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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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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 PTPβ 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
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 PDGFinduced 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 phosphorylation is poorly understood. In addition to SH-2, several phosphatases, including a low-molecular-weight PTP (PTP-1B) and a receptor-like tyrosine phosphatase (DEP-1), interact with and dephosphorylate the PDGFB receptor (4, 13, 18, 22). More recently, in-gel PTP assays were used to identify PDPGFB receptor-associating PTPs and revealed that PTP-PEST and TC-PTP also could be recovered in PDPGFB receptor immunoprecipitates (26).

Site-selective dephosphorylation of the PDGFB receptor by SH-2 and PTP-1B has been demonstrated (5, 21). Analyses of DEP-1 dephosphorylation of PDGFB 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 PDGFB receptor and other receptor tyrosine kinases. It was recently demonstrated that murine embryonic fibroblasts (MEFs) lacking the TC-PTP displayed a defective PDGFB-induced IKK/NF-κB activation pathway, whereas PDGFB-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 PDGFB receptor signaling. We demonstrate that TC-PTP directly controls PDGFB receptor phosphorylation in a site-selective manner and also negatively regulates the mitogenic response to PDGFB.

**MATERIALS AND METHODS**

**Antibodies.** Polyclonal antisera 958 against the PDGFB receptor, monoclonal antibodies PY99, and polyclonal antisera against Akt1/2 were obtained from Santa Cruz Biotechnologies (Santa Cruz, Calif.). Polyclonal antisera recognizing Akt 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 PDGFB receptors from mouse embryos.** TC-PTP wild-type (wt) and knockout (ko) mice were obtained from Cell Signaling Technologies, Ltd. (Beverly, Mass.). The TC-PTP monoclonal antibody clone 6F3 was described previously (17).

**Materials and Methods.** The peptides were conjugated to keyhole limpet hemocyanin by using m-maleimidobenzoyl-N-hydroxysuccinimide 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 keyhole limpet hemocyanin by using m-maleimidobenzoyl-N-hydroxysuccinimide 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).

**Isolation of PDGFB receptors from mouse embryos.** TC-PTP wild-type (wt) and knockout (ko) embryonic day 14.5 (E14.5) mouse embryos were homogenized in lysis buffer, resolved by SDS–7% polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore). PDGFB 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 PDGFB 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). Recombining cells were created by transfecting TC-PTP ko MEFs with pEIA4 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 PDGFB (ko/wt1 and ko/wt2) or the CS mutant (ko/CS1 and ko/CS2) were generated. Additionally, wt TC-PTP and the CS mutant (ko/CS1 and ko/CS2) were transfected into TC-PTP ko MEFs using Lipofectamine- Plus reagent according to the manufacturer’s protocol (Invitrogen Life Technologies, Carlsbad, Calif.). PTP-1B ko and wt MEFs (31), PTP-1B ko MEFs (13), and MEFs reconstituted with human wt PDGFB (P1P-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 PDGFB receptor (6) and PDGFB 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 μg 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 PDGFB receptor (Table 1) were synthesized by 9-fluorenlymethoxycarbonyl 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-maleimidobenzoyl-N-hydroxysuccinimide 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 phosphotyrosine was coupled 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 lines, 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 PDGFB-IB/ml or 50 ng of PDGFB-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% Trasylol for 15 min on ice. The lysates were cleared by centrifugation at 16,000 × g for 15 min at 4°C. PDGFB receptor antibodies were precipitated with wheat germ agglutinin (WGA)-Sepharose (Pharacia). 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 phospho-tyrosine 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 and supplemented with 10 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 AEEDYGEFEAKKKK) as a substrate as previously described (37). Assays were performed in duplicate, and phosphatase activity was expressed as the relative amount of 32P-labeled radioactive release 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 precipitated 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 loaded with 4 μCi of myo-[3H]inositol (Amer sham) 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 were supplemented 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 presoncinated phosphotydylinositol/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 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 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 adding 150 μl of Trasylol/ml of cell suspension. The cells were pelleted and diluted to a final concentration of 2.5 × 10^5 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 spectrophotometrically by using a Biomek1000 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.

