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## Site-Selective Regulation of Platelet-Derived Growth Factor $\beta$ Receptor Tyrosine Phosphorylation by T-Cell Protein Tyrosine Phosphatase

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**The platelet-derived growth factor (PDGF)  $\beta$  receptor mediates mitogenic and chemotactic signals. Like other tyrosine kinase receptors, the PDGF  $\beta$  receptor is negatively regulated by protein tyrosine phosphatases (PTPs). To explore whether T-cell PTP (TC-PTP) negatively regulates the PDGF  $\beta$  receptor, we compared PDGF  $\beta$  receptor tyrosine phosphorylation in wild-type and TC-PTP knockout (ko) mouse embryos. PDGF  $\beta$  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  $C\gamma 1$  binding site Y1021, previously implicated in chemotaxis, displayed the largest increase in phosphorylation. The increase in Y1021 phosphorylation was accompanied by increased phospholipase  $C\gamma 1$  activity and migratory hyperresponsiveness to PDGF. PDGF  $\beta$  receptor tyrosine phosphorylation in PTP-1B ko MEFs but not in PTP $\epsilon$  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  $\beta$  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  $\alpha$  and  $\beta$  receptors (16). The binding of PDGF to its receptors results in receptor dimeriza-

tion, promoting phosphorylation in *trans* between the two receptors in the complex. PDGF-AA forms  $\alpha\alpha$  receptor dimers, PDGF-AB forms  $\alpha\alpha$  and  $\alpha\beta$  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  $\alpha\alpha$  receptor and  $\beta\beta$  receptor dimers, respectively, but also may activate both receptor types in cells coexpressing  $\alpha$  and  $\beta$  receptors (12, 24).

Phosphorylation of tyrosine 857 (Y857) in the catalytic loop of the PDGF  $\beta$  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  $C\gamma 1$  (PLC $\gamma 1$ ), 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

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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 p52<sup>Shc</sup> 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  $\beta$  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  $\beta$  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  $\beta$  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  $\beta$  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- $\kappa$ B 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  $\beta$  receptor signaling. We demonstrate that TC-PTP directly controls PDGF  $\beta$  receptor phosphorylation in a site-selective manner and also negatively regulates the migratory response to PDGF.

#### MATERIALS AND METHODS

**Antibodies.** Polyclonal antiserum 958 against the PDGF  $\beta$  receptor, monoclonal antibody PY99, and polyclonal antiserum against Akt1/2 were obtained from Santa Cruz Biotechnologies (Santa Cruz, Calif.). Polyclonal antisera 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  $\beta$  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 (50 mM Tris, 50 mM NaCl, 0.25% Na deoxycholate, 1% NP-40, 1 mM EGTA, 10 mM NaF, 50 mM disodium  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1% sodium dodecyl sulfate [SDS]). One milligram of total cell extract from each embryo was precleared with protein A-agarose for 1 h at 4°C. PDGF  $\beta$  receptors then were immunoprecipitated with 2  $\mu$ g of anti-PDGF  $\beta$  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 polyvinylidene difluoride membranes (Immobilon; Millipore). PDGF  $\beta$  receptor phosphorylation was detected with monoclonal phosphotyrosine antibody PY99 (1  $\mu$ g/ml), followed by stripping in 0.4 M NaOH and

TABLE 1. Sequences of phosphopeptides used for antibody production

Phosphopeptide	Sequence <sup>a</sup>
pY579.....	CSSDGH <b>p</b> YTYVDPM
pY751.....	CKDESVD <b>p</b> YVPLDM
pY771.....	CADIESSN <b>p</b> YMAPYDNYVPS
pY1021.....	CNEGDND <b>p</b> YIIPLD

<sup>a</sup> Bold type indicates the phosphorylated tyrosine.

probing with 2  $\mu$ g of PDGF  $\beta$  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 TC-PTP (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 TC-PTP ko MEFs by using Lipofectamine/Plus reagent according to the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, Calif.). PTP $\epsilon$  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  $\beta$  receptor (6) and PDGF  $\beta$  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  $\mu$ g of streptomycin/ml.

