The non-receptor protein-tyrosine phosphatases (PTPs) 1B and T-cell phosphatase (TCPTP) have been implicated as negative regulators of multiple signaling pathways including receptor-tyrosine kinases. We have identified PTP1B and TCPTP as negative regulators of the hepatocyte growth factor receptor, the Met receptor-tyrosine kinase. In vivo, loss of PTP1B or TCPTP enhances hepatocyte growth factor-mediated phosphorylation of Met. Using substrate trapping mutants of PTP1B or TCPTP, we have demonstrated that both phosphatases interact with Met and that these interactions require phosphorylation of twin tyrosines (Tyr-1234/1235) in the activation loop of the Met kinase domain. Using confocal microscopy, we show that trapping mutants of both PTP1B and the endoplasmic reticulum-targeted TCPTP isoform, TC48, colocalize with Met and that activation of Met enables the nuclear-localized isoform of TCPTP, TC45, to exit the nucleus. Using small interfering RNA against PTP1B and TCPTP, we demonstrate that phosphorylation of Tyr-1234/1235 in the activation loop of the Met receptor is elevated in the absence of either PTP1B or TCPTP and further elevated upon loss of both phosphatases. This enhanced phosphorylation of Met corresponds to enhanced biological activity and cellular invasion. Our data demonstrate that PTP1B and TCPTP play distinct and non-redundant roles in the regulation of the Met receptor-tyrosine kinase.

The receptor for hepatocyte growth factor/scatter factor, Met, controls a program of epithelial growth and remodeling through the coordination of cell proliferation and survival, cell migration, and epithelial morphogenesis (1). This process is important both during embryogenesis and for organ regeneration in the adult (2–4). In response to HGF binding, the catalytic kinase activity of the Met receptor is elevated, leading to intermolecular autophosphorylation and recruitment of signaling molecules. These, through a series of protein-protein interactions, transduce the extracellular signal to the interior of the cell. Although numerous studies have addressed mechanisms resulting in elevation of Met RTK activity (5), little is known about the mechanisms involved in down-regulation of the Met receptor.

Biological consequences resulting from RTK activation are determined by the duration, intensity, and specificity of the signals activated downstream of the receptor. Regulation of signals occurs on multiple levels, including the RTK itself. Ligand activation of the Met receptor promotes internalization of the receptor into the endocytic pathway with subsequent degradation in the lysosome (6). Termination of RTK signaling has been correlated with receptor dephosphorylation, degradation, or sequestration from the cytoplasm (5). In addition to receptor internalization, trafficking, and degradation in the lysosome as mechanisms of down-regulation, the Met receptor has been identified as a substrate for several PTPs. The receptor PTP LAR (leukocyte antigen-related) targets the Met receptor in confluent cells. This interaction is initiated after cell-cell contact and suppresses the mitogenic response of primary rat hepatocytes, resulting in contact inhibition (7). Another receptor PTP, DEP-1, has been proposed to negatively regulate Met RTK signaling via dephosphorylation of the carboxyl-terminal tyrosine residues in Met that are involved in substrate binding (8). Although DEP-1 suppresses Met signaling, it does not dephosphorylate the tyrosine residues in the activation loop (Tyr-1234/1235) that are required for Met catalytic activity. Because Met continues to signal after internalization (9), other PTPs are likely to be involved in the suppression and/or down-regulation of Met receptor catalytic activity.

5 The abbreviations used are: HGF, hepatocyte growth factor; RTK, receptor-tyrosine kinase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; KO, knock out; WT, wild type; GFP, green fluorescent protein; JNK, c-Jun NH2-terminal kinase; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; PTP, protein-tyrosine phosphatase; TCPTP, T-cell phosphatase; siRNA, small interfering RNA; ER, endoplasmic reticulum; IR, insulin receptor; PFA, paraformaldehyde; WCL, whole cell lysates; IP, immunoprecipitate.
We have recently established that phosphorylation of Met is elevated in PTP1B-null mice after treatment with Fas ligand, suggesting that the Met receptor may be a target for this phosphatase (10). PTP1B is a non-receptor tyrosine phosphatase that has been shown to either genetically or biochemically interact with multiple RTKs, including the insulin, insulin-like growth factor, EGF, and PDGFβ receptors (11). PTP1B possesses a phosphatase catalytic domain and a proline-rich domain. It interacts with and dephosphorylates multiple proteins including p130Cas, p62Dok, Jak2, and Tyk2 (12–17). At its carboxyl terminus, it contains an endoplasmic reticulum (ER)-targeting signal that anchors PTP1B to the cytoplasmic face of the ER (18, 19). The role of PTP1B in the down-regulation of the insulin receptor (IR) has been extensively studied. Liver and muscle from PTP1B knock-out (KO) mice show hyperphosphorylation of the IR upon stimulation with insulin. These mice are resistant to weight gain caused by a high fat diet, demonstrating that loss of PTP1B has physiological consequences in vivo (20, 21).

