The Receptor-Type Protein Tyrosine Phosphatase J Antagonizes the Biochemical and Biological Effects of RET-Derived Oncoproteins

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Abstract

Thyroid cancer is frequently associated with the oncogenic conversion of the RET receptor tyrosine kinase. RET gene rearrangements, which lead to the generation of chimeric RET/papillary thyroid carcinoma (PTC) oncogenes, occur in PTC, whereas RET point mutations occur in familial multiple endocrine neoplasia type 2 (MEN2) and sporadic medullary thyroid carcinomas (MTC). We showed previously that the expression of the receptor-type protein tyrosine phosphatase J (PTPRJ) is suppressed in neoplastically transformed follicular thyroid cells. We now report that PTPRJ coimmunoprecipitates with wild-type RET and with the MEN2A-associated RET(C634R) oncoprotein but not with the RET/PTC1 and RET-MEN2B isoforms. Using mutated forms of PTPRJ and RET-MEN2A, we show that the integrity of the respective catalytic domains is required for the PTPRJ/RET-MEN2A interaction. PTPRJ expression induces dephosphorylation of the RET (C634R) and, probably via an indirect mechanism, RET/PTC1 oncoproteins on two key RET autophosphorylation sites (Tyr^{1062} and Tyr^{905}). This results in a significant decrease of RET-induced Shc and extracellular signal-regulated kinase 1/2 phosphorylation levels. In line with this finding, adoptive PTPRJ expression reduced the oncogenic activity of RET(C634R) in an *in vitro* focus formation assay of NIH3T3 cells. As expected from the coimmunoprecipitation results, the RET(M918T) oncoprotein, which is associated to MEN2B and sporadic MTC, was resistant to the dephosphorylating activity of PTPRJ. Taken together, these findings identify RET as a novel substrate of PTPRJ and suggest that PTPRJ expression levels may affect tumor phenotype associated with RET/PTC1 and RET(C634R) mutants. On the other hand, resistance to PTPRJ may be part of the mechanism of RET oncogenic conversion secondary to the M918T mutation. (Cancer Res 2006; 66(12): 6280-7)

Introduction

The *Ptprj* gene encodes a receptor-type tyrosine phosphatase, previously designated *density-enhanced phosphatase-1, PTPη*, or *CD148*, which is composed of an extracellular domain containing eight fibronectin type III repeats, a transmembrane domain, and a single cytoplasmic catalytic domain (1, 2). A growing body of evidence implicates PTPRJ in the inhibition of cell growth and cell migration (3–5). In fact, PTPRJ can dephosphorylate tyrosine kinase receptors that are crucial for cell growth and migration [i.e., platelet-derived growth factor (PDGF; refs. 6, 7), hepatocyte growth factor (HGF; refs. 8, 9), and vascular endothelial growth factor (VEGF; ref. 10)].

Ptprj is a candidate for the mouse colon cancer susceptibility locus *Scc1* (11, 12). A considerable *Ptprj* loss of heterozygosity has been identified in human colon, mammary, lung, and thyroid carcinomas (13, 14). Moreover, the Gln^{276}Pro *Ptprj* polymorphism is implicated in susceptibility to various human cancers (11), whereas the Gln^{276}Pro, Arg^{326}Gln, and Asp^{872}Glu polymorphisms have been associated with thyroid carcinoma (15). *Ptprj* expression was found to be reduced in *in vitro*–transformed thyroid follicular cells (2) and in primary specimens of human papillary thyroid carcinomas (PTC; ref. 14), whereas reintroduction of *Ptprj* in thyroid (16) and breast cancer (17) cells suppressed the neoplastic phenotype. Not surprisingly, viral-delivered *Ptprj* was successful in the treatment of preclinical models of human thyroid (14) and pancreatic (18) carcinomas.

RET is a single-pass transmembrane tyrosine kinase receptor (19). In PTC, which account for 80% of all thyroid carcinomas (20), RET is rearranged with other genes to generate chimeric RET/PTC oncogenes. The most frequent fusions are RET/PTC1, H4-RET, RET/PTC3, and RFG-RET (21). Germ-line point mutations in RET are responsible for the inheritance of multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B) and familial medullary thyroid carcinoma (MTC; ref. 22). MEN2A and MEN2B predispose to MTC and pheochromocytoma. MEN2B is characterized by additional disease phenotypes and is clinically more aggressive than MEN2A and familial MTC. In most MEN2A cases, the mutations target specific cysteine residues (usually Cys^{634}) in the extracellular domain of the RET receptor (22). These mutations result in unpaired cysteine residues, thereby leading to the formation of intermolecular disulfide bridges and ligand-independent dimerization (21). A single point mutation, which results in a threonine for methionine substitution at codon 918 within the RET catalytic domain (M918T), is the most frequent mutation in MEN2B and in most RET mutation-positive (up to 50% of cases) sporadic MTC cases (22).

