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Published in:
Molecular and Cellular Biology

DOI:
10.1128/MCB.24.5.2190-2201.2004

2004

Link to publication

Citation for published version (APA):

Total number of authors:
13

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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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Received 25 August 2003/Returned for modification 8 October 2003/Accepted 8 December 2003

The platelet-derived growth factor (PDGF) β receptor mediates mitogenic and chemotactic signals. Like other tyrosine kinase receptors, the PDGF β receptor is negatively regulated by protein tyrosine phosphatases (PTPs). To explore whether T-cell PTP (TC-PTP) negatively regulates the PDGF β receptor, we compared PDGF β receptor tyrosine phosphorylation in wild-type and TC-PTP knockout (ko) mouse embryos. PDGF β receptors were hyperphosphorylated in TC-PTP ko embryos. Fivefold-higher ligand-induced receptor phosphorylation was observed in TC-PTP ko mouse embryo fibroblasts (MEFs) as well. Reexpression of TC-PTP partly abolished this difference. As determined with site-specific phosphotyrosine antibodies, the extent of hyperphosphorylation varied among different autophosphorylation sites. The phospholipase Cγ1 binding site Y1021, previously implicated in chemotaxis, displayed the largest increase in phosphorylation. The increase in Y1021 phosphorylation was accompanied by increased phospholipase Cγ1 activity and migratory hyperresponsiveness to PDGF. PDGF β receptor tyrosine phosphorylation in PTP-1B ko MEFs but not in PTPs ko MEFs was also higher than that in control cells. This increase occurred with a site distribution different from that seen after TC-PTP depletion. PDGF-induced migration was not increased in PTP-1B ko cells. In summary, our findings identify TC-PTP as a previously unrecognized negative regulator of PDGF β receptor signaling and support the general notion that PTPs display site selectivity in their action on tyrosine kinase receptors.

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 site-specific recruitment of signal transduction molecules containing SH2 domains to the activated receptor (16); these molecules include adaptor proteins such as Shc 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
lines (19). TC-PTP has been implicated in the regulation of growth factor receptor signaling, both at the level of receptor tyrosine phosphorylation and in the regulation of downstream signaling events. The overexpression of a truncated, active form of TC-PTP has been shown to reduce the tyrosine phosphorylation of several proteins in PDGF-stimulated cells (7). Both the epidermal growth factor (EGF) receptor and the adaptor protein p52Shc have been identified as substrates for TC-PTP (38). The association between the EGF receptor and the 45-kDa TC-PTP takes place at the plasma membrane (38), whereas the 48-kDa TC-PTP colocalizes with the EGF receptor in the endoplasmic reticulum (ER) (39). In addition, TC-PTP has been linked to the dephosphorylation of the insulin receptor (11) and acts as a negative regulator of cytokine signaling through dephosphorylation of the Jak family of tyrosine kinases (36).

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). More recently, in-gel PTP assays were used to identify PDGF receptor-associating PTPs and revealed that PTP-PEST and TC-PTP also could be recovered in PDGF receptor immunoprecipitates (26).

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.

### MATERIALS AND METHODS

**Antibodies.** Polyclonal antiserum 958 against the PDGF β receptor, monoclonal antibody PY99, and polyclonal antiserum against Akt1/2 were obtained from Santa Cruz Biotechnologies (Santa Cruz, Calif.). Polyclonal antiseria recognizing Akt phosphorylated at Thr308, total Erk1/2, and Erk1/2 phosphorylated at Thr202/Tyr204 were obtained from Cell Signaling Technologies, Ltd. (Beverly, Mass.). The TC-PTP monoclonal antibody clone 6F3 was described previously (17).