Recent studies show that internalization of the EGF and PDGF receptors is required for their colocalization with PTP1B (22), supporting a role for PTP1B in the dephosphorylation and down-regulation of internalized RTKs. The mechanism by which PTP1B interacts with its substrates has been elucidated. Biochemical studies have demonstrated that the affinity of PTP1B for a substrate containing twin tyrosines is 70-fold higher than for a substrate in which one tyrosine is substituted with phenylalanine (23). Moreover, using peptides corresponding to the activation loop of the kinase domain of the IR, the catalytic domain of PTP1B has been shown to associate with the phosphorylated twin tyrosines present in this sequence (23). Additionally, recent studies have shown that PTP1B docks on the kinase domain of the IR in a region opposite to the activation loop that contains these tyrosine residues (24).

A given RTK can be the substrate of several phosphatases, emphasizing the importance of this mechanism. For example, the EGF receptor is thought to be negatively down-regulated by PTP1B, LAR, SHP-1, PTP1B, and TCPTP (25–28), and the IR has been shown to associate with and be dephosphorylated by PTP1B (20, 21, 29), TCPTP (30, 31), and PTPζ (32), among others.

PTP1B is most closely related to the non-receptor TCPTP, which is expressed in all tissues and at all stages of development (33). TCPTP is alternatively spliced, yielding two isoforms that differ in their non-catalytic carboxyl termini: the ER-localized 48 kDa form (TC48) and the nuclear-localized 45 kDa form (TC45) (34). Both TC48 and TC45 are present in humans (35), but only TC45 has been identified in mouse (36). TCPTP can regulate signaling pathways by dephosphorylating its substrates, which include the EGF (37), PDGF-β (38, 39) and insulin receptors (30, 31, 40), JAK1 and JAK3 (41), p52Shc (37), STAT1 (42), and Src (43). Although PTP1B and TCPTP are closely related and are structurally similar (44), they possess differential specificities for recognition of substrates (30, 43). Moreover, siRNA against TCPTP or PTP1B in vivo has demonstrated that PTP1B and TCPTP do not act additively on the IR in mouse liver (45).

The interaction of a wild-type (WT) phosphatase with its substrate is transient. Substrate trapping mutants of PTPs have been engineered in which a single aspartic acid residue in the catalytic domain is replaced with alanine (D/A mutants) (27). These proteins are capable of binding to PTP substrates but are slow to catalyze the dephosphorylation reaction and, thus, form a stable intermediate with the substrate (46). Hence, such mutants compete when co-expressed with WT PTPs. In this study we demonstrate a role for PTP1B and TCPTP in the dephosphorylation of
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The Met RTK and show that substrate trapping mutants of either PTP1B or TCPTP (TC48) are recruited to an internalized Met receptor. Moreover, Met activation promotes nuclear exit of TCPTP (TC45) and recruitment to the membrane, indicating a role for each phosphatase in the dephosphorylation and modulation of the signal from the Met receptor.

EXPERIMENTAL PROCEDURES

DNA Constructs and siRNA—GFP-TC48 or GFP-TC45 WT and D/A constructs were created by cloning TC45 or TC48 WT or D/A into the BglII and HindIII sites of the pEGFPc2 vector (Clontech, Mountain View, CA). PTP1B WT and D/A was inserted into the BglII and EcoRI sites of the pEGFPc2 vector. TC48 was a kind gift from N. Tonks. Tpr-Met and its various mutants, Met and Met-Y1003F, have been described previously (9, 47). siRNA duplexes against the PTP1B target sequence CACGAGGTATTTTAATGAA and TCPTP target sequence CACAAAGAAGTTACATCTTAA were obtained from Qiagen (Mississauga, ON, Canada) and transfected into HeLa cells for 48 h using Hiperfect (Qiagen) following the manufacturer’s instructions.

