RECEPTOR TYPE PROTEIN TYROSINE PHOSPHATASE BETA (RPTP-ß) DIRECTLY DEPHOSPHORYLATES AND REGULATES HEPATOCYTE GROWTH FACTOR RECEPTOR (HGFR/MET) FUNCTION.

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Running title: RPTP-ß regulation of Met

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Protein tyrosine phosphorylation is a ubiquitous, fundamental biochemical mechanism that regulates essential eukaryotic cellular functions. The level of tyrosine phosphorylation of specific proteins is finely tuned by the dynamic balance between protein tyrosine kinase (PTK) and protein tyrosine phosphatases (PTP) activities. Hepatocyte growth factor receptor (HGFR, also known as Met), a receptor PTK, is a major regulator of proliferation, migration, and survival for many epithelial cell types. We report here that receptor type protein tyrosine phosphatase-beta (RPTP-ß) specifically dephosphorylates Met, and thereby regulates its function. Expression of RPTP-ß, but not other RPTP family members or catalytically inactive forms of RPTP-ß, reduces HGF-stimulated Met tyrosine phosphorylation in HEK293 cells. Expression of RPTP-ß in primary human keratinocytes reduces both basal and HGF-induced Met phosphorylation at tyrosine 1356 and inhibits downstream MEK1/2 and Erk activation. Furthermore, shRNA-mediated knockdown of endogenous RPTP-ß increases basal and HGF-stimulated Met phosphorylation at tyrosine 1356, in primary human keratinocytes. Purified RPTP-ß intracellular domain preferentially dephosphorylates purified Met at tyrosine 1356 in vitro. In addition, substrate-trapping mutant of RPTP-ß specifically interacts with Met in intact cells. Expression of RPTP-ß in human primary keratinocytes reduces HGF induction of VEGF expression, proliferation, and motility. Taken together, the above data indicate that RPTP-ß is a key regulator of Met function.

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is expressed in numerous tissues (1-5), and participates in the regulation of angiogenesis, organogenesis, tissue repair and neural induction (6). HGF induces random movement/scattering in epithelial cells as well as dissociation, migration, and invasion of cells through the extracellular matrix in vivo (7,8). HGF is mitogenic in many normal cell types, including epithelial cells, vascular endothelial cells, and melanocytes. HGF is also a morphogen that induces transition of epithelial cells into a mesenchymal morphology and formation of branched tubule-like structures (9,10). In keratinocytes, HGF has been shown to promote motility and proliferation (11,12). Each of these biological effects exerted by HGF is triggered by stimulation of its cell surface receptor Met (also known as HGF receptor, HGFR), with concomitant activation of downstream effector molecules (8,13,14).

Upon ligand binding, Met auto-phosphorylates several tyrosine residues in its carboxyl terminal domain. Met function is controlled by its state of tyrosine phosphorylation (15-17). Among the tyrosines within the carboxyl-terminus, phosphorylation of tyrosines 1234 and 1235, located within the catalytic domain, is required for tyrosine kinase activity (15-17). Phosphorylation of tyrosines 1349 and 1356, which are highly conserved among other members of Met family such as Sea and Ron, is required for tyrosine kinase activity (15-17). Phosphorylation of tyrosines 1349 and 1356, when phosphorylated, serve as multifunctional binding sites for Gab1, Grab2, PI3 kinase, phospholipase C (PLC)γ, SHP2, and Cbl proto-oncogene. Phosphorylation of tyrosine 1356 is essential for transducing signals for cell motility and morphogenesis (19-21).
In addition to its roles in many normal physiological processes, Met also plays important roles in human malignancy. Met was originally identified as TPR-Met oncogene, which possesses ligand-independent tyrosine kinase activity. TPR-Met arises from chromosomal fusion of translocated promoter region (TPR) in chromosome 1 with Met carboxyl terminal sequence on chromosome 7 (22,23). This rearrangement has been observed in patients with gastric carcinoma (24). A large body of evidence demonstrates that mis-regulation of Met signaling pathway is involved in many types of human cancers. Inappropriate expression of HGF/Met autocrine signaling confers increased tumorigenesis and metastatic activity in vivo, and Met expression often correlates with poor prognosis (8,25,26). Met pathway also plays key roles in epithelial-mesenchymal transition (EMT), which is involved in tumor invasion (6).

Met is one member of a large family of protein tyrosine kinases (PTK). PTK-mediated tyrosine phosphorylation is balanced by the family of protein tyrosine phosphatases (PTPs) (27-29). Aberrant regulation of either PTKs or PTPs can lead to abnormal cellular behavior and diseases such as cancer and autoimmunity (30). There are 107 genes in the human genome that encode for PTPs (81 active enzymes) and 90 genes encode for PTKs (85 active enzymes). Similar levels of complexity suggest that the two families have comparable substrate specificity (31). PTKs have been implicated in controlling the amplitude of a signaling response, while PTPs are thought to have important roles in controlling the rate and duration of the response (32,33).