**Isolation of PDGF β receptors from mouse embryos.** TC-PTP wild-type (wt) and knockout (ko) embryonic day 14.5 (E14.5) mouse embryos were homogenized in ice-cold PBS and lysed in 20 mM Tris-HCl (pH 7.5) – 1% Trasylol for 15 min on ice. The lysates were cleared by centrifugation at 16,000 g for 15 min at 4°C. PDGF β receptors then were immunoprecipitated with 2 μg of anti-PDGF β receptor antibodies at 4°C for 2 h. Protein complexes were washed three times in lysis buffer, resolved by SDS–7% polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidine difluoride membranes (Immobilon; Millipore). PDGF β receptor phosphorylation was detected with monoclonal phosphotyrosine antibody PY99 (1 μg/ml), followed by stripping in 0.4 M NaOH and reprobing 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). Reciprocating cells were created by transfecting TC-PTP ko MEFs with pCNA4 vectors containing constructs of either the 45-kDa isoform of mouse wt-PTP (17) or a catalytically inactive mutant form of PDGF-PTP in which the catalytic cysteine is mutated to serine (CS). Two mass cultures stably expressing wt PDGF-PTP (ko/wt1 and ko/wt2) or the CS mutant (ko/CS1 and ko/CS2) were generated. Additionally, wt-PTP and the CS mutant were transiently expressed in PDGF-PTP ko MEFs using Lipofectamine Plus reagent according to the manufacturer’s protocol (Invitrogen Life Technologies, Carlsbad, Calif.). PTP-PEST and wt MEFS (31), PTP-1B ko MEFS (13), and the CS mutant were 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% penicillin/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-maleimido benzoyl-N-hydroxysuccinimide ester (Pierce), and the conjugates were used to immunize rabbits. The antibodies were affinity purified by passing the antiserum over three consecutive columns of immobilized nonphosphorylated peptide, phosphotyrosine-agarose, and phosphopeptide modified peptide as previously described (9). The peptides were coupled to Sulfolink (Pierce) and phosphotyrosine was coupled to Affigel 10 (Bio-Rad) according to the manufacturer's 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 then 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 Na₂VO₃ - 1% Trasylol 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 peroxidase-conjugated secondary antibodies by using an LAS-100Plus charge-coupled device camera (Fujifilm). Densitometric analysis of the bands was performed by using advanced image data analyzer software (Fujifilm).

**Phosphatase assay.** Cells were lysed in the buffer described above but supplemented with 10 mM 1,4-dithiothreitol and lacking Na₂VO₃. 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 \( ^{32} \text{P} \)-labeled peptide (amino acid sequence AEIDGFEAKKKK) as a substrate as previously described (37). Assays were performed in duplicate, and phosphatase activity was expressed as the relative amount of \( ^{32} \text{P} \)-labeled radioactivity released from the peptide after 7 min of incubation at 30°C.

In vitro PDGF \( \beta \) receptor dephosphorylation. PAE cells stably expressing the PDGF \( \beta \) 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 precipitated with WGA-Sepharose, followed by four washes in phosphatase assay buffer. The precipitates were resuspended in a total volume of 100 \( \mu \)l 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 10³ cells per well in 12-well plates and incubated with serum-free M199 medium supplemented with 1 mg of BSA/ml overnight. After being washed with 4 ml of cold M199 medium, 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 \( \beta \) receptor-associated PI 3-kinase activity.** Cells were starved overnight in medium supplemented with 1 mg of BSA/ml and then were stimulated with 50 ng of PDGF-BB/ml at room temperature for 10 min. PDGF \( \beta \) receptors were immunoprecipitated with 2 \( \mu \)g of anti-PDGFR \( \beta \) receptor antibody at 4°C for 3 h. Aliquots of the corresponding immunoprecipitates were subjected to analysis of PDGF \( \beta \) receptor levels by immunoblotting. The collected beads were subjected to a kinase reaction in the presence of 20 \( \mu \)Ci of [\( ^{32} \text{P} \)]ATP and with 0.2 \( \mu \)g of presonicated phosphatidylinositol- \( \mu \)l as a substrate as previously described (18). The kinase reaction was performed at 37°C for 30 min and was stopped with 100 \( \mu \)l 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 Gel 60; Whatman LKGF). 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 ChemoTX (Neuroprobe) cell migration microplate with a pore size of 3.2 \( \mu \)m. The filters were coated with 50 \( \mu \)g 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 \( \mu \)l of Trasylol/ml of cell suspension. The cells were pelleted and diluted to a final concentration of 2.5 \( \times \)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 Biomek1000 automated laboratory workstaton (Beckman) with a 600-nm filter. All experiments were performed in quadruplicate.

**RESULTS**

**PDGF \( \beta \) receptor hyperphosphorylation in vivo following TC-PTP deletion.** The effect of TC-PTP deletion on in vivo phosphorylation of the PDGF \( \beta \) receptor was determined with TC-PTP \( ^{+/+} \) (wt) and TC-PTP \( ^{−/−} \) (TC-PTP ko) E14.5 mouse embryos. Immunoprecipitation of PDGF \( \beta \) 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 \( \beta \) receptor tyrosine phosphorylation in vivo.