Cell Culture and DNA Transfections—All cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics. For expression of cDNAs, 1 × 10⁶ HEK 293 cells were seeded 24 h before transient transfection using Lipofectamine Plus (Invitrogen), carried out following the manufacturer’s instructions. Media was replaced 3 h post-transfection, and cells were lysed 24 h post-transfection in lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 10% glycerol) lacking sodium vanadate unless otherwise indicated in the figure legends. 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride were added to the lysis buffer before use.

Antibodies and Reagents—A rabbit polyclonal antibody raised against a peptide from the carboxyl terminus of human (48) or mouse (49) Met was used for anti-Met immunoprecipitations and Western blots, and anti-Met p-Tyr-1234/35, p-Tyr-1003, anti-pJNK, and JNK were purchased from Cell Signaling Technologies (Pickering, ON, Canada). Anti-Met B2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-p-Tyr-20 was from Berkeley Antibody Co., Inc./Bio-Can (Mississauga, ON, Canada), and anti-p-Tyr-1234/35 from R&D Systems (Minneapolis, MN). Mouse anti-Met antibody was purchased from R&D Systems (Minneapolis, MN). The Met receptor was immunoprecipitated from 3 mg of protein lysate using the mouse-specific anti-Met antibody (49). Blots were probed with antibodies as indicated in figure legends and quantified using the NIH ImageJ software. Each experiment was carried out a minimum of three times.

Confocal Immunofluorescence Microscopy—2 × 10⁴ HeLa cells or 1 × 10⁶ T47D cells were seeded on glass coverslips (Bellco Glass Inc. Vineland, NJ) in 24-well plates (Nalgene NUNC, Rochester, NY) and transfected with the indicated DNA using Lipofectamine Plus (Invitrogen) following the man-
ufacturer’s instructions 16 h post-plating. 16 h later, cells were serum-starved for 2 h either with or without cycloheximide (0.1 mg/ml) before HGF treatment (1.5 nM) as indicated. Coverslips were washed once with PBS and then fixed in 2% paraformaldehyde (PFA; Fisher) in PBS for 20 min. Coverslips were then washed 4 times in PBS, and residual PFA was removed by three 5-min washes with 100 mM glycine/PBS. Cells were permeabilized with 0.3% Triton X-100/PBS and blocked for 30 min with blocking buffer (5% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween 20, PBS). Coverslips were incubated with primary and secondary antibodies diluted in blocking buffer for 1 h and 40 min, respectively, at room temperature. Coverslips were washed four times in IF buffer (0.5% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween 20, PBS) between primary and secondary antibodies. Coverslips were mounted with Immumount (Thermo-Shandon, Pittsburgh, PA). Confocal images were taken using a Zeiss 510 Meta laser scanning confocal microscope (Carl Zeiss Canada Ltd, Toronto, ON, Canada) with a 100× objective. Image analysis was carried out using the LSM 5 image browser (Empix Imaging, Mississauga, ON, Canada).

Invasion Assays—3 × 10⁴ HeLa cells were seeded directly onto 6.5-mm Corning Costar transwells coated with 100 μg/cm² Matrigel (BD Biosciences). Complete media was added to both the top and bottom wells, and cells were incubated at 37 °C overnight. For HGF stimulations, 0.15 nM HGF was added to the bottom wells. After the overnight incubation, cells on both sides of the transwells were fixed using formalin phosphate for 20 min at room temperature. After washing with PBS, cells were stained with 0.1% crystal violet in 20% methanol for 20 min at room temperature. Cells on the top layer were scraped, and membranes were left to dry overnight. Images were captured using a Retiga 1300 digital camera (QIMAGING, Burnaby, BC, Canada) and a Zeiss AxioVert 135 microscope (Carl Zeiss Canada Ltd.). Imaging of these assays was carried out with a 10× objective, and images were analyzed using the ImageScope software (Aperio Technologies, Vista, CA). Each experiment was carried out a minimum of three times.