Among the PTP super family, there are 38 classical, tyrosine-specific PTPs. These classical PTPs can be further subdivided into 21 receptor type PTPs (RPTPs) and 17 non-transmembrane PTPs. RPTPs contain an intracellular region containing catalytic activity, a transmembrane region, and an extracellular region. All PTPs, regardless of their subtype, contain at least one catalytic domain with a highly conserved active site signature motif ([I/V]HCXAGXXR[S/T]G), where the cysteine residue is absolutely required for catalytic activity. The specificity of PTPs towards substrates can be achieved by their tissue/cell specific expression, subcellular localization/compartmentalization, post-translational modification, and/or specific interaction between PTP active sites and target sequences (34,35).

We report here, that Met tyrosine phosphorylation and function are regulated by receptor type protein tyrosine phosphatase-beta (RPTP-β). RPTP-β directly dephosphorylates Met tyrosine 1356, the major binding site for multiple effector molecules that drive down-stream signaling pathways.

**Materials and Methods**

**Materials** Adult human primary keratinocytes were purchased from Cascade Biologies Inc., (Portland, OR). Human embryonic kidney 293 (HEK293) cells and A431 cells were purchased from ATCC. Met, Erk, and RPTP-β antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-Met (pY1234/1235), phospho-Met (pY1349), phospho-MEK1/2, and phospho-Erk antibodies were purchased from Cell Signaling Technology (Beverly, MA). Phospho-Met (pY1349/1356) was purchased from Rockland (Gilbertsville, PA). Phospho-Met (pY1356) was purchased from Abgent (San Diego, CA). RPTP-κ antibody has been previously described (36). Sodium orthovanadate, hydrogen peroxide, and α-actin antibody were purchased from Sigma. TPR-Met cDNA was obtained from Addgene Inc. (Cambridge, MA). Full length human Met cDNA was obtained from Open Biosystems (Huntsville, AL). Met kinase inhibitor II was purchased from Calbiochem (San Diego, CA). Pervanadate was made by mixing 0.5 M sodium orthovanadate with 0.5 M hydrogen peroxide at room temperature for 30 minutes.

**Cell culture** Adult human primary keratinocytes were expanded in modified MCDB153 media (EpiLife, Cascade Biologies, Inc., Portland, OR) under 5% CO2 at 37°C. HEK293 cells and A431 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 1.5 g/ml sodium bicarbonate, supplemented with 10% FBS under 5% CO2 at 37°C.

**Plasmid construction** Full-length human RPTP-β cDNA was subcloned into pShuttle mammalian expression vector (pShuttle RPTP-β, Clontech Laboratories, Inc., Palo Alto, CA). cDNA fragment encoding human TPR-Met fusion protein was generated by PCR from pBABE-puro TPR-Met (Addgene Inc.) as a template using the following sequence:

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*Note: The sequence needs to be converted into a readable format*
primers (Forward primer: 5’-ACCATGGCGGCG- GTGTTGCAAGCA-3’; Reverse primer: 5’-TGATG- TCTCCAGAAAGGAGG-3’). PCR products were then cloned into pcDNA3.1-V5-Myc-TOPO with V5 epitome and 6XHis Tag in-frame to generate pcDNA3.1 TRP-Met. cDNA sequences corresponding to the intracellular domain of RPTP-β (amino acid residue 1647 - 1997) was amplified by PCR, using pShuttle RPTP-β as template, with forward primer: 5’-TAGGATCCAGCCATGG- TCGAGAAAGACCCT-3’; and reverse primer: 5’- ATGCGGCCGCTCAATGCCTTGAATAGACTGG -3’. The PCR products were digested and cloned into BamHI/NotI sites of pGEX-6P-3 to fuse the GST Tag in-frame. All clones were confirmed by direct DNA sequencing.

Site-directed mutagenesis  The highly conserved cysteine residues (C1904) in the active site of the PTP domain of human RPTP-β were identified by homology to consensus sequence ([I/V]HCXAGXXR[ST]G) alignment using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The highly conserved aspartate residue (D1870) was identified by Laser Gene MegAlign sequence alignment program. QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to generate cysteine 1904 to serine mutation to generate catalytically inactive form of RPTP-β (C1904S). Aspartate 1870 was mutated to alanine to generate the trapping mutant of RPTP-β (D1870A) (37). Oligonucleotides used for mutagenesis of D1870A and C1904S were: D1870A forward primer: 5’-CGGTGTGGCCAGCCCATGGAGTCCC-3’, D1870A reverse primer: 5’-GGGACTCCAGGCACCTTGAATAGCTGG -3’. The PCR products were digested and cloned into BamHI/NotI sites of pGEX-6P-3 to fuse the GST Tag in-frame. All mutations were confirmed by direct DNA sequencing.

Adeno-X expression vector construction and adenovirus production  pShuttle RPTP-β was used to generate Adeno-X expression vector using Adeno-X expression system (Clontech Laboratories, Inc., Palo Alto, CA). HEK293 cells were used for adenovirus production (38).