Here, we show that PTPRJ can dephosphorylate the RET(C634R) (RET-MEN2A) and RET/PTC1 oncoproteins at Tyr^{905} and Tyr^{1062} and that this process impairs RET downstream signaling. For RET(C634R), RET dephosphorylation was accompanied by RET and

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PTPRJ coimmunoprecipitation. Accordingly with this finding, PTPRJ reduced the *in vitro*–transforming ability of RET(C634R).

Materials and Methods

Materials and plasmids. Anti-hemagglutinin (HA) monoclonal antibody (mAb) was purchased from Boehringer Mannheim (Mannheim, Germany). Anti-RET rabbit polyclonal antibodies were raised against the recombinant kinase domain of the RET protein (23). Anti-phosphotyrosine mAb was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (Thr^{202}/Tyr^{204}) and anti-phosphorylated AKT (Ser^{473}) antibodies were from Cell Signaling Technology, Inc. (Beverly, MA), and anti-ERK antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). All the RET constructs used in this study encode the short RET isoform, RET-9, and were cloned in pCDNA3(Myc-His) (Invitrogen, Groningen, the Netherlands). Wild-type (WT) RET and the RET/PTC1, RET(M918T) (RET-MEN2B), RET(C634R) (RET-MEN2A), and RET(A883F) constructs are described elsewhere (23). The RET-MEN2A(ΔK) construct that lacks the entire cytosolic domain (from amino acid 712 to the C terminus) and the RET-MEN2A(4F) construct that carries tyrosine-to-phenylalanine substitutions at Y826, Y1015, Y1029, Y1062, and RET-MEN2A(Y1062F) were generated by PCR or site-directed mutagenesis. The PTPRJ expression construct and the phosphatase-dead (C1239S) PTPRJ-C/S mutant cloned in pCEFL-HA are described elsewhere (16). The substrate-trapping (D1205A) PTPRJ-D/A mutant (9) was generated by site-directed mutagenesis using the QuickChange kit (Stratagene, La Jolla, CA), and the mutation was confirmed by DNA sequencing.

Cell culture and transfection. HEK293T cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin (Life Technologies, Inc., Paisley, PA). NIH3T3 cells were maintained in DMEM supplemented with 10% calf serum. Human thyroid carcinoma-derived cell lines, TPC1 and FB2, carrying the RET/PTC1 fusion protein (24) were cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, and 100 units/mL penicillin-streptomycin. Where indicated, cells were transfected with LipofectAMINE (Life Technologies) according to the manufacturer's instructions in Opti-MEM medium (Life Technologies).

Cell lysis, immunoprecipitation, and Western blot analysis. Cells were washed with ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100] containing 1 mmol/L phenylmethylsulfonyl fluoride and 1 mmol/L sodium orthovanadate. The lysates were clarified by centrifugation (15,000 × *g*) for 1 hour and incubated with 1 to 2 μg of primary antibodies for 2 hours, and immunocomplexes were precipitated with protein A-Sepharose or protein G-Sepharose beads (Amersham Pharmacia Biotech, Little Chalfort, United Kingdom) for 1 hour at 4°C. After immunoprecipitation, beads were washed four times with RIPA buffer, suspended in sample buffer [2% (w/v) SDS, 20 mmol/L Tris-HCl (pH 6.8), 2 mmol/L EDTA, 10% (w/v) sucrose, 20 μg/mL bromphenol blue] in the absence or presence of 80 mmol/L DTT, and boiled for 3 minutes. The lysates were subjected to SDS-PAGE, separated on