**TC-PTP ko MEFs display increased PDGF \( \beta \) receptor phosphorylation.** The role of TC-PTP in PDGF \( \beta \) 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 \( \beta \) 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 \( \beta \) 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 deletion, 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 TC-PTP 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 \( \beta \) receptor phosphorylation, reconstitution of TC-PTP ko MEFs with wt TC-PTP led to a decrease in PDGF \( \beta \) 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 \( \beta \) 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 \( \beta \) 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 \( \beta \) 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-
investigated PDGF receptor phosphorylation differed between different sites. Densitometric quantitation revealed that Y579 displayed a larger increase in phosphorylation than Y579 and Y751 in TC-PTP ko MEFs that these two sites are preferred substrates for TC-PTP. To further investigate whether TC-PTP displayed site selectivity against the different autophosphorylation sites in the PDGF receptor, we performed an in vitro phosphatase assay in which recombinant TC-PTP was allowed to dephosphorylate a WGA-immobilized PDGF 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 receptor phosphorylated in an in vitro kinase assay. The rates of dephosphorylation of the different sites were the same as those observed with PDGF 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 receptor phosphorylation. To investigate the effects of losses of other PTPs on the phosphorylation of selected sites in the PDGF receptor, we used MEFs from PTP-1B and PTPε ko mice. MEFs from PTP-1B ko mice displayed greater phosphorylation of the PDGF 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 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 not shown).
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 pattern of phosphorylation of the PDGF β receptor by recombinant TC-PTP. Phosphorylated PDGF β receptors were obtained by WGA-Sepharose precipitation from stimulated PAE cells stably expressing the PDGF β receptor. The phosphorylated receptors were incubated with buffer only or with 3 ng of recombinant TC-PTP for 10 min at 30°C. Following SDS-PAGE, the proteins were transferred to nitrocellulose membranes; phosphorylation of the indicated sites was detected with site-specific phosphotyrosine antibodies followed by densitometric analysis. The in vitro dephosphorylation of each site by recombinant TC-PTP is expressed as the percent reduction (mean and standard error of the mean; n = 3) in the signal after in vitro dephosphorylation.

from Fig. 4C inserted into Fig. 6B), a loss of PTP-1B led to a marked increase in the phosphorylation of Y579, whereas Y771 was found to be less affected than the other sites (Fig. 6B). As expected from the findings shown in Fig. 6A, a loss of PTPε did not affect the phosphorylation of any of the sites investigated (Fig. 6B).

TC-PTP deletion selectively modulates PDGF-induced signal transduction. The finding that TC-PTP depletion alters the pattern of phosphorylation of the PDGF β receptor implies that this phosphatase could selectively affect the signaling outcome of receptor ligation. Therefore, we investigated the signaling pathways downstream of Y1021 and Y751, the two sites that were most and least affected by deletion of TC-PTP, respectively.

PLCγ1 is activated following binding to Y1021 of the PDGF β receptor. Stimulation of wt cells with PDGF-BB dose-dependently activated PLCγ1, measured as the production of inositol phosphate (Fig. 7A). Consistent with the observed hyperphosphorylation of Y1021 in TC-PTP ko cells, ligand-induced PLCγ1 activation was increased in these cells (Fig. 7A). PLCγ1 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 PLCγ1 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 PLCγ1 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 depletion 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 depletion 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 PLCγ1 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 dephosphorylating the PDGF β 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 consequently might be able to modulate growth factor-induced signals and fine-tune its response to the surrounding environment. In addition, regulation of the expression and activity of site-selective PDGF β receptor-directed phosphatases by other receptors would provide a way for cross talk between different classes of receptors.

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 PDGFα and PDGFβ heterodimeric complexes (9). Also, DEP-1 dephosphorylation of the PDGF β 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.
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 increase in Erk activation following PDGF β receptor activation in PTP-1B ko cells. In the context of discussing PTP specificity, it is also noteworthy that depletion of PTPs has no effects on PDGF β receptor phosphorylation (Fig. 6). This observation presents clear evidence that not all PTPs are involved in the control of PDGF receptor signaling.

Phosphorylation of Y1021 and subsequent activation of PLCγ1 have been linked to PDGF-induced chemotaxis (15, 23, 35). It is therefore noteworthy that TC-PTP ko cells display hyperphosphorylation of Y1021, enhanced PLCγ1 activation, and an increased migratory response whereas, in contrast, PTP-1B ko cells are characterized by less than a twofold increase in the phosphorylation of Y1021 and no increase in the migratory response to PDGF. These findings support the possibility 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.
REFERENCES