Preparation of membrane extract and membrane Met kinase assay  Human primary keratinocytes were washed twice with ice-cold hypotonic buffer (20 mM Tris-HCl, pH7.6 with 10 mM NaCl), and scraped from the culture plates in hypotonic buffer supplemented with protease inhibitor cocktail (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM phenylmethysulfonyl fluoride). Cell suspensions were disrupted in a Dounce homogenizer. Unbroken cells were separated from membrane fraction by centrifuge at 500 x g for 10 minutes. Supernatant was centrifuged at 20,000 x g for 30 minutes. Membrane pellet was resuspended in hypotonic buffer with protease inhibitor cocktail and homogenized by passing the suspension back and forth repeatedly through a 25 gauge needle. Membrane suspension was then supplemented with 100 μM ATP, 0.2% β-mercaptoethanol, and 30 mM MgCl2. Kinase reaction was carried out by addition of phosphatase inhibitors and incubating at room temperature for 30 minutes. Laemmlı sample buffer was added to stop the reactions. Tyrosine phosphorylation of Met was analyzed by Western blotting using phospho-Met (pY1349/1356) antibody.

Transient transfection of HEK293 cells  pShuttle RPTPs were transiently transfected into HEK293 cells using Superfection, according to manufacturer’s protocol (Qiagen, Chatsworth, CA). Cells were treated 24 hours after transfection.

Lentivirus mediated shRNAi silencing of RPTP-β in primary human keratinocytes  MISSION TurboGFP shRNA Control Vector and shRNA constructs targeting RPTP-β (5’- CCGGCGGGTGTATCA- GACTAATTATCTCGAGATAATTAGTCTGACCCGTTTTT -3, were purchased from Sigma-Aldrich (St. Louis, MO). Lentivirus was produced in 293FT cells after transfection of RPTP-β shRNA construct and helper plasmids using Superfection method as described by the manufacturer (Qiagen). Two days after transfection, media from 293FT cells was collected and used to infect human primary keratinocytes to knockdown endogenous RPTP-β.

Preparation of whole cell lysates and Western blot  Cells were washed twice with ice-cold PBS, scraped from the culture dishes in WCE buffer (25 mM Hepes, pH7.2, 75 mM NaCl, 2.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate), supplemented with protease inhibitor cocktail and 1 mM sodium
orthovanadate, and transferred to microfuge tubes. Following 10 minutes incubation at 4°C, cell homogenates were centrifuged at 14,000 x g for 10 minutes, and supernatants were collected and used as whole cell extracts. Equal amounts of protein from whole cell extracts were resolved by 10% SDS-polyacrylamide gel (Invitrogen, Carlsbad, CA), and transferred to Immobilon-P filter paper (Millipore, Bedford, MA). Immunoreactive proteins were visualized by enhanced chemifluorescence (ECF) according to manufacturer’s protocol (GE Healthcare, Piscataway, NJ). Quantification of chemofluorescence was performed using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**GST-RPTP-β-IC fusion protein expression and purification** GST-RPTP-β-IC fusion protein was expressed in *E. coli* strain BL21 and purified by GST affinity column as previously described (39). Purity was at least 90%, as judged by SDS PAGE. The concentration of purified protein was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA).

**Preparation of His-Tagged, tyrosine-phosphorylated TPR-Met** Human embryonic kidney HEK293 cells were transfected with pcDNA3.1 TPR-Met using Superfection method as described by the manufacturer (Qiagen). Twenty four hours after transfection, cells were treated with 0.5 mM orthovanadate for 30 minutes at 37°C, washed with ice-cold PBS and lysed with TGH lysis buffer (50 mM HEPES, 20 mM NaCl, 1% Triton X-100, 10% glycerol, pH 7.5) supplemented with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and 1 mM Sodium orthovanadate (Sigma-Aldrich, St. Louis, MO). Lysates were mixed with Ni-NTA Agarose Nickel-Chelating Resin (Invitrogen, Carlsbad, CA) and incubated overnight at 4°C. Ni-NTA Agarose was washed three times with wash buffer (50 mM HEPES, 20 mM NaCl, 0.1% Triton X-100, 10% glycerol, pH 7.5) and His-Tagged TPR-Met was eluted from the beads by addition of Laemmli sample buffer. RPTP-β associated with TPR-Met was detected by Western blot probed with RPTP-β antibody.

**Real time reverse transcriptase-polymerase chain reaction (RT-PCR)** Total cellular RNA was purified using EZgene Total RNA Purification Kit according to manufacturer’s protocol (Biomiga, San Diego, CA). Reverse transcription of total RNA was carried out using a Taqman Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on a 7300 Sequence Detector (Applied Biosystems), using Taqman Universal PCR Master Mix kit (Applied Biosystems). Primer/probe combinations were purchased from Applied Biosystems. Target gene mRNA levels were normalized to endogenous housekeeping gene 36B4 mRNA levels.