RESULTS

Met RTK Is a Substrate for PTP1B and TCPTP

Loss of PTP1B Results in Increased Levels of Met Phosphorylation—To test if Met is a physiological substrate for PTP1B, PTP1B-null mice were injected via the hepatic portal vein with 15 nM HGF, the ligand for the Met receptor. Livers were harvested at the times indicated, and proteins from lysates were subjected to immunoprecipitation using an antibody specific to murine Met protein. Increase in phosphorylation of Met was normalized to the levels of Met receptor as evaluated by densitometry (Fig. 1). Notably, we observe no significant differ-
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Enhance in basal tyrosine phosphorylation of the Met receptor in livers from WT or PTP1B-null mice (Fig. 1A). However, in response to HGF, tyrosine phosphorylation of the Met receptor was enhanced significantly in PTP1B-null animals when compared with livers from WT animals (Fig. 1, A and B). Using general anti-phosphotyrosine antibodies (p-Tyr-100), we observe that the overall tyrosine phosphorylation of Met in PTP1B-null animals is 4-fold higher than Met phosphorylation in WT animals when stimulated with HGF for similar time periods (Fig. 1, A and B).

To provide insight into the specificity of enhanced Met phosphorylation in PTP1B-null animals, we utilized Met tyrosine phosphorylation specific antibodies. Upon binding HGF, Met is autophosphorylated on tyrosine 1234 and 1235 in the activation loop of the Met catalytic domain (47). In response to HGF, phosphorylation of these tyrosines is required for activation of Met kinase and subsequent phosphorylation of other sites in the Met receptor, including Tyr-1003 in the juxtamembrane domain and Tyr-1003 and -1356 in the Met carboxyl terminus (47, 50–52). Utilizing phosphotyrosine-specific antibodies to compare phosphorylation of Tyr-1234/1235, Tyr-1003 or Tyr-1365 of Met in response to HGF in livers from WT and PTP1B-null mice, we observed an overall increase in phosphorylation of these tyrosines of 4–6-fold when compared with lysates of livers from WT mice (Fig. 1, A and B). In response to HGF, phosphorylation of tyrosines 1234/35 is elevated in PTP1B-null mice when compared with WT mice, demonstrating for the first time in vivo that the phosphorylation state of Tyr-1234/1235, required for the catalytic activity of the Met receptor, is regulated by PTP1B (Fig. 1, A and B). As expected, enhanced phosphorylation of Tyr-1234/1235 coincides with enhanced phosphorylation of non-catalytic tyrosines, Tyr-1003 in the juxtamembrane domain and Tyr-1365 in the carboxyl terminus (Fig. 1, A and B). To examine whether loss of PTP1B also impacts on downstream signaling pathways, the engagement of the JNK and ERK pathways were studied using phosphospecific antibodies. Consistent with enhanced phosphorylation of Met, increased phosphorylation of the Met-activated pathway components ERK1/2 in HGF-injected livers from KO versus WT mice was observed (Fig. 1C). Interestingly, we saw no induction of p46 Jnk1, whereas p54 Jnk2 was elevated in PTP1B KO livers after HGF portal vein injection when compared with WT (Fig. 1, C and D), consistent with an increase in Jnk2/3 activation post-HGF stimulation in rat hepatoma cells (53). These results are consistent with the Met receptor being a physiological substrate for PTP1B in vivo, which regulates its catalytic activity and subsequent downstream signaling.

**Dephosphorylation of Met in Response to HGF**—Previous data from our laboratory and others has shown that down-regulation of Met can take place via internalization followed by degradation of the receptor (6, 9). To study the role of dephosphorylation in down-regulation of the Met receptor, the endogenous Met receptor in HeLa cells was stimulated with HGF at 4 °C for 60 min, and activation of the receptor as visualized through anti-phosphotyrosine Western blotting was followed during a chase in medium lacking HGF at 37 °C. Under these conditions, loss of tyrosine phosphorylation (t_b) of the receptor was detectable as early as 15 min post-chase, whereas Met itself was stable (Fig. 2, A and B). These data demonstrate that under these conditions Met is significantly dephosphorylated before degradation.