**Peptide synthesis and generation of site-selective phosphotyrosine antibodies.** Peptides corresponding to tyrosine phosphorylation sites of the human PDGF  $\beta$  receptor (Table 1) were synthesized by 9-fluorenylmethoxy carbonyl 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 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 MgCl<sub>2</sub> and immediately diluted in an equal volume of double-distilled H<sub>2</sub>O. 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 Na<sub>3</sub>VO<sub>4</sub>-1% Trasylol for 15 min on ice. The lysates were cleared by centrifugation at 16,000  $\times$  g for 15 min at 4°C. PDGF  $\beta$  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  $\mu$ g/ml) or PY99 (1  $\mu$ g/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 Na<sub>3</sub>VO<sub>4</sub>. Following immunoprecipitation of TC-PTP, the beads were washed three times in lysis buffer and once in phosphatase 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 AEEIYGEFEAKKKK) 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  $\mu\text{l}$  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.

**Inositol 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  $\mu\text{Ci}$  of *myo*-[ $^3\text{H}$ ]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 inositol 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  $\mu\text{g}$  of anti-PDGF  $\beta$  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  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ]ATP and with 0.2  $\mu\text{g}$  of presonicated phosphatidylinositol/ $\mu\text{l}$  as a substrate as previously described (18). The kinase reaction was performed at 37°C for 30 min and was stopped with 100  $\mu\text{l}$  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 LK6DF). 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  $\mu\text{m}$ . The filters were coated with 50  $\mu\text{g}$  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  $\mu\text{l}$  of Trasylol/ml of cell suspension. The cells were pelleted and diluted to a final concentration of  $2.5 \times 10^6$  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%  $\text{CO}_2$ . 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  $\beta$  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.

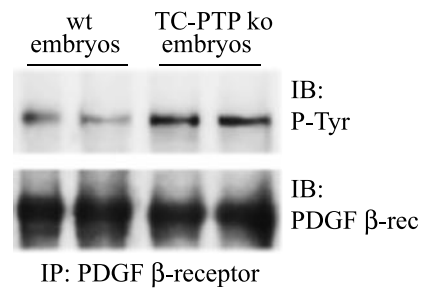


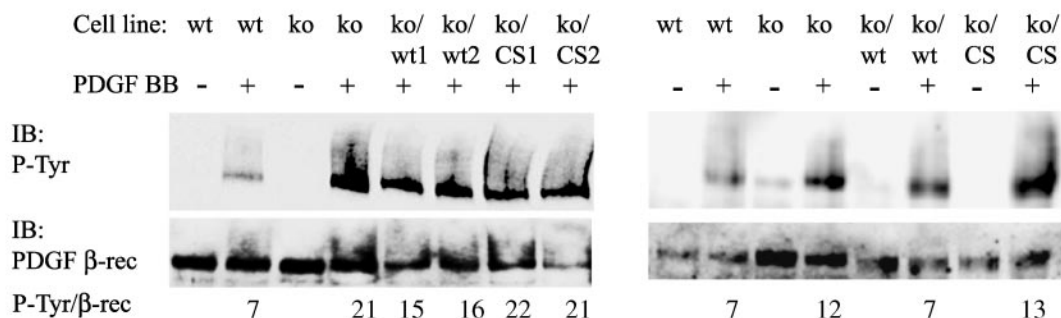
FIG. 1. Increased PDGF  $\beta$  receptor phosphorylation in TC-PTP ko E14.5 mouse embryos. E14.5 embryos were harvested from TC-PTP ko and wt mice, and cell lysates were prepared. The PDGF  $\beta$  receptor ( $\beta$ -rec) was immunoprecipitated (IP) from precleared lysates, separated by SDS-PAGE,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

**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 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 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.