**Results**

**Inhibition of membrane-associated protein tyrosine phosphatase activity increases Met tyrosine phosphorylation.** We initially investigated regulation of Met by RPTP activity in the membrane fraction from primary human keratinocytes. Inhibition of membrane-associated PTP activity by addition of PTP inhibitors orthovanadate or pervanadate significantly elevated

**Association of TPR-Met with RPTP-β trapping mutant** pcDNA3.1 TPR-Met mammalian expression vector was co-transfected into HEK293 cells with pShuttle vector containing either wild type RPTP-β, or active site cysteine mutant RPTP-β (C1904S), or trapping mutant RPTP-β (D1870A) by Superfection method. One day post transfection, HEK293 cells were lysed in TGH lysis buffer supplemented with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN). Cell lysates were mixed with Ni-NTA Agarose and incubated overnight at 4°C. Ni-NTA Agarose was washed three times with wash buffer (50 mM HEPES, 20 mM NaCl, 0.1% Triton X-100, 10% glycerol, pH 7.5) and purified His-Tagged TPR-Met was eluted from the beads by addition of Laemmli sample buffer. RPTP-β associated with TPR-Met was detected by Western blot probed with RPTP-β antibody.

Purified tyrosine phosphorylated TPR-Met was mixed with purified GST-RPTP-β-IC fusion protein in PTP assay buffer (150 mM NaCl, 1% IGEPAL CA-630, 50 mM Tris, pH 8.0), supplemented with 10 mM DTT, and incubated at 37°C for the indicated times. Dephosphorylation reactions were terminated by addition of Laemmli sample buffer, and the levels of TPR-Met tyrosine phosphorylation were analyzed by Western blot probed with total and phospho-Met antibodies.
tyrosine phosphorylation of Met. Tyrosine phosphorylation of tyrosines 1349/1356 was elevated 4-6 fold. (Figure 1). Similar elevations of phosphorylation of tyrosines 1234/1235 were also observed (data not shown). These data suggest that keratinocytes express membrane-associated PTP activity that dephosphorylates Met.

Specific dephosphorylation of Met by RPTP-β in HEK293 cells.

In order to identify membrane-associated RPTPs that dephosphorylate Met, we performed expression screening in HEK293 cells. Based expression profiling of RPTPs in human keratinocytes (36), we chose to characterize six candidate RPTPs. These six RPTPs were separately expressed in HEK293 cells, and their ability to reduce the level of HGF-induced phosphorylation of tyrosine 1349/1356 in endogenous Met was determined. The expression of each candidate RPTP has been confirmed by Western blot (Figure 2A). Among the six RPTPs, only RPTP-β significantly decreased the level of Met phosphorylation (Figure 2A). Over-expression of two different catalytically inactive mutant forms of RPTP-β (D1870A and C1904S) or wild-type RPTP-κ, did not decrease Met tyrosine phosphorylation, indicating that reduction of Met tyrosine 1349/1356 requires catalytic activity of RPTP-β (Figure 2B).

Over-expression of RPTP-β reduces Met phosphorylation at tyrosine 1356, but not 1349, in primary human keratinocytes.

We next investigated regulation of HGF-induced Met phosphorylation by RPTP-β in primary human keratinocytes. Adenovirus-mediated expression of RPTP-β substantially reduced the level of HGF-stimulated Met tyrosine phosphorylation, as detected by phospho-Met tyrosine 1349/1356 (Figure 3A) and phosphor-Met tyrosine 1356 (Figure 3B) antibody. Expression of RPTP-β had no effect on HGF-induced phosphorylation of Met tyrosine 1349 (Figure 3A). Similarly, expression of RPTP-β had no effect on HGF-induced phosphorylation of Met tyrosines 1234/1235 (Figure 3C). Expression of RPTP-β in human epidermoid ovarian carcinoma A431 cells also specifically reduced HGF-induced Met phosphorylation at tyrosine 1356 (data not shown). Finally, dephosphorylation of Met by RPTP-β was specific, since expression of RPTP-κ, which dephosphorylates EGFR (36), had no effect on HGF-induced Met phosphorylation at tyrosines 1234/1235, 1349, or 1356 (Figures 3A-3C). Based on these data, we conclude that RPTP-β specifically dephosphorylates tyrosine 1356.

Over-expression of RPTP-β inhibits HGF-induced MEK1/2 and Erk activation and downstream gene expression.

Phosphorylated tyrosine 1356 can serve as a docking site for Grb-2 binding (19,20), which leads to activation of Ras/MAP kinase pathway. Since over-expression of RPTP-β reduces tyrosine 1356 phosphorylation, we examined the effects of RPTP-β expression on HGF-induced phosphorylation of MEK1/2 and Erk, in primary human keratinocytes. As shown in Figure 4, expression of RPTP-β inhibits both MEK1/2 and Erk1/2 phosphorylation. In contrast, over-expression of RPTP-κ does not suppress HGF-induced MEK1/2 or Erk1/2 activation (Figures 4A and 4B, respectively).