**Knockdown of TCPTP or PTP1B Results in Increased Phosphorylation of the Met RTK**—To establish if PTP1B and its closely related enzyme TCPTP dephosphorylate the Met RTK, Met phosphorylation was examined in HeLa cells transfected with scrambled or siRNA designed to target either PTP1B, TCPTP, or both TCPTP and PTP1B (Fig. 3). Upon stimulation with HGF, Met tyrosine phosphorylation was elevated in cells in which the levels of either PTP1B or TCPTP were knocked down by specific siRNA but not in cells transfected with scrambled siRNA (Fig. 3A). Met tyrosine phosphorylation increased up to 2-fold in the presence of siRNA for either PTP1B or TCPTP alone (Fig. 3B). Notably, when both PTP1B and TCPTP are targeted together, up to 3-fold higher tyrosine phosphorylation of Met was observed, suggesting an additive role for these phosphatases in the regulation of tyrosine phosphorylation of Met (Fig. 3, A and B).
Interaction of Met with PTP1B or TCPTP requires tyrosines Tyr-1234 and Tyr-1235 of the Met receptor. A, protein lysates from HEK 293 cells co-expressing a combination of either GFP-tagged trapping mutants of PTP1B or TC48 or Myc-tagged TC45 and 1234/1235 twin tyrosine substitutions of Tpr-Met were subjected to immunoprecipitation using an anti-GFP or anti-Myc antibody as indicated, and immunoblot analysis was performed on both the immunoprecipitates and the corresponding WCL with antibodies as indicated. B, immunoprecipitation was performed with anti-GFP antibody, and blots were probed with antibodies specific for p-Tyr-100, Met, Met p-Tyr-1349, and GFP in lysates of cells expressing GFP-tagged PTP1B, TC45 or TC48, and Met mutants as indicated. Met receptor was immunoprecipitated, and immunoblot analysis was performed with antibodies raised against p-Tyr-100, Met, or Met p-Tyr-1349. WCL were probed with an antibody against GFP to examine levels of phosphatase present in each lane. C, immunoprecipitations were performed with anti-GFP and anti-Met antibodies in lysates of cells expressing GFP-tagged phosphatases and Met mutants where indicated, and immunoblot analysis was performed with antibodies against Met, Met p-Tyr-1234/1235, Met p-Tyr-1003, and GFP. D, densitometric analysis of amount of Met trapped by PTP1B, TC45, or TC48. Analysis was performed using NIH ImageJ. Results shown are the average of three independent experiments. The error bars D represent S.E. Immunoblot analysis of WCL with actin was used as a loading control.

Knockdown of TCPTP or PTP1B Results in Enhanced HGF-dependent Cell Invasion—To study the physiological relevance of the Met-PTP1B/TCPTP interaction, HeLa cells transfected with scrambled or siRNA designed to target PTP1B, TCPTP, or both TCPTP and PTP1B were trypsinized and seeded on Matrigel in the presence or absence of HGF. The HGF-dependent invasion of HeLa cells treated with siRNA to PTP1B or TCPTP was 1.3 and 1.6-fold higher, respectively, than that of cells treated with scrambled siRNA alone (Fig. 4). These data represent an average of four independent experiments. Moreover, knockdown of both phosphatases results in enhanced invasion (1.8-fold) supporting a role for TCPTP and PTP1B in Met dephosphorylation and negative regulation of Met biological activity.

TCPTP or PTP1B (D/A) Substrate Trapping Mutants Interact with the Met RTK—PTPs contain a conserved aspartate that is required as a proton acceptor in the phosphate hydrolysis reaction. Substitution of this residue with alanine (D/A) results in a PTP that can form complexes with tyrosine-phosphorylated proteins in a cellular context (27). These mutants, termed trapping mutants, have provided a powerful mechanism to identify putative substrates for tyrosine phosphatases (54, 55). To establish if PTP1B or TCPTP can interact with the Met RTK as a cellular substrate and if they interact with similar tyrosines in the Met RTK, GFP fusion proteins of PTP1B, TC45 or TC48 WT or D/A (trapping mutants) were co-expressed with a constitutively active variant of the Met RTK (Tpr-Met) (56, 57). The trapping mutants, but not WT PTP1B or either isoform of WT TCPTP, were able to associate with Tpr-Met as evident from their coimmunoprecipitation (Fig. 5A). To establish if association of either phosphatase with Met is dependent on the ability of the catalytic domain of TC45, TC48, or PTP1B to interact with Met, the ability of Met to be trapped by these phosphatases was tested in the presence of sodium orthovanadate, a potent nonspecific phosphatase inhibitor that binds to the phosphatase catalytic domain (58). The addition of sodium orthovanadate abrogated the ability of TC45, TC48, and PTP1B (D/A) to coimmunoprecipitate with Tpr-Met, indicating that this interaction is specific to the ability of the phosphatase to bind to its substrate via the phosphatase catalytic domain (Fig. 5B).