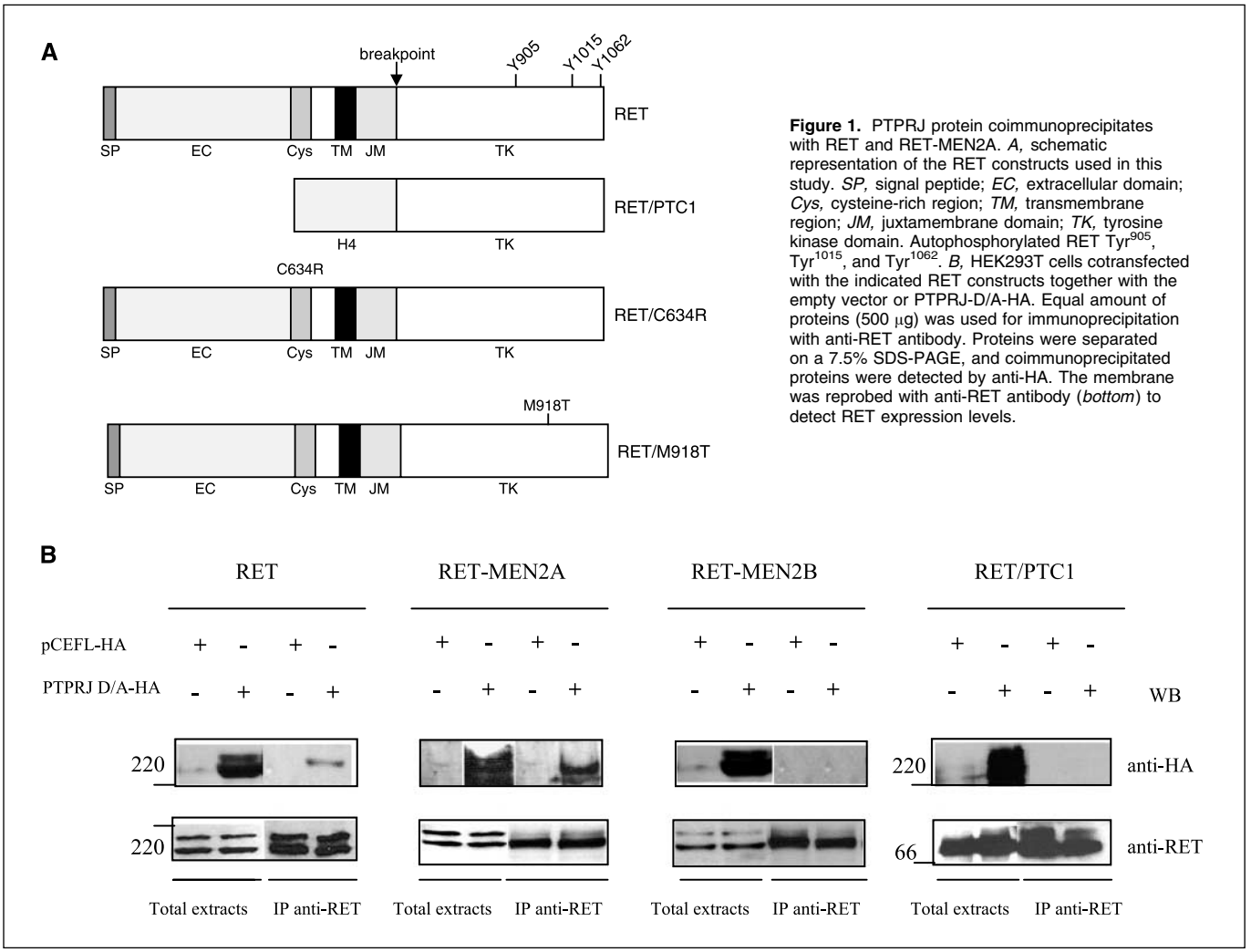

Figure 1. PTPRJ protein coimmunoprecipitates with RET and RET-MEN2A. *A,* schematic representation of the RET constructs used in this study. *SP,* signal peptide; *EC,* extracellular domain; *Cys,* cysteine-rich region; *TM,* transmembrane region; *JM,* juxtamembrane domain; *TK,* tyrosine kinase domain. Autophosphorylated RET Tyr^{905}, Tyr^{1015}, and Tyr^{1062}. *B,* HEK293T cells cotransfected with the indicated RET constructs together with the empty vector or PTPRJ-D/A-HA. Equal amount of proteins (500 μg) was used for immunoprecipitation with anti-RET antibody. Proteins were separated on a 7.5% SDS-PAGE, and coimmunoprecipitated proteins were detected by anti-HA. The membrane was reprobed with anti-RET antibody (*bottom*) to detect RET expression levels.