HGF has been shown to modulate angiogenesis through induction of vascular endothelial growth factor (VEGF) (40,41). This induction is mediated by activation of the Ras/MAP kinase pathway (42,43). Given the ability of RPTP-β to reduce HGF-induced Erk activation, we investigated the ability of RPTP-β to inhibit HGF-induced VEGF gene expression, in primary human keratinocytes. Consistent with the above results, expression of RPTP-β abolishes HGF-induced VEGF mRNA expression (Figure 4C).

Knockdown of endogenous RPTP-β increases Met phosphorylation.

We next investigated the effect of knock-down of endogenous RPTP-β on basal and HGF-induced tyrosine phosphorylation of Met, in primary human keratinocytes. Lentivirus mediated expression of shRNA reduced the level of RPTP-β protein approximately 70% (Figure 5A). This knock-down of endogenous RPTP-β elevated both basal and HGF-stimulated phosphorylation of Met tyrosine 1356 (Figure 5B), but has no effect on HGF-induced phosphorylation of Met tyrosine 1349 (Figure 5C). Furthermore, knock-down of RPTP-b potentiates both basal and HGF-stimulated Erk activation (Figure 5D).
RPTP-β directly dephosphorylates Met tyrosine 1356

RPTP-β may act directly or indirectly to reduce phosphorylation of Met tyrosine 1349/1356, in intact cells. To distinguish between these possibilities, we performed in vitro phosphatase assays, using purified intracellular domain of RPTP-β and purified phosphorylated TPR-Met as substrates. In these assays, RPTP-β reduced Met phosphorylation in a dose and time dependent manner. As shown in Figure 6, phosphorylation of Met tyrosine 1356 was reduced 75% within 15 minutes by RPTP-β. In contrast, RPTP-β had no significant effect on the level of tyrosine 1349 phosphorylation (Figure 6). These data indicate that RPTP-β directly dephosphorylates Met tyrosine 1356 in vitro and is consistent with our findings in intact cells (Figures 3A and 3B).

RPTP-β substrate-trapping mutant specifically binds Met in intact cells

PTPs and their substrates normally do not form stable complexes. Mutation of a conserved active site aspartic acid (D1870A in RPTP-β) to alanine prevents completion of phosphate ester hydrolysis and therefore traps PTP and substrate in a stable complex (37). Substrate-trapping mutants have been employed to identify physiological substrates for several PTPs (36,37,44). To further substantiate the role of RPTP-β in Met regulation in intact cells, wild type, substrate-trapping mutant, and active site cysteine mutant RPTP-β were separately co-expressed with His-tagged TRP-Met in HEK293 cell. Pull-down assays were performed with Ni-NTA beads to purify His-tagged TRP-Met from HEK293 lysates and TRP-Met associated proteins were analyzed by Western blot. As shown in Figure 7, substrate-trapping mutant RPTP-β bound to TRP-Met. In contrast, neither wild type nor active site cysteine mutant RPTP-β bound to Met. These data indicate that RPTP-β forms a catalytically active complex with Met in intact cells and Met is a physiological substrate for RPTP-β.

RPTP-β regulates Met-mediated proliferation and migration in human primary keratinocytes.

HGF/Met has been shown to be a key mediator of wound healing through its ability to promote keratinocyte proliferation and migration (11,12,45,46). Therefore, we investigated the role of RPTP-β in the regulation of keratinocyte proliferation and migration. We found that blockade of Met tyrosine kinase activity by a specific inhibitor (47,48) suppressed proliferation of primary human keratinocytes (Figure 8A). Expression of RPTP-β similarly inhibited cell proliferation (Figure 8B). Furthermore, expression of RPTP-β abolished HGF-dependent migration of primary human keratinocytes in a scratch assay (Figure 8C).

Taken together, the above data demonstrate that RPTP-β negatively regulates several important biological functions of HGF/Met via direct dephosphorylation of Met tyrosine 1356.

Discussion

The present study demonstrates regulation of Met tyrosine phosphorylation and function by RPTP-β via specific dephosphorylation of Met tyrosine 1356. Tyrosine 1356 is the major binding site for multiple Met effector molecules, which mediate down-stream pathways, including Ras/MAP kinase pathway. Over-expression of RPTP-β suppresses multiple Met functions including proliferation and migration, and inhibits HGF-induced MAP kinase activation and gene expression. Using multiple techniques including substrate-trapping and direct in vitro phosphatase assay, we demonstrate that Met is a bona fide substrate for RPTP-β.