**A**



**B**

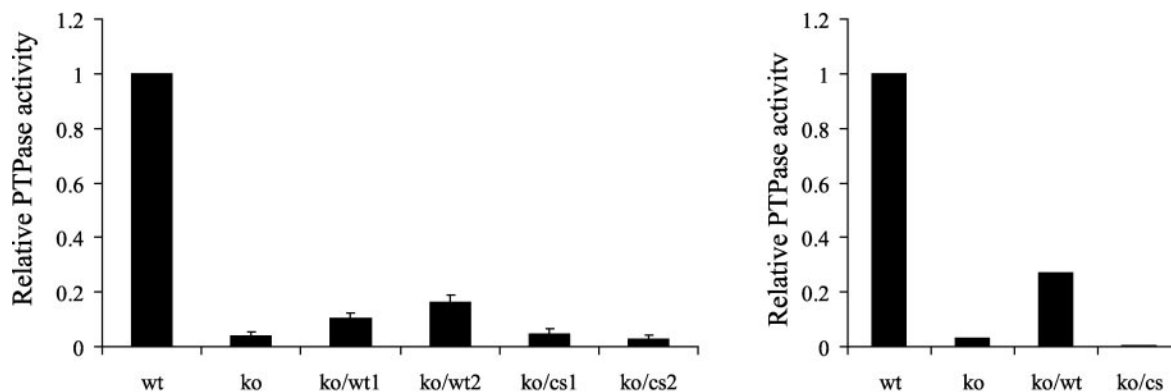


FIG. 2. Increased PDGF  $\beta$  receptor phosphorylation in TC-PTP ko cells. (A) TC-PTP wt and ko cells, together with TC-PTP ko cells stably expressing wt TC-PTP (ko/wt1 and ko/wt2) or a CS mutant form of TC-PTP (ko/CS1 and ko/CS2), were left untreated or were stimulated with 10 ng of PDGF-BB/ml and 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  $\beta$  receptor antibodies. Densitometric analysis of the immunoreactivity was performed; reactivity with the PY99 antibody/reactivity with the PDGF  $\beta$  receptor ( $\beta$ -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  $^{32}$ P-labeled peptide (amino acid sequence AEEEIpYGEFEAKKKK, where pY indicates the phosphorylated tyrosine) as a substrate. Phosphatase activity is expressed as relative amounts of  $^{32}$ P radioactivity released after 7 min. Each assay was performed in duplicate, and the data are given as mean  $\pm$  standard error of the mean from three separate experiments (left panel) or as the mean from one representative experiment (right panel).

**Generation of a panel of site-specific PDGF  $\beta$  receptor antibodies.** To study the phosphorylation of the individual tyrosine residues of the PDGF  $\beta$  receptor, we generated antibodies against phosphopeptides derived from the human PDGF  $\beta$  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  $\beta$  receptor were used. As a control for site selectivity, we used a series of PAE cell lines each expressing PDGF  $\beta$  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  $\beta$  receptor in an im-

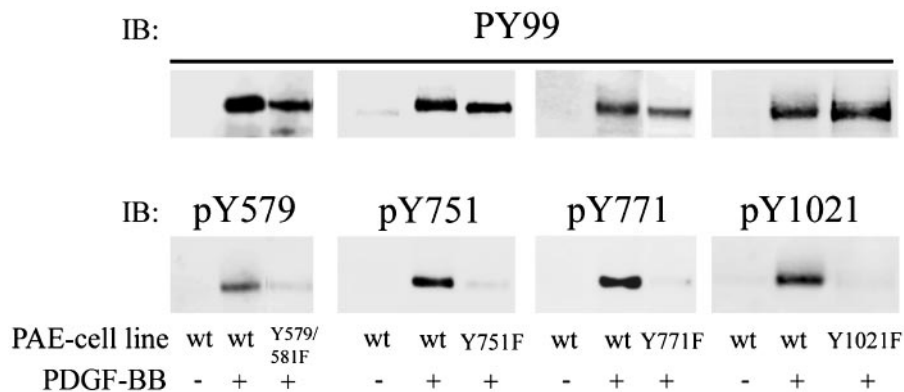


FIG. 3. Specificity of site-selective phosphotyrosine antibodies in immunoblotting. PAE cells stably expressing the wt PDGF  $\beta$  receptor or PDGF  $\beta$  receptors carrying tyrosine-to-phenylalanine point mutations at the indicated tyrosine residues were left untreated or were stimulated with 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.

munoblotting assay, whereas all of the antibodies recognized the phosphorylated wt PDGF  $\beta$  receptor in an immunoblotting assay (Fig. 3). The antibodies also recognized the phosphorylated PDGF  $\alpha$  receptor, a result which was expected because several phosphorylated tyrosines are conserved between the two PDGF receptors (data not shown). Low-affinity recognition of the PDGF  $\alpha$  receptor also was detected with the pY771 antibody, despite the fact that this site is not conserved (data not shown). Mutations of individual phosphorylation sites of the PDGF  $\beta$  receptor to which the antibodies were raised specifically abolished binding, demonstrating that the antibodies are site selective (Fig. 3).