Interaction of PTP1B and TCPTP with Met Requires Activation Loop Tyrosines—Crystallographic analysis of PTP1B in complex with a peptide corresponding to the tandem tyrosines present in the activation loop of the IR revealed that PTP1B engages both of these tyrosines, one at the PTP1B catalytic pocket and the second at an adjacent p-Tyr binding pocket (23). The second site is conserved in TCPTP (44). Similarly, analysis of the crystal structure of the catalytic domain of TCPTP shows a high degree of structural similarity to PTP1B (44). Both TCPTP and PTP1B recognize the twin tyrosines of the IR in vivo (30, 31); hence, they may interact with analogous tandem tyrosines (located at Tyr-1234 and Tyr-1235) in the Met receptor. To test this, the ability of Tpr-Met or Tpr-Met carrying phenylalanine substitutions of Tyr-1234, Tyr-1235, or Tyr-1234/1235 in the activation loop to coimmunoprecipitate with TC45, TC48, or PTP1B (D/A) mutants was examined after transient co-transfections. Coimmunoprecipitation of tyrosine-phosphorylated Met was monitored with antibodies spe-
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Our data establish that tyrosines within the activation loop of Met are required for interaction with PTP1B. However, because substitution of these residues decreases the kinase activity of the receptor (47), other tyrosine residues will exhibit decreased phosphorylation. To determine whether tyrosine residues outside the kinase domain and within the carboxyl terminus of Met, previously shown to play important roles in Met signaling, are targets for PTP1B or TCPTP, we performed further coimmunoprecipitation experiments. When PTP1B D/A or TCPTP D/A mutants were coexpressed with Tpr-Met mutants Y1349F, Y1356F, or Y1349/56F, we observed no significant decrease in the ability of PTP1B D/A or TCPTP D/A to coimmunoprecipitate with the Met mutants (Fig. 6B). Previous studies have suggested that Met-associated phosphatase activity requires Tyr-1003, a residue located in the juxtamembrane domain that is absent in Tpr-Met (59, 60). To determine whether TCPTP or PTP1B interacts with the full-length Met RTK, the ability of WT Met or the mutant Met-Y1003F to coimmunoprecipitate with the D/A mutants of each PTP was examined after transient transfection. Tyrosine phosphorylation of Met was monitored utilizing antibodies specific to Met Tyr-1234/1235 and Met Tyr-1003. Both WT Met and Met-Y1003F coimmunoprecipitated with D/A mutants of PTP1B and TCPTP (Fig. 6C), although the interaction of Met-Y1003F was decreased when compared with WT Met with TCPTP (Fig. 6, C and D). The small decrease in trapping of Y1003F by TCPTP suggests that Tyr-1003 may be involved in the interaction of TCPTP with Met and is consistent with previous studies demonstrating a decrease in phosphatase recruitment by this mutant (59). Taken together, our results demonstrate that tyrosines 1234 and 1235 of Met are essential for the interaction of Met with TCPTP and PTP1B, whereas Tyr-1003 may play an accessory role.