RPTP-β is encoded by the ptprb gene, and is composed of an extracellular domain with cell adhesion molecule-like (CAM-like) motif (multiple fibronectin type III-like domains), a transmembrane domain, and a single intracellular PTP domain (49,50). RPTP-β is localized to chromosome 12 of the human genome, in a region associated with tumors whose cells have lost contact inhibition (51). Due to historical reasons (e.g. cloned and named by different groups), RPTP-ζ (encoded by the gene ptprz) is sometimes referred as RPTP-β/ζ or even RPTP-β in the literature. While there are many published studies regarding RPTP-β/ζ, there are only a few published studies on true RPTP-β. Two studies describe in vitro analysis of RPTP-β intracellular domain (52,53), and a very recent study indicates that RPTP-β regulates angiopoietin-Tie2 signaling pathway in human endothelial cells. This latter study did not elucidate mechanism of action or identify any RPTP-β substrates (54). Interestingly, knockout of mouse homologue of human RPTP-β, VE-PTP, is embryonic lethal due to defective angiogenesis (55,56). This observation is consistent
with the important role that Met plays in angiogenesis (57).

Most RPTPs have two intracellular PTP domains, referred to as membrane-proximal (D1) and membrane distal (D2) domains. Usually the membrane-proximal PTP (D1) domain is catalytically active, while the membrane-distal PTP (D2) domain possesses little, if any, enzymatic activity (58). The D2 domain is thought to play important roles in substrate recognition or protein-protein interactions (59). RPTP-β has only a D1 domain; it lacks a D2 domain. We found that purified RPTP-β maintains its catalytic specificity towards Met tyrosine 1356 in vitro, indicating that a D2 domain is not necessary to confer substrate recognition and specificity.

Biological function of Met is determined by phosphorylation of multiple C-terminal tyrosines. In addition to our finding that RPTP-β dephosphorylates Met, other RPTPs have been shown to influence Met tyrosine phosphorylation (44,60,61). DEP-1 has been shown to preferentially dephosphorylate tyrosines 1349 and 1365 (44). However, DEP-1 expression did not affect MET-dependent MAP kinase activation (44). PTP1B and TCPTP have been shown to dephosphorylate tyrosine 1234/1235 of Met (61). Using only antisense method, Kulas et al. have shown that knockdown of RPTP-LAR increases tyrosine phosphorylation of insulin receptor, insulin receptor substrate-1, EGFR, and Met. The mechanism for the observed increased phosphorylation was not determined (60). Our data demonstrate that RPTP-β directly and specifically dephosphorylates Met tyrosine 1356. This finding raises the possibility that fine tuning of ligand-induced Met signaling specificity can be achieved by the levels and activities of sub-sets of RPTPs in different cell types and tissues.

We have previously described direct and specific dephosphorylation of EGFR by RPTP-κ (36). Therefore, the functions of two important RPTK pathways are specifically controlled by distinct RPTPs. Activation of EGFR or Met leads to both common and distinct cellular responses. There is also cross-talk between these two RTKs. For example, activation of EGFR leads to ligand-independent constitutive activation of Met (62). These overlapping functions of EGFR and Met allow lung cancer to develop resistance towards EGFR-targeting therapy by switching to Met-dependent pathways for proliferation and survival (63). On the other hand, in Met-amplified gastric cancer cells, selective blocking of Met abolishes the cross-talk activation of EGFR. However, EGFR and downstream signaling pathways (e.g. Ras/MAPK and PI3k/Akt) can still be activated by EGFR ligand in a Met-independent manner (64). Our studies suggest that the availability and abundance of RPTP-κ and RPTP-β will play important roles in regulation of EGFR and Met functions in human physiology and cancer.

Acknowledgments

We thank Vladimir Grachtchouk for technical assistance, Diane Fiolek for graphic preparation and administrative support. Human RPTP-β, RPTP-δ, RPTP-κ and RPTP-ξ cDNA was generously provided by Dr. H. Sato (Dana-Farber Cancer Institute, USA). This work is supported in part by 5R01 ES012920 (to G. J. F.).

References

Figure 1. Protein tyrosine phosphatase inhibitors increase tyrosine phosphorylation of Met in keratinocyte plasma membrane fraction. Plasma membrane fraction, from primary human keratinocytes, was supplemented with 100 μM ATP, 30 mM MgCl₂, and 0.2% β-mercaptoethanol, and incubated with 1 mM orthovanadate (VO₄), or 1 mM pervanadate (PV) for 30 minutes at room temperature. The reaction mixture was analyzed by Western blot with antibodies that recognizes Met dual phosphorylated at both tyrosines 1349 and 1356 (Phospho-Met 1349/1356) and total Met. Band intensities were quantified by chemifluorescence. Data are means±SEM. Inset shows a representative Western blot. N=3, *p<0.05

Figure 2. RPTP-β specifically reduces Met phosphorylation in HEK293 cells. (A) HEK293 cells were transfected with empty pShuttle mammalian expression vector (V) or pShuttle containing protein coding sequences for human full-length RPTP-β, RPTP-δ, RPTP-κ, RPTP-μ, RPTP-π, or RPTP-ζ. RPTP protein expressions were confirmed by antibodies specific for each RPTP. Twenty-four hours post transfection, cells were treated with vehicle (-HGF) or 10 ng/ml HGF (+HGF) for 30 minutes. Equal amount of whole cell lysates were analyzed by Western blot with antibodies that recognizes Met dual phosphorylated at both tyrosines 1349 and 1356 (Phospho-Met 1349/1356) and total Met. Band intensities were quantified by chemifluorescence. Data are means±SEM. Inset shows a representative Western blot. N=3, *p<0.05. (B) HEK293 cells were transfected with wild type (WT) RPTP-β or wild type RPTP-κ, or D1870A, or C1904S mutants of RPTP-β. Twenty-four hours post transfection, cells were treated with 10 ng/ml HGF for 30 minutes and analyzed for Met phosphorylation as described above. Results are representative of three experiments.

Figure 3. RPTP-β inhibits Met phosphorylation at tyrosine 1356, but not 1349, in primary human keratinocytes. Keratinocytes were infected with empty (Ept), RPTP-β, or RPTP-κ-expressing adenovirus. Twenty-four hours post infection, cells were treated with control (Ctrl) or 10 ng/ml HGF for 30 minutes. Equal amount of whole cell lysates were analyzed by Western blots, which were probed with antibodies indicated in the figures. (A) Phospho-Met 1349/1356 antibody (closed bars) specifically recognizes Met that is dual phosphorylated at both tyrosines 1349 and 1356. Phospho-Met 1349 antibody (open bars) recognizes Met that is phosphorylated at tyrosine 1349, independent of phosphorylation status of tyrosine 1356. Band intensities were quantified by chemifluorescence. Data are means±SEM. Inset shows a representative Western blot. N=3, *p<0.05 for RPTP-β Ctrl vs. Ept Ctrl or RPTP-β/HGF vs. Ept/HGF. (B). Keratinocytes were treated and lysates were analyzed by Western blots as described above. Phospho-Met 1356 antibody recognizes Met that is phosphorylated at tyrosine 1356, independent of phosphorylation status of tyrosine 1349. N=3, *p<0.05 for RPTP-β/HGF vs. Ept/HGF. (C). Keratinocytes were treated and lysates were analyzed by Western blots as described above. Phospho-Met 1234/1235 antibody recognizes Met that is dual phosphorylated at tyrosines 1234 and 1235. Results are representative of three experiments.

Figure 4. RPTP-β inhibits HGF-induced MEK1/2 and Erk activation and VEGF induction in primary human keratinocytes. Keratinocytes were infected with empty (Ept), RPTP-β, or RPTP-κ-expressing adenovirus. Twenty-four hours post infection; cells were treated with control (Ctrl) or 10 ng/ml HGF for 30 minutes. (A and B) Equal amounts of whole cell lysates were analyzed by Western blot, which were probed with antibodies that recognize (A) phosphorylated MEK1/2 (p-MEK 1/2) or total MEK1/2, or (B) phosphorylated Erk p44 (open bars)/p42 (closed bars) (p-Erk) or total Erk. Band intensities were quantified by chemifluorescence. Inset shows representative Western blots. Data are means±SEM, N=3; *p<0.05 for RPTP-β/HGF vs.
Ept/HGF. (C) Total RNA was isolated and VEGF mRNA levels were determined by real-time RT-PCR analysis. N=3, *p<0.05 for RPTP-β/HGF vs. Ept/HGF.

**Figure 5.** Knock-down of endogenous RPTP-β increases Met phosphorylation at tyrosine 1356, but not 1349, in human primary keratinocytes. Keratinocytes were infected with non-targeting (NT shRNA) or RPTP-β-targeting shRNA lentivirus. Forty eight hours post infection; cells were treated with vehicle (Ctrl) or 10 ng/ml HGF for 30 minutes. Equal amounts of whole cell lysates were analyzed by Western blot, which were probed with antibodies indicated in the figures. (A) RPTP-β-targeting shRNA reduces expression of RPTP-β. N=3. (B) Knock-down of endogenous RPTP-β increases Met phosphorylation at tyrosine 1356. Band intensities were quantified by chemifluorescence. Inset shows representative Western blots. Data are means±SEM, N=3; *p<0.05 for Ctrl/RPTP-β shRNA vs. Ctrl/NT shRNA and for HGF/RPTP-β shRNA vs. HGF/NT shRNA. (C) Knock-down of endogenous RPTP-β does not alter Met phosphorylation at tyrosine 1349. Band intensities were quantified by chemifluorescence. Inset shows representative Western blots. Data are means±SEM, N=3. (D) Knock-down of endogenous RPTP-β increases Erk activation. Band intensities were quantified by chemifluorescence. Inset shows representative Western blot. N=3, *p<0.05.

**Figure 6.** RPTP-β preferential dephosphorylates Met tyrosine 1356 in vitro. Phosphorylated purified His-tagged TPR-Met was incubated with purified GST-RPTP-β-intracellular domain, at 37°C for indicated times. Reactions were terminated by addition of SDS-loading buffer and samples were analyzed by Western blots probed with phospho-Met 1356 antibody (closed squares) or phospho-Met 1349 antibody (open circles). Inset shows representative Western blot. Band intensities were quantified by chemifluorescence. Data are means±SEM, N=5; *p<0.05.

**Figure 7.** RPTP-β interacts with Met in HEK293 cells. Cells were co-transfected with His-tagged TPR-Met and wild type (WT), substrate trapping mutant (D1870A), or catalytically inactive mutant (C1904S) RPTP-β. Twenty four hours after transfection, whole cell lysates were prepared and Ni-NTA beads were added to pull-down His-tagged TPR-Met. TPR-Met-associated proteins were analyzed by Western blots probed with RPTP-β antibody. A representative Western blot from four independent experiments is shown.

**Figure 8.** RPTP-β regulates Met-dependent cell proliferation and motility, in primary human keratinocytes. (A) Keratinocytes were treated with vehicle (open circles) or Met kinase inhibitor II (1 M) (closed squares). Cells were harvested at the indicated times post-treatment and counted by a hemocytometer. Results are means±SEM of six independent experiments; *p<0.05. (B) Keratinocytes were infected with empty (open circles) or RPTP-β (closed squares) adenovirus. Cells were harvested at the indicated times post-treatment and counted by a hemocytometer. Results are means±SEM of six independent experiments; *p<0.05. (C) Confluent keratinocytes were infected with empty or RPTP-β adenovirus. Twenty four hours later, cells were treated with vehicle (-HGF) or 10ng/ml HGF (+HGF) and the bottom surface of the culture dishes were scraped with a pipette tip to create a cell free zone. Migration of cells into the cell-free zone was monitored, eight hours after scraping, by phase contrast light microscopy. A representative image of four independent experiments is shown.
Figure 1

Phospho-Met 1349/1356 / Total Met

Fold Change

Ctrl VO₄ PV

Phospho-Met 1349/1356

Total Met

*
Figure 2

A

Phospho-Met 1349/1356
RPTP
Total Met

V V β δ κ μ π ζ

- HGF  + HGF

Phospho-Met 1349/1356 (Fold Change)

RPTP V V β δ κ μ π ζ

- HGF  + HGF

B

RPTP-β  RPTP-κ

WT D1870A C1904S WT

Phospho-Met 1349-1356
Total Met
Figure 3

A

Phospho-Met 1349/1356

HGF

Ept

RPTP-β

RPTP-κ

Adeno

G331_B309_DF

Phospho-Met 1349

Total Met

RPTP-β

RPTP-κ

Abs

B

Phospho-Met 1356

HGF

Ept

RPTP-β

RPTP-κ

Adeno

G314v4_B308v2_DF

Phospho-Met 1356

Total Met

RPTP-β

RPTP-κ

Abs

C

Phospho-Met 1234/1235

Total Met

RPTP-β

RPTP-κ

Abs
Figure 4

A

B

C

VEGF mRNA (Fold Change)

p-MEK 1/2 / Total MEK 1/2

p-p44-p42 Erk / Total p44-p42 Erk

VEGF mRNA (Fold Change)

Ctrl | HGF | Ctrl | HGF | Ctrl | HGF
---|---|---|---|---|---
Ept |  |  |  |  |  |
RPTP-β |  |  |  |  |  |
RPTP-κ |  |  |  |  |  |
Adeno |  |  |  |  |  |
HGF |  |  |  |  |  |

Abs
Figure 5

A

<table>
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<th>shRNA</th>
<th>Ctrl</th>
<th>RPTP-β</th>
<th>RPTP-β</th>
<th>RPTP-β</th>
<th>β-Actin</th>
<th>Abs</th>
</tr>
</thead>
</table>

B

Veh  RPTP-β shRNA

Phospho-Met 1356

Total Met

Abs

C

Ctrl  RPTP-β shRNA

Phospho-Met 1349

Total Met

D

Veh  RPTP-β shRNA

p-p44 Erk / Total Erk

p-p42 Erk / Total Erk

Abs

NT shRNA  RPTP-β shRNA
Figure 7

Lysate
Pull-Down
Pull-Down

WT D1870A C1904S
RPTP-β
RPTP-β
Total Met
Figure 8

A

Cell Number (x 100,000)

0

2

4

6

8

10

0 1 2 3

Days

B

Cell Number (x 100,000)

0

5

10

15

20

0 1 2 3

Days

C

-HGF 0 Hours   -HGF 8 Hours   +HGF 8 Hours

Empty

RPTP-ß
Receptor type protein tyrosine phosphatase beta (RPTP-β) directly dephosphorylates and regulates hepatocyte growth factor receptor (HGFR/MET) function

Yiru Xu, Wei Xia, Dustin Baker, Jin Zhou, Hyuk Chol Cha, John J. Voorhees and Gary J. Fisher

J. Biol. Chem. published online March 15, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.212597

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