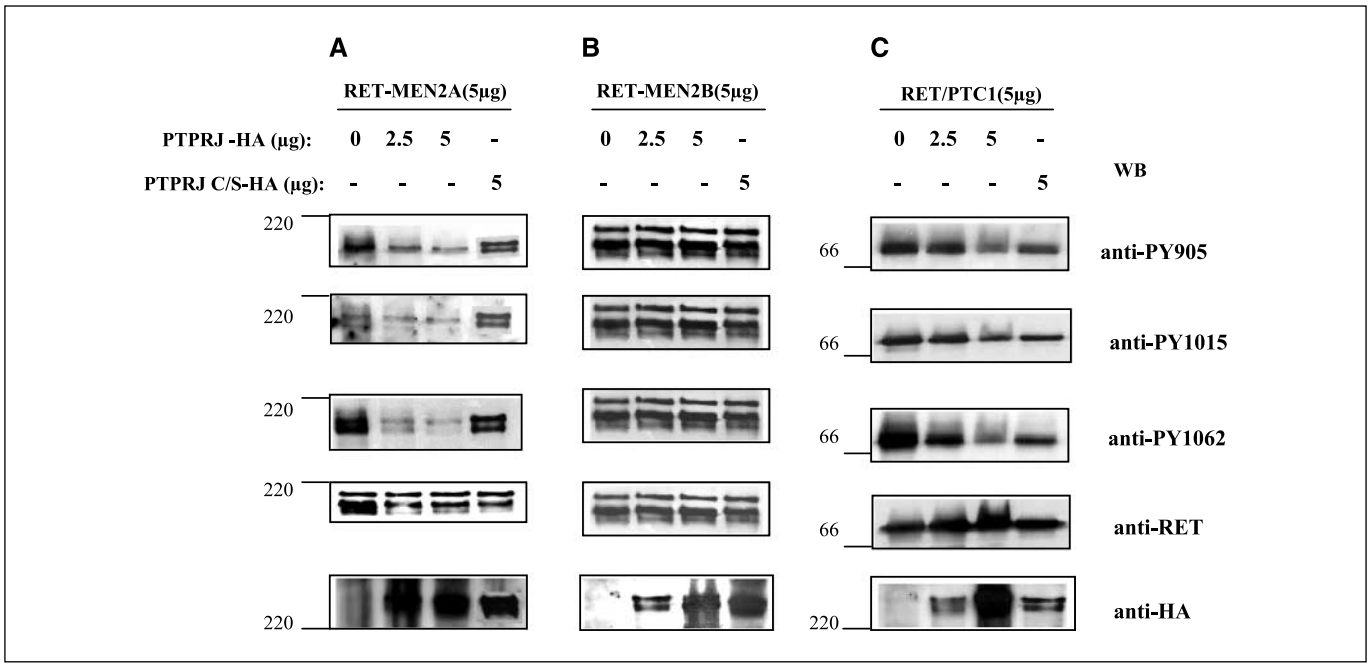

Figure 2. PTPRJ reduces tyrosine phosphorylation levels of RET/PTC1 and RET-MEN2A. HEK293T cells were transiently cotransfected with 5 μg of the indicated RET expression plasmids and different amounts of PTPRJ cDNA or 5 μg of the PTPRJ-C/S mutant. Whole-cell lysates were separated by 7.5% SDS-PAGE, and RET phosphorylation was analyzed by immunoblotting with the phosphorylated-specific RET antibodies. Equal gel loading was assessed by reprobing with anti-RET and anti-HA antibodies. Data are representative of three independent experiments.

5% to 10% gradient gels, and transferred onto polyvinylidene difluoride membranes (Nihon Millipore Kogyo KK, Tokyo, Japan) overnight at 100 mA. The transferred proteins were treated with 0.5% (v/v) Tween 20-PBS containing 3% (w/v) ovalbumin for 1 hour. Then, the membrane was incubated overnight at 4°C with different primary antibodies. As secondary antibodies, we used peroxidase-conjugated anti-mouse (1:1,000) or anti-rabbit (1:1,000) immunoglobulins (DAKO, Glostrup, Denmark). The filters were washed four times with Tween 20-PBS and examined with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Munich, Germany).

Subcellular fractionation. HEK293T-transfected cells (10^7) were washed in PBS and allowed to swell in 250 μL hypotonic buffer [20 mmol/L HEPES (pH 7.4), 10 mmol/L EDTA, 2 mmol/L DTT, protease and phosphatase inhibitors] on ice for 15 minutes. Cells were ruptured by repeated passage through a 27-gauge needle. Nuclei were removed by centrifugation at 700 × *g* for 10 minutes. Membranes were isolated from the postnuclear supernatant by centrifugation at 35,000 rpm for 45 minutes in a 90TI rotor (in 800-μL tube) on a Beckman ultracentrifuge (Beckman Coulter, Fullerton, CA). The supernatant portion of the latter centrifugation was the soluble cytosolic fraction. The pellet (membranes) was resuspended in RIPA lysis buffer (100 μL) and centrifuged at 13,000 rpm for 30 minutes. Aliquots from each fraction were subjected to immunoblotting and immunoprecipitation.

Expression and purification of His_6 fusion protein. A cDNA sequence corresponding to the PTPRJ catalytic domain (amino acids 1067-1297) was inserted into the pET21c plasmid. *Escherichia coli* BL21 DE3 was transformed with the expression plasmids and grown at 37°C; protein synthesis was induced by the addition of 0.1 mmol/L isopropyl-L-thio-B-D-galactopyranoside. The bacterial pellet was suspended in ice-cold NETN buffer [1% NP40, 20 mmol/L Tris-HCl (pH 8), 1 mmol/L EDTA, 100 mmol/L NaCl] and sonicated six times (15 seconds each time). Bacterial debris was removed by centrifugation, and fusion proteins in the supernatant were bound to Ni-NTA agarose beads (Qiagen, Hilden, Germany), washed thrice with 10 volumes of buffer [1% NP40, 10 mmol/L Tris-HCl (pH 7.5), 250 mmol/L NaCl], and resuspended in NETN buffer.

***In vitro* dephosphorylation assay.** RET proteins were purified from 500 μg of cellular proteins by immunoprecipitation. The immunoprecipitated RET proteins were washed thrice with the RIPA buffer and twice with the PTP buffer [50 mmol/L Tris-HCl (pH 6.8), 150 mmol/L NaCl, 10 mmol/L DTT, protease inhibitors without phosphatase inhibitors] and divided into three portions. Then, they were suspended in 50 μL PTP buffer containing 1 and 3 μg of purified PTPRJ-His_6 and incubated for 30 minutes at 30°C. The reactions were terminated by adding an equal volume of 5× SDS sample buffer, boiled for 5 minutes, and subjected to immunoblotting as described above.

Colony assay. Cells were seeded at a density of 2×10^6 per 100-mm diameter dish. The next day, cells were transiently transfected with pCEFL or PTPRJ by the calcium phosphate procedure. Forty-eight hours after transfection, cells were split and selected in G418 (Life Technologies). Two weeks later, cells were stained with 500 μg crystal violet/mL in 20% methanol and the colonies were counted.