**Deletion of TC-PTP causes a site-selective increase in PDGF  $\beta$  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  $\beta$  receptor, MEFs were stimulated with 50 ng of PDGF-DD/ml (2). Stimulation with PDGF-DD induced fivefold more PDGF  $\beta$  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  $\beta$  receptor phosphorylation sites, we used the site-specific phosphotyrosine antibodies directed against the PDGF  $\beta$  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). Also, the 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  $\beta$  receptor in an in vitro assay.** The fact that two of the investigated PDGF  $\beta$  receptor sites, Y1021 and Y771, dis-

played 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  $\beta$  receptor, we performed an in vitro phosphatase assay in which recombinant TC-PTP was allowed to dephosphorylate a WGA-immobilized PDGF  $\beta$  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  $\beta$  receptors phosphorylated in an in vitro kinase assay. The rates of dephosphorylation of the different sites were the same as those observed with PDGF  $\beta$  receptors phosphorylated by stimulation with PDGF-BB (data not shown).

**Deletion of PTP-1B but not of PTP $\epsilon$  is associated with site-selective effects on PDGF  $\beta$  receptor phosphorylation.** To investigate the effects of losses of other PTPs on the phosphorylation of selected sites in the PDGF  $\beta$  receptor, we used MEFs from PTP-1B and PTP $\epsilon$  ko mice. MEFs from PTP-1B ko mice displayed greater phosphorylation of the PDGF  $\beta$  receptor following stimulation with PDGF-DD than did MEFs reconstituted with human PTP-1B (Fig. 6A), in agreement with published results (13). In contrast, PTP $\epsilon$  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  $\beta$  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