PTP1B D/A Trapping Mutant Colocalizes with an Activated Met Receptor—The ability of PTP1B to act as a phosphatase for the Met receptor in vivo in PTP1B KO mice (Fig. 1) and in HeLa cells (Fig. 3) and that of PTP1B D/A trapping mutants to coimmunoprecipitate with Met (Figs. 5 and 6) prompted us to examine the subcellular location at which this interaction occurs. PTP1B possesses an ER-targeting signal and localizes to the ER (22, 61). A role for PTP1B has been proposed in the dephosphorylation of RTKs after stimulation (18) as well as from the synthetic pathway (62). To examine the colocalization of Met and PTP1B in the absence of stimulation, HeLa cells were transfected with GFP-tagged WT or the substrate trapping mutant of PTP1B (D/A), and their localization was analyzed by confocal microscopy. In the absence of HGF, we observed localization of PTP1B D/A trapping mutants to coimmunoprecipitate with Met and is consistent with previous studies demonstrating a decrease in phosphatase recruitment by this mutant (59).

FIGURE 7. Localization of Met with PTP1B D/A. A, HeLa cells were seeded on coverslips and transfected with GFP-PTP1B D/A. 8, 16 h post-transfection, cells were treated with cycloheximide (0.1 mg/ml) for 2 h and stimulated with 1.5 nM HGF at 37 °C for the indicated times. A and B, coverslips were fixed in 2% PFA and stained with Met AF276 and EEA1 antibodies (B only). Confocal images were taken with a 100× objective and 1× zoom. Yellow staining represents colocalization between endogenous Met (red) and TC48 (green) or EEA1 (green or purple, as shown). The scale bar is as indicated in the figure.
overexpressed, is active in the absence of its ligand (48, 65). In Met or its kinase dead variant (K1110A). Met, when transiently transfected with the TC45 D/A trapping mutant were pretreated with cycloheximide to deplete the synthetic pool of Met receptor. As observed for PTP1B D/A in HeLa cells, in the absence of cycloheximide (Fig. 7A), TC48 D/A localizes with Met in a perinuclear compartment (Fig. 9A). However, in the presence of cycloheximide where Met is no longer present in the synthetic pathway, this colocalization is abrogated, and instead, under these conditions, TC48 shows colocalization with Met only after stimulation with HGF (Fig. 9B and supplemental Fig. 1). The GFP vector- and GFP TC48 WT-expressing cells do not show any colocalization with Met (Fig. 9B and supplemental Fig. 1).

**DISCUSSION**

A common theme in the regulation of RTKs via dephosphorylation is the involvement of multiple phosphatases, which specifically target different residues phosphorylated during RTK activation (66). Although several PTPs have been associated with Met dephosphorylation, to date none has been shown to act as a negative regulator of the major autophosphorylation sites of the Met receptor catalytic domain (Tyr-1234/1235). Here we show that two closely related enzymes of the PTP family, PTP1B and TCPTP, target the Met RTK. Our results demonstrate that Met is a physiological target for these enzymes and that they engage with the twin tyrosine residues (1234/35) within the activation loop of Met. Both PTP1B and TCPTP colocalize with endogenous Met after ligand-induced Met activation (Figs. 7 and 9). In addition, targeted loss of PTP1B or TCPTP leads to increased HGF-dependent phosphorylation of Met and increased cell invasion in response to HGF (Figs. 1 and 3).

We demonstrate enhanced Met phosphorylation in PTP1B-null animals and elevated downstream signaling in response to HGF (Fig. 1). This is similar to what was observed for the IR, where livers from PTP1B-null mice exhibited higher IR phosphorylation than livers from PTP1B-WT mice in response to insulin (20). However, unlike the IR, there was no difference in basal phosphorylation of the Met receptor in the absence of PTP1B (Fig. 1). These data provide additional evidence that PTP1B is not the only phosphatase negatively regulating the Met receptor. Supporting this hypothesis, we have shown that knockdown of either TCPTP or PTP1B leads to higher tyrosine phosphorylation of the activated Met receptor and that knockdown of both phosphatases results in an additive increase in Met tyrosine phosphorylation in response to stimulation (Fig. 3). In agreement with the observed increase in Met tyrosine phosphorylation, pathways downstream of Met, i.e. pERK and
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![Image](http://example.com/image.png)