Focus formation assay. For primary focus formation assays, NIH3T3 cells were cotransfected with 1 μg of the required plasmid DNA with the calcium phosphate precipitation method as described elsewhere (23). Transformed foci were scored at 3 weeks. Cells were then fixed in 10% acetic acid and 10% methanol, stained with crystal violet, and counted. Transforming efficiency was expressed as focus-forming units per pmol of added DNA.

Results

PTPRJ forms a complex with the RET WT and RET-MEN2A-mutant proteins. RET WT cDNA was transfected into HEK293T cells together with the empty vector or a HA-tagged substrate-trapping mutant of PTPRJ (PTPRJ-D/A). The D/A (D1205A) PTPRJ mutant, like the corresponding mutants of other PTPs, is not able to dephosphorylate the substrate. It acts as a substrate-trapping mutant because of the absence of the aspartate acid role and the flipping of the "WPD" loop that binds to substrate and blocks it into the catalytic pocket. Hence, its affinity for substrates is

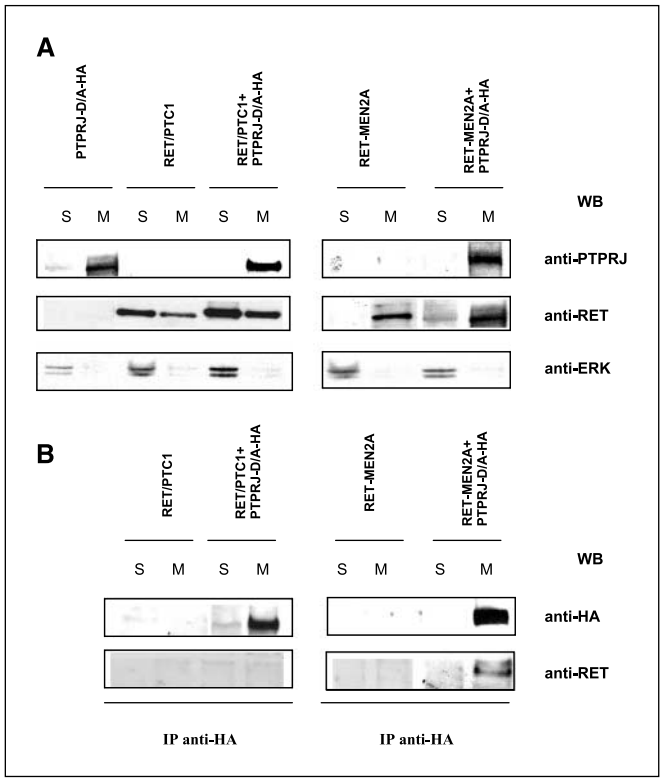

Figure 3. Subcellular localization of RET proteins. *A,* subcellular fractions of HEK293T cells cotransfected with the indicated RET constructs together with the empty vector or PTPRJ-D/A-HA were obtained as described in Materials and Methods. The amount of the soluble (*S*) with respect to the membrane fraction (*M*) was calculated after separation from the nuclear fraction (which was negative for RET expression; data not shown). For the soluble and membrane fractions, protein amounts deriving from comparable numbers of cells were analyzed by immunoblotting with anti-HA and anti-RET antibodies. Aliquots of each fraction representative of the same number of cells were analyzed with anti-ERK antibody as a control of the cytosolic purity. *B,* 1 mg of the soluble and membrane extracts was immunoprecipitated (*IP*) with anti-HA antibody and immunoblotted (*WB*) with anti-RET antibody (*bottom*). The blots were reprobed with anti-HA antibody (*top*) to determine the level of PTPRJ.

severalfold higher with respect to WT phosphatase (8, 9). Equal protein amounts (500 μg) were immunoprecipitated with anti-RET polyclonal antibody. Western blot hybridization with anti-HA antibody identified the PTPRJ protein in RET WT/PTPRJ-D/A cotransfected cells (Fig. 1*B*), indicating that RET and PTPRJ formed a protein complex. We carried out the same experiment with oncogenic RET mutants (i.e., MEN2A-associated C634R, MEN2B-associated M918T, and PTC-associated RET/PTC1). The RET constructs are indicated in Fig. 1*A*. As shown in Fig. 1*B*, RET-MEN2A formed a complex with PTPRJ, whereas RET/PTC1 and RET-MEN2B did not. This effect was unrelated to the amount of proteins loaded, which was the same in all samples (Fig. 1*B, bottom*). Intriguingly, also the rare MEN2B-associated RET mutant Ala^{883}Phe (21) failed to form complexes with PTPRJ (data not shown).