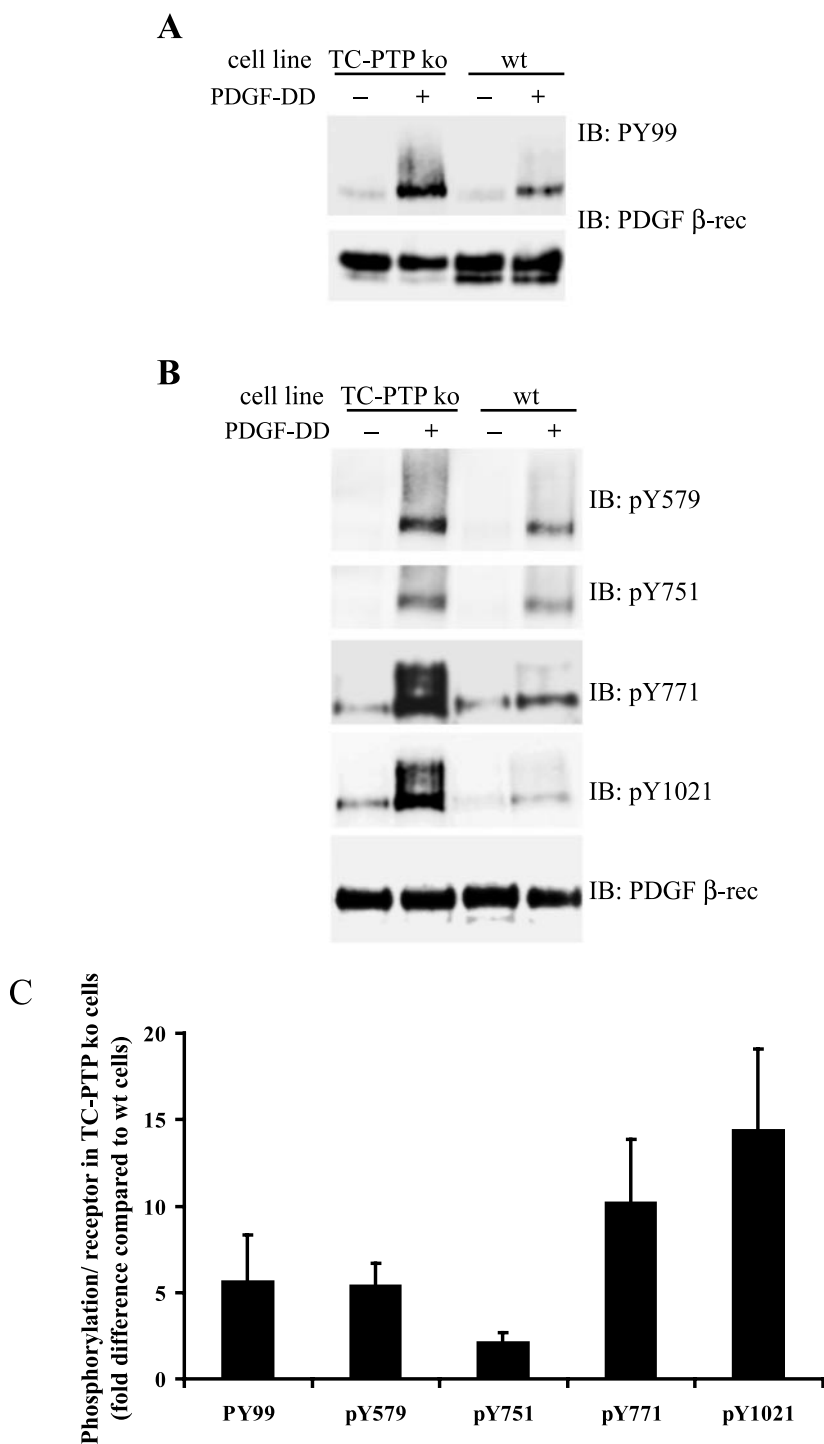


FIG. 4. Site-selective increase in tyrosine phosphorylation of the PDGF  $\beta$  receptor in TC-PTP ko cells. (A) Cells were stimulated with 50 ng of PDGF-DD/ml and lysed. Receptors were precipitated with WGA-Sepharose, and the precipitated proteins were separated by SDS-PAGE. Receptor phosphorylation was detected by immunoblotting with PY99 and PDGF  $\beta$  receptor ( $\beta$ -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  $\beta$  receptors immunoprecipitated were determined. (C) Densitometric analysis of changes in site-selective PDGF  $\beta$  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.

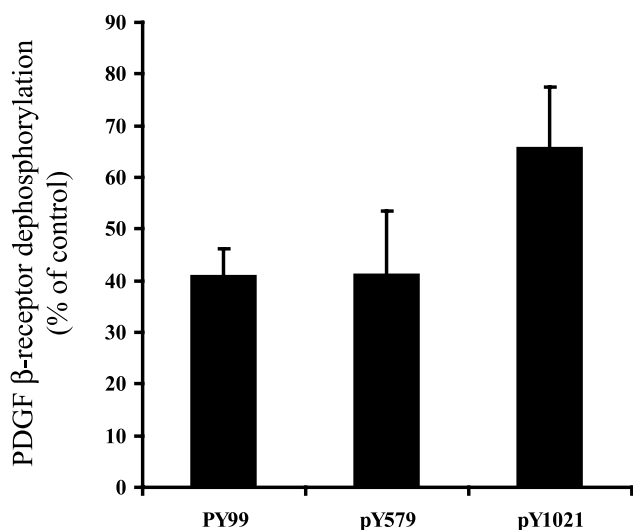


FIG. 5. Site-selective in vitro dephosphorylation of the PDGF  $\beta$  receptor by recombinant TC-PTP. Phosphorylated PDGF  $\beta$  receptors were obtained by WGA-Sepharose precipitation from stimulated PAE cells stably expressing the PDGF  $\beta$  receptor. The phosphorylated receptors were incubated 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 PTP $\epsilon$  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  $\beta$  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.