**FIGURE 9. Localization of Met with TC48 D/A.** A. HeLa cells were plated on coverslips and transfected with GFP-TC48 D/A. Yellow staining represents colocalization between endogenous Met (red) and TC48 (green). B. 16 h post-transfection cells were treated with cycloheximide (0.1 mg/ml) for 2 h and stimulated with 1.5 mM HGF at 37 °C for the indicated times. A and B, coverslips were fixed in 2% PFA and stained with Met AF276 and EEA1 antibody (B only). Confocal images were taken with a 100× objective and 1× zoom. Yellow staining represents colocalization between endogenous Met (red) and TC48 (green) or EEA1 (green or purple). The Scale bar is indicated in the figure.

pJNK2/3, are more highly phosphorylated in livers from PTP1B-null mice in response to HGF (Fig. 1). This is consistent with the observed elevation of pERK downstream from insulin, EGF, and PDGF RTKs (30, 67) in PTP1B-null MEFs. However, because no increase was observed in basal phosphotyrosine levels of Met, our data indicate that other PTPs may be involved. PTPs selectively recognize specific tyrosines on their target proteins. Here we show that neither TCPTP (D/A) nor PTP1B (D/A) mutants is able to trap a Met mutant protein in which the twin tyrosines located within the activation loop of the kinase domain (Tyr-1234/1235) are substituted with phenylalanine residues (Fig. 5). Phosphorylation of Tyr-1234/1235 is required for full catalytic activity of Met for phosphorylation of tyrosines outside the catalytic domain (47). Our data, therefore, demonstrates that both TCPTP and PTP1B can interact with the activated Met receptor. Hence, the mechanism through which TCPTP and PTP1B bind to Met may be similar to the interaction of PTP1B with the IR, where PTP1B interacts with the dual tyrosines in the activation loop of the receptor (23, 30, 31, 37, 63). In support of this, tyrosine to phenylalanine substitution mutants outside the kinase domain (Tyr-1003/1349/1356) retained the ability to bind trapping mutants of either PTP1B or TCPTP (Fig. 5, B–D). This is in contrast to DEP-1 receptor PTP, which specifically dephosphorylates Met Tyr-1349 (8). However, the ability of TCPTP but not PTP1B to trap Met was abrogated by ~40% when Met Tyr-1003 was substituted with phenylalanine (Fig. 5, C and D), supporting previous reports of a role for Tyr-1003 in interacting with TCPTP (59).

Down-modulation of RTKs via degradation is a widely accepted mechanism by which a receptor signal is regulated. The Met RTK continues to signal upon internalization, demonstrating that the receptor is active within the trafficking compartment, and signal termination has been associated with Met degradation (9). However, we provide evidence supporting dephosphorylation of Met before degradation (Fig. 2), demonstrating that dephosphorylation of Met may modulate Met signaling in the cytoplasm. In support of this, after knockdown of either PTP1B, TCPTP, or both, there is a statistically significant increase in the ability of cells to invade in response to HGF (Fig. 4). Similarly, knockdown of PTP1B resulted in increased Met activation in the presence of the tyrosine phosphatase inhibitor, bpV(phen) (68). In addition, treatment with bpV(phen) delayed wound healing in rabbit corneas (68).

Previous studies have shown that interaction of EGF receptor and PDGF receptor with PTP1B requires internalization of the receptor (22). In agreement with this, we did not observe any colocalization between Met and either PTP1B or TCPTP in the absence of Met stimulation or when cycloheximide was used to block de novo protein synthesis, but we saw colocalization after HGF stimulation (Figs. 7 and 9). TC45, the nuclear isoform of TCPTP, is shown to interact with the insulin and EGF receptors only upon stimulation with ligand (31, 63). Consistent with this, we observed robust translocation of TC45 from the nucleus upon HGF stimulation (Fig. 9A) and its relocation to the vicinity of puncta that contain activated Met after stimulation (Fig. 8B). The release of TC45 from the nucleus into the cytosol in response to HGF was as early as 5 min post-HGF stimulation, suggesting that this is likely the result of Met signaling, independent of Met trafficking (Fig. 8 and data not shown), supporting a role for modulation of the Met signal during trafficking (9).

In this study, we have demonstrated that in addition to DEP-1 and LAR, TCPTP and PTP1B dephosphorylate the Met RTK, colocalize with Met after stimulation, and modulate Met receptor-mediated cellular invasion. These studies provide new evidence of mechanisms of down-regulation of the Met RTK and highlight the need for a fuller understanding of the role of tyrosine phosphatases in this process.

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