FIG. 1. Increased PDGF β receptor phosphorylation in TC-PTP ko E14.5 mouse embryos. E14.5 embryos were harvested from TC-PTP ko and wt mice, and cell lysates were prepared. The PDGF β receptor (β-rec) was immunoprecipitated (IP) from precleared lysates, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Receptor phosphorylation was detected by immunoblotting (IB) with a monoclonal phosphotyrosine antibody (PY99) followed by PDGF β receptor antibodies. Immunoprecipitates from two separate embryos each of TC-PTP ko and wt mice are shown.

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 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 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-

**FIG. 2.** Increased PDGF β receptor phosphorylation in TC-PTP ko cells. (A) TC-PTP wt and ko cells, together with TC-PTP ko cells stably expressing wt TC-PTP (ko/wt1 and ko/wt2) or a CS mutant form of TC-PTP (ko/cs1 and ko/cs2), were left untreated or were stimulated with 10 ng of PDGF-BB/ml and lysed (left panel). Alternatively, TC-PTP wt and ko MEFs were mock transfected or TC-PTP ko MEFs were transfected with wt TC-PTP (ko/wt) or the CS mutant form of TC-PTP (ko/CS). At 24 h after transfection, the cells were left untreated or were stimulated with 10 ng of PDGF-BB/ml and lysed (right panel). Receptors were precipitated with WGA-Sepharose, and the precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. Phosphorylated receptors were detected by consecutive immunoblotting (IB) with a monoclonal phosphotyrosine antibody (PY99) and PDGF β receptor antibodies. Densitometric analysis of the immunoreactivity was performed; reactivity with the PY99 antibody/reactivity with the PDGF β receptor (β-rec) antibodies is indicated below each lane. Representative results from three experiments are shown. (B) TC-PTP was immunoprecipitated from cell lysates containing equal amounts of proteins from TC-PTP wt cells, TC-PTP ko cells, and TC-PTP ko cells stably reconstituted with wt TC-PTP (ko/wt1 and ko/wt2) or a CS mutant form of TC-PTP (ko/cs1 and ko/cs2) by using monoclonal TC-PTP antibody clone 6F3 (left panel). Alternatively, TC-PTP wt and ko MEFs were mock transfected or TC-PTP ko MEFs were transfected with wt TC-PTP (ko/wt) or the CS mutant form of TC-PTP (ko/CS). At 24 h after transfection, the cells were lysed and TC-PTP was immunoprecipitated by using monoclonal TC-PTP antibody clone 6F3 (right panel). The immunoprecipitated phosphatase activity was determined by using a 32P-labeled peptide (amino acid sequence AEEEIpYGEFEAKKKK, where pY indicates the phosphorylated tyrosine) as a substrate. Phosphatase activity is expressed as relative amounts of 32P radioactivity released after 7 min. Each assay was performed in duplicate, and the data are given as mean ± standard error of the mean from three separate experiments (left panel) or as the mean from one representative experiment (right panel).
investigated PDGF/H9252 phosphorylation differed between different sites. Thus, the increases in total tyrosine phosphorylation (Fig. 4C). Also, the increase in Y771 phosphorylation greatly exceeded the increase seen in total tyrosine phosphorylation of the receptor, whereas the increase in Y751 phosphorylation was higher than the increase in total tyrosine phosphorylation. In contrast, the phosphorylation of Y751 changed less than the increase in Y579 phosphorylation was similar to the increase in total tyrosine phosphorylation of the receptor, whereas the increase in Y579 occurred to the same extent as total receptor dephosphorylation (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 TC-PTP 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 not shown).

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 a larger increase in phosphorylation than Y579 and Y751 in TC-PTP ko MEFS indicated 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 β receptor, we performed an in vitro phosphatase assay in which recombinant TC-PTP was allowed 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 phosphotyrosine 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).

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 indicated 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 β receptor, we performed an in vitro phosphatase assay in which recombinant TC-PTP was allowed 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 phosphotyrosine 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 TC-PTP 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 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.
Pattern of phosphorylation of the PDGF 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-selective 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 PTPt 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 deletion 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 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 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 PDGFβ/H9251 and H9252 receptor heterodimeric complexes (9). Also, DEP-1 dephosphorylation of the PDGFβ/H9252 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.
A

![Graph showing InsP production (fold of control) for cell lines wt and TC-PTP ko.](image)

B

![Image of blot for 32P-PIP and IB: PDGF β-rec.](image)

C

![IB: P-Akt and IB: Akt](image)
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 depletion on PDGF-induced migration 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.