PTPRJ overexpression leads to reduced tyrosine phosphorylation of RET-MEN2A and RET/PTC1. To determine whether PTPRJ dephosphorylates RET, we used phosphorylated-specific antibodies to detect the phosphorylation levels of three RET intracellular tyrosines: Y905, Y1015, and Y1062 (25). These sites are constitutively phosphorylated in oncogenic RET mutants. Y905 maps in the activation loop of the RET kinase, and its phosphorylation is required to maintain the enzyme in the active conformation. Y1015 is the docking site for phospholipase Cγ, and Y1062 in the COOH-terminal tail of the receptor is the binding site for the Shc, FRS2, IRS1/2, DOK1/4/5, and Enigma proteins (26). Binding to Shc and FRS2 mediates recruitment of Grb2-SOS complexes, so leading to GTP exchange consequent to RAS and RAS/ERK stimulation (21). We transfected HEK293T cells with a fixed amount of RET-MEN2A (5 μg) and increasing amounts of WT PTPRJ (0, 2.5, and 5 μg) or 5 μg of the catalytically inactive PTPRJ-C/S mutant cDNA. As shown in Fig. 2*A*, phosphorylation of Y905 and Y1015 gradually and dose dependently decreased as the amount of PTPRJ increased. The effect was more pronounced for Y1062. In fact, Y1062 phosphorylation was significantly decreased in the presence of a low amount of PTPRJ (Fig. 2*A*). These effects required intact PTPRJ catalytic activity because they were not observed with PTPRJ-C/S (Fig. 2*A*).

We next measured PTPRJ activity on the RET-MEN2B and RET/PTC1 proteins. Consistent with the lack of coimmunoprecipitation (Fig. 1*C*), RET-MEN2B was not dephosphorylated when coexpressed with PTPRJ (Fig. 2*B*). This suggests that the MEN2B mutation, which targets the P+1 loop of the RET kinase domain, may cause conformational changes that prevent PTPRJ and RET from interacting. Differently, despite the lack of coimmunoprecipitation (Fig. 1*B*), RET/PTC1 was dephosphorylated when coexpressed with catalytically active PTPRJ (Fig. 2*C*). However, PTPRJ had a less pronounced on RET/PTC1 than on RET-MEN2A. In fact, 2.5 μg PTPRJ reduced Y1062 phosphorylation by ~50% for RET/PTC1 (Fig. 2*C*) and ~95% for RET-MEN2A (Fig. 2*A*). RET/PTC proteins are delocalized to the cytosol consequent to truncation of the RET extracellular and transmembrane domains (26). It is likely that the altered subcellular localization reduces the stoichiometry or stability of the RET/PTC1-PTPRJ interaction to levels that are undetectable by coimmunoprecipitation. This, notwithstanding, PTPRJ remained capable of dephosphorylating RET/PTC1 albeit to a lesser extent compared with RET-MEN2A.

To investigate whether the lack of coimmunoprecipitation between RET/PTC1 and PTPRJ is depending on the RET/PTC1 protein subcellular localization, we obtained cytosolic and membrane extracts of RET/PTC1-transfected cells and assessed RET/PTPRJ-D/A-HA coimmunoprecipitation. As it can be observed

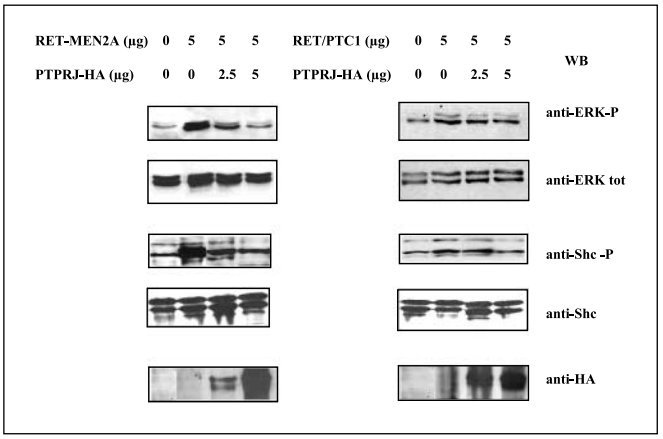

Figure 4. PTPRJ expression impairs activation of Shc and ERK1/2 by RET/PTC1 and RET-MEN2A. Cell lysates were analyzed by immunoblotting with phosphorylated ERK1/2 (*ERK-P*; Thr^{202}/Tyr^{204}) antibody and phosphorylated Shc (Tyr^{239}/Tyr^{240}) antibodies. Total ERK (*ERK tot*) and Shc amounts are also shown. Data are representative of three independent experiments.