PLC $\gamma$ 1 is activated following binding to Y1021 of the PDGF  $\beta$  receptor. Stimulation of wt cells with PDGF-BB dose dependently activated PLC $\gamma$ 1, measured as the production of inositol phosphate (Fig. 7A). Consistent with the observed hyperphosphorylation of Y1021 in TC-PTP ko cells, ligand-induced PLC $\gamma$ 1 activation was increased in these cells (Fig. 7A). PLC $\gamma$ 1 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  $\beta$  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 PLC $\gamma$ 1 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 PLC $\gamma$ 1 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  $\beta$  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  $\beta$  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  $\beta$  receptor (Fig. 3 to 6). Importantly, the increase in tyrosine phosphorylation of the PDGF  $\beta$  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  $\beta$  receptor phosphorylation in cells from TC-PTP ko mice was associated with increased PDGF-induced activation of PLC $\gamma$ 1 and increased cell migration in response to PDGF (Fig. 7 and 8).



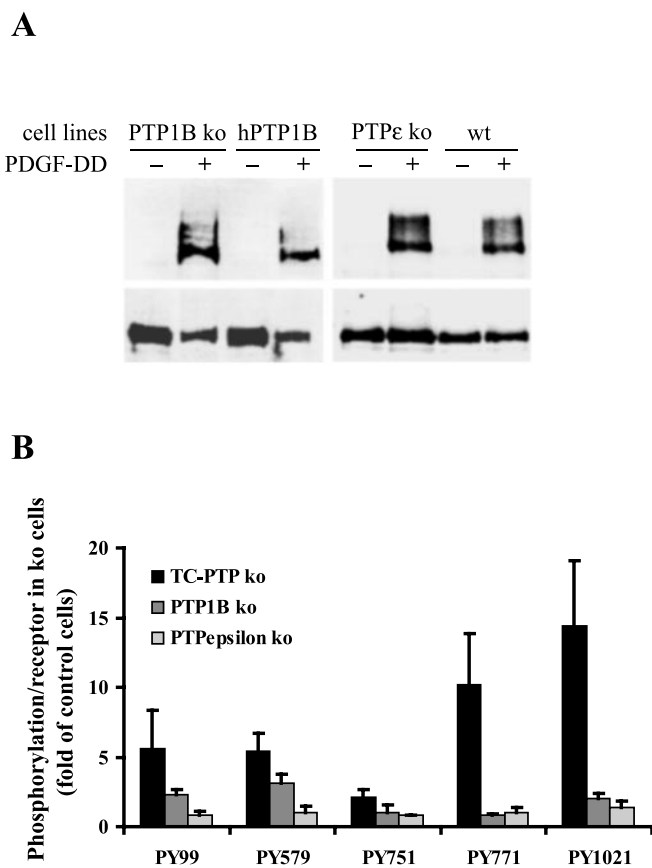


FIG. 6. Deletion of PTP-1B but not deletion of PTP $\epsilon$  induces a site-selective increase in tyrosine phosphorylation of the PDGF  $\beta$  receptor. (A) Cells were stimulated with 50 ng of PDGF-DD/ml and lysed. Receptors were precipitated with WGA-Sepharose and subjected to SDS-PAGE. Phosphorylation of the PDGF  $\beta$  receptor was detected with phosphotyrosine antibodies. (B) Densitometric analysis of changes in site-selective PDGF  $\beta$  receptor phosphorylation in TC-PTP, PTP-1B, and PTP $\epsilon$  ko MEFs compared to wt MEFs (TC-PTP and PTP $\epsilon$ ) 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.

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.

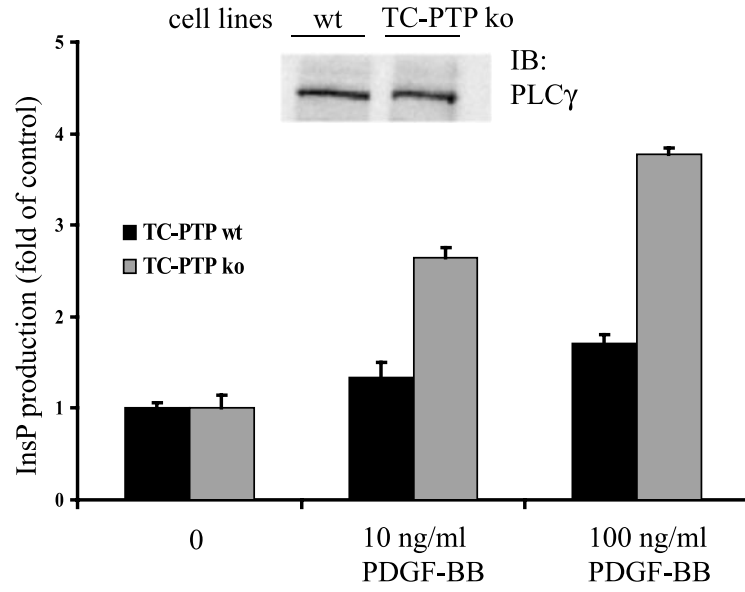
Both TC-PTP and PTP-1B were found to display site selectivity in dephosphorylating the PDGF  $\beta$  receptor and, importantly, each phosphatase regulated the phosphorylation of a distinct set of tyrosine residues (Fig. 3 and 6). The notion that different phosphatases regulate the phosphorylation status 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  $\beta$  receptor-directed phosphatases by other

receptors would provide a way for cross talk between different classes of receptors.

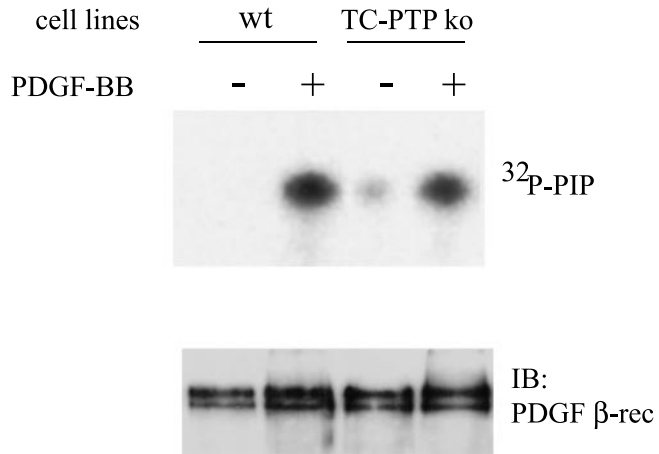
In general, the issue of site selectivity in the dephosphorylation of tyrosine kinase receptors by PTPs remains poorly explored. However, studies that have been done so far on PDGF receptor dephosphorylation support the notion that selectivity in dephosphorylation is a common feature. Deletion of the binding site for SHP-2 results in a specific increase in the phosphorylation of Y771 in PDGF  $\alpha$  and  $\beta$  receptor heterodimeric complexes (9). Also, DEP-1 dephosphorylation of the PDGF  $\beta$  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  $\beta$  receptor phosphorylation than did a loss of PTP-1B.

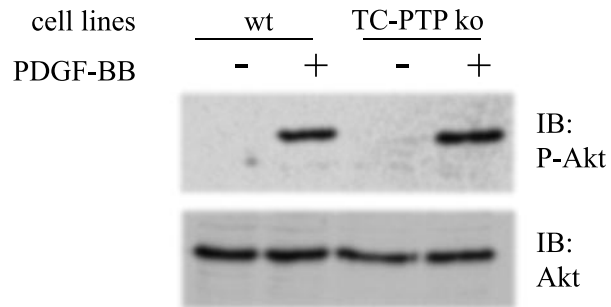
A



B



C



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  $\beta$  receptor activation in PTP-1B ko cells. In the context of discussing PTP specificity, it is also noteworthy that depletion of PTP $\epsilon$  has no effects on PDGF  $\beta$  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 PLC $\gamma$ 1 have been linked to PDGF-induced chemotaxis (15, 23, 35). It is therefore noteworthy that TC-PTP ko cells display hyperphosphorylation of Y1021, enhanced PLC $\gamma$ 1 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 dem-

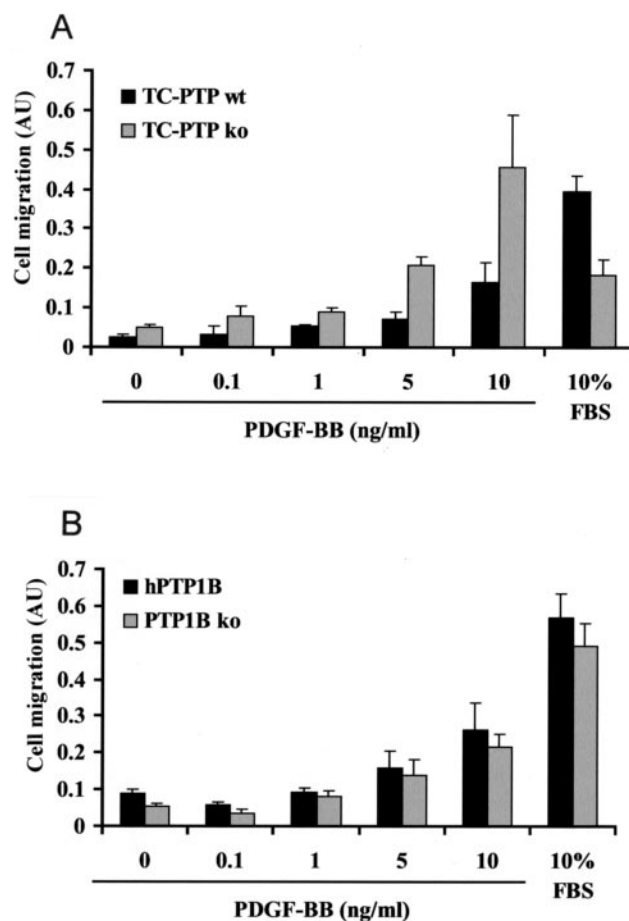


FIG. 8. Increased cell migration in response to PDGF in TC-PTP ko cells. Cell migration experiments were carried out with a 96-well ChemoTX cell migration microplate with a pore size of 3.2  $\mu$ m. The wells of the microplate were filled with the indicated stimuli, and the filters were placed on top of the wells. TC-PTP ko and wt MEFs (A) or PTP-1B ko and reconstituted MEFs (hPTP-1B) (B) were placed on top of the filters and allowed to migrate across the filters for 4 h at 37°C. Cells that migrated and that adhered to the filters were fixed, stained with crystal violet, and quantitated by using a Biomek1000. The amounts of cells that migrated are given as absorbance units (AU; mean and standard error of the mean for four experiments, each performed in quadruplicate). FBS, fetal bovine serum.

onstrated 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

FIG. 7. Effects of loss of TC-PTP on PDGF-induced signaling pathways. (A) MEFs from TC-PTP wt or ko mice were labeled with *myo*-[ $^3$ H]inositol for 24 h and then stimulated with the indicated amounts of PDGF-BB in the presence of LiCl at 37°C for 30 min. PLC $\gamma$ 1 activity was monitored by measuring the release of inositol phosphate (InsP). Data are given as the mean and standard error of the mean ( $n = 3$  or 4). The inset shows immunoblotting (IB) analysis of PLC $\gamma$ 1 expression in total cell lysates from TC-PTP ko and wt MEFs. (B) Cells were stimulated with 50 ng of PDGF-BB/ml at room temperature for 10 min. PDGF  $\beta$  receptors ( $\beta$ -rec) were immunoprecipitated, and coprecipitating PI 3-kinase activity was measured by incubating beads with 0.2  $\mu$ g of presonicated phosphatidylinositol (PIP)/ $\mu$ l in the presence of [ $\gamma$ - $^{32}$ P]ATP. The kinase reaction was performed at 37°C for 30 min, and phosphatidylinositol was extracted and subjected to thin-layer chromatography. Phosphatidylinositol phosphorylation was detected with a PhosphorImager. The amounts of PDGF  $\beta$  receptors in the immunoprecipitates were assessed by immunoblotting. (C) Cells were left untreated or were stimulated with 10 ng of PDGF-BB/ml for 7 min and lysed. Activation of the Akt kinase was assessed by immunoblotting with antisera to Akt phosphorylated at Thr308 (P-Akt) in total cell lysates.

(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  $\beta$  receptor signaling. Further studies should be aimed at elucidating the physiological relevance of this regulatory mechanism.

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