). Most of these studies have associated individual PTPs with PDGF receptor signaling after analyses of cells with heterologous PTP expression or by characterization of PTPs coprecipitating with PDGF receptors. In the present study, we identified TC-PTP as a negative regulator of PDGF receptor phosphorylation by analyses of the consequences of PTP depletion.

Both TC-PTP and PTP-1B were found to display site selectivity in dephosphorylation of the PDGF receptor and, importantly, each phosphatase regulated the phosphorylation of a distinct set of tyrosine residues (Fig. 3 and 6). The notion that different phosphatases regulate the phosphorylation status of different tyrosine residues could account for cell type-specific effects of growth factor stimulation. By regulating the expression and/or activation of tyrosine phosphatases, the cell subsequently might be able to modulate growth factor-induced signals and fine-tune its response to the surrounding environment.

In general, the issue of site selectivity in the dephosphorylation of tyrosine kinase receptors by PTPs remains poorly explored. However, studies that have been done so far on PDGF receptor dephosphorylation support the notion that selectivity in dephosphorylation is a common feature. Deletion of the binding site for SHP-2 results in a specific increase in the phosphorylation of Y771 in PDGF receptor heterodimeric complexes (9). Also, DEP-1 dephosphorylation of the PDGF receptor displays site selectivity, with Y1021 and Y857 occurring as preferred and nonpreferred sites, respectively (22, 32). Finally, the phosphorylation of regulatory Y857 was dramatically increased after the overexpression of the catalytically inactive form LMW-PTP, suggesting that this site is a preferred site for dephosphorylation by LMW-PTP (5). Support for the general notion of the site-selective action of PTPs was also recently provided by the demonstration that DEP-1 preferentially dephosphorylates Y1349 and Y1365 of the hepatocyte growth factor receptor/c-Met (30).

A loss of TC-PTP resulted in a larger increase in overall PDGF β receptor phosphorylation than did a loss of PTP-1B.

**FIG. 6. Deletion of PTP-1B but not deletion of PTPε induces a site-selective increase in tyrosine phosphorylation of the PDGF β receptor.**

(A) Cells were stimulated with 50 ng of PDGF-DD/ml and lysed. Receptors were precipitated with WGA-Sepharose and subjected to SDS-PAGE. Phosphorylation of the PDGF β receptor was detected with phosphotyrosine antibodies. (B) Densitometric analysis of changes in site-selective PDGF β receptor phosphorylation in TC-PTP, PTP-1B, and PTPε ko MEFs compared to wt MEFs (TC-PTP and PTPε) or reconstituted cells (hPTP-1B). Data are given as the relative increase (mean and standard error of the mean; n = 3 to 5) in phosphorylation/receptor. The total phosphorylation/receptor and the phosphorylation of each site/receptor in the respective control cells were set to 1.
A

Graph showing the production of InsP (fold of control) with different treatments:
- **0 ng/ml PDGF-BB**
- **10 ng/ml PDGF-BB**
- **100 ng/ml PDGF-BB**

**Cell Lines:**
- **wt**
- **TC-PTP ko**

B

**Cell Lines:**
- **wt**
- **TC-PTP ko**

**PDGF-BB:**
- **-**
- **+**

**32p-PIP**

**IB:**
- PDGF β-rec

C

**Cell Lines:**
- **wt**
- **TC-PTP ko**

**PDGF-BB:**
- **-**
- **+**

**IB:**
- P-Akt
- Akt
It should be noted, however, that the experimental systems are not identical. TC-PTP ko MEFs were compared to MEFs from littermate wt mice, whereas PTP-1B ko MEFs were compared to reconstituted cells. Furthermore, the immortalization of the MEFs used in this study is likely to affect signaling pathways, thereby affecting the intracellular response to growth factors. However, the fact that the distributions of the increases in tyrosine phosphorylation between the sites differ between TC-PTP and PTP-1B ko MEFs implies that these PTPs regulate different responses to PDGF. Also, the absence of effects of PTP-1B depletion on PDGF-induced migration is in agreement with the results of Haj et al. (13), who detected a selective PTP-1B depletion on PDGF-induced migration is in agreement with the results of Haj et al. (13), who detected a selective PTP-1B depletion on PDGF-induced migration.

The absence of effects of PTP-1B depletion on PDGF-induced migration is in agreement with the results of Haj et al. (13), who detected a selective PTP-1B depletion on PDGF-induced migration.

These findings suggest that site-selective dephosphorylation by PTPs translates into alterations in specific cellular responses. The physiological relevance of these findings should be further explored, e.g., by comparing the patterns of PDGF receptor phosphorylation in tissues where PDGF mediates predominantly proliferative or migratory responses.

TC-PTP also has been linked to the dephosphorylation of other tyrosine kinase receptors, including the EGF and insulin receptors (11, 38, 39). In addition, TC-PTP acts as a negative regulator of cytokine signaling through dephosphorylation of the Jak family of tyrosine kinases (36). The target specificity of TC-PTP is presently unclear, and more studies are required for identification of the functional role(s) of this enzyme.

Recent observations indicate spatially restricted tyrosine kinase dephosphorylation by PTPs. Dephosphorylation of the EGF receptor by the 48-kDa isoform of TC-PTP occurs in the ER, whereas nuclear 45-kDa TC-PTP translocates to the cell periphery, where it dephosphorylates the EGF receptor in response to EGF (38). PTP-1B is also located in the ER, where it dephosphorylates the EGF receptor and the PDGF receptor (14). From this perspective, it will be interesting to elucidate whether the effects on PDGF receptor phosphorylation demonstrated in this study occur on the entire receptor population or on spatially restricted receptor subsets.

PDGF receptor signaling has well-documented important functions in developmental as well as pathological processes...
(3, 33). The identification of TC-PTP as a negative regulator of PDGF receptor signaling will be important in the further elucidation of the pathophysiological role of the receptor. One obvious topic for future studies is to investigate whether the bone marrow stroma-dependent defect in hematopoiesis seen in TC-PTP ko mice involves perturbed PDGF receptor signaling (40). This possibility is supported by previous studies which implicated PDGF receptor signaling in diseases of the bone marrow stroma.

In conclusion, we have obtained evidence that both TC-PTP and PTP-1B act as distinct site-selective modulators of PDGF β receptor signaling. Further studies should be aimed at elucidating the physiological relevance of this regulatory mechanism.

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REFERENCES