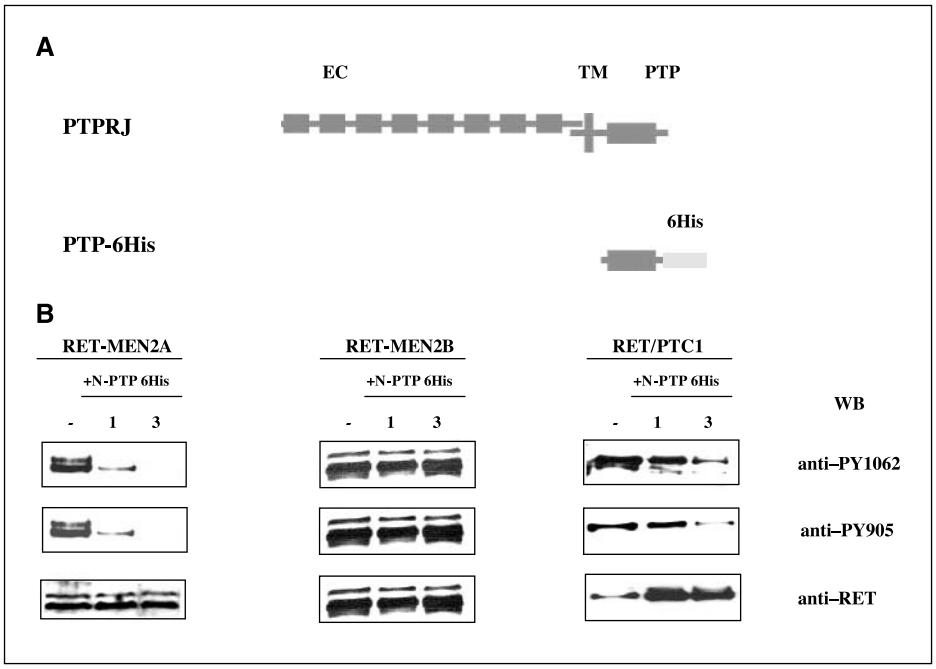

Figure 5. *In vitro* dephosphorylation of RET/PTC1 and RET-MEN2A by PTPRJ. *A,* schematic representation of the full-length PTPRJ protein and PTP-His_6 corresponding to the PTP domain expressed as His_6 fusion protein. *B,* total proteins from HEK293T cells transiently transfected with the indicated RET expression plasmids were immunoprecipitated with anti-RET antibody. The immunocomplexes were incubated with purified PTP-His_6 in phosphatase buffer for 30 minutes at 37°C. After incubation, the proteins were separated by 10% SDS-PAGE and immunoblotted with antibodies directed against RET phosphotyrosines and with anti-RET antibody.

in Fig. 3*A*, PTPRJ and RET-MEN2A were distributed at the membrane level, whereas RET/PTC1 protein was mostly present at the cytosolic level. No coimmunoprecipitation was observed between RET/PTC1 and PTPRJ-D/A using either the membrane or the cytosolic extracts (Fig. 3*B*). Conversely, coimmunoprecipitation was observed when the membrane extracts from the RET-MEN2A-transfected cells were used (Fig. 3*B*). Therefore, it is likely that the low levels of the RET/PTC1 protein present at the membrane level do not allow the detection of the interaction between RET/PTC1 and PTPRJ-D/A.

Down-regulation of RET-MEN2A-induced signal transduction by PTPRJ. We next investigated the effect of PTPRJ expression on RET-MEN2A and RET/PTC1 downstream signaling targets. HEK293T cells were cotransfected with RET-MEN2A or RET/PTC1 and increasing amounts of WT PTPRJ (0, 2.5, and 5 μg). Cell extracts were immunoblotted with site-specific phosphoantibodies to determine the phosphorylation levels of p42/44 ERKs (T202/Y204) and Shc (Y239/Y240). Shc undergoes tyrosine phosphorylation on binding to RET pY1062 (27). As shown in Fig. 4, phosphorylation of ERKs and Shc was readily induced by RET oncoproteins. Interestingly, ERK and Shc phosphorylation levels decreased as PTPRJ concentrations increased (Fig. 4).

PTPRJ dephosphorylates RET-MEN2A *in vitro*. To obtain more information about the PTPRJ/RET interaction, we constructed a His_6-tagged fusion protein containing the catalytic

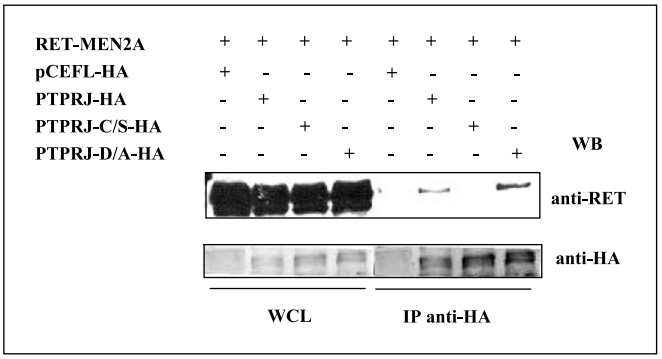

Figure 6. RET-MEN2A interaction with PTPRJ WT and mutated proteins. HEK293T cells were cotransfected with 5 μg of the empty pCEFL-HA vector or carrying either PTPRJ/WT, PTPRJ-C/S (catalytically inactive PTPRJ mutant carrying the C1239S mutation), or PTPRJ-D/A (carrying the D1205A mutation) together with 5 μg of RET/MEN2A. Total cell extracts were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-RET antibody (*top*). The same membranes were reprobed with anti-HA antibody (*bottom*) to determine the level of transfected PTPRJ protein expression. To show the expression of the transfected RET protein, whole-cell lysates (*WCL*; 50 μg) were immunoblotted with anti-RET antibody (*bottom*). Data are representative of three independent experiments.

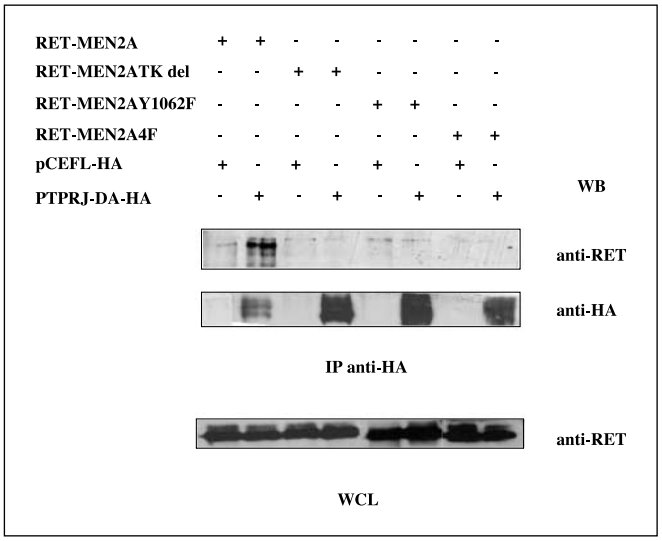

Figure 7. Tyr^{1062} is required for RET binding to PTPRJ. Cell lysates (1 mg) prepared from HEK293T cells transfected with 5 μg of the indicated RET plasmids together with 5 μg of PTPRJ-D/A were immunoprecipitated with anti-HA and immunoblotted with anti-RET antibody (*top*). Membranes were reprobed with anti-HA antibody (*middle*). Expression levels of the transfected RET proteins were detected by immunoblotting whole-cell lysates (100 μg) with anti-RET (*bottom*). Data are representative of three independent experiments.

Table 1. Focus-forming ability of RET-MEN2 oncogenes coexpressed with different amounts of PTPRJ

Experiment	Foci no.						
	Vector	RET-MEN2A	RET-MEN2A/ PTPRJ (1:1)	RET-MEN2A/ PTPRJ (1:3)	RET-MEN2B	RET-MEN2B/ PTPRJ (1:1)	RET-MEN2B/ PTPRJ (1:3)
1	5	68	30	6	73	80	78
2	7	75	25	12	85	65	89

NOTE: NIH3T3 fibroblasts were transfected with 1 μg of RET-MEN2A or RET-MEN2B and different amounts of PTPRJ cDNA. Transformed foci were scored 3 weeks later. Two independent experiments are reported.

domain (PTP) of PTPRJ (Fig. 5*A*). Protein extracts from HEK293T cells transfected with RET-MEN2A, RET/PTC1, or RET-MEN2B were immunoprecipitated with anti-RET antibody and incubated with 1 or 3 μg of the purified PTP-His_6 protein; RET phosphorylation was then assessed by phosphorylated immunoblot. RET-MEN2A and RET/PTC1 Y1062 and Y905 were efficiently dephosphorylated in the presence of PTP-His_6 in a dose-dependent manner (Fig. 5*B*). In the *in vivo* experiments (Fig. 2), PTPRJ exerted a stronger effect on RET-MEN2A than on RET/PTC1. It is noteworthy that the RET-MEN2B protein was insensitive to the N-PTPRJ-His_6 phosphatase activity (Fig. 5*B*).

Molecular determinants of the RET-MEN2A/PTPRJ interaction. We next evaluated the effects of different point mutations of PTPRJ on its interaction with RET-MEN2A. RET-MEN2A was cotransfected with HA-tagged WT, substrate-trapping (D/A), or catalytically inactive (C/S) mutant forms of PTPRJ cDNA in HEK293T cells. The cell extracts were immunoprecipitated with anti-HA and blotted with anti-RET. RET-MEN2A readily coimmunoprecipitated with WT and substrate-trapping (D/A) PTPRJ (Fig. 6). Importantly, the catalytic-dead C1239S PTPRJ mutant did not associate to RET-MEN2A (Fig. 6). Thus, Cys^{1239} is required for the interaction between PTPRJ and the RET-MEN2A protein.

To identify the RET-MEN2A domains required for PTPRJ binding, we transfected the PTPRJ-D/A-HA construct into HEK293T cells together with RET-MEN2A TK del that lacks the entire cytosolic domain (from amino acid 712 to the C terminus), RET-MEN2A(4F) that carries tyrosine-to-phenylalanine substitutions at Y826, Y1015, Y1029, and Y1062 or RET-MEN2A(Y1062F) that is mutated in the multiple docking site. Cell extracts were immunoprecipitated with anti-HA and blotted with anti-RET. As shown in the Fig. 7, coimmunoprecipitation was observed with the RET-MEN2A but not with the RET-MEN2A TK del, RET-MEN2A(4F), and with the MEN2A Y1062F mutant. This result indicates that RET cytosolic portion and, particularly, Y1062 were critical for the binding of RET to PTPRJ.

PTPRJ inhibits RET-MEN2A-mediated neoplastic transformation. We used a focus formation assay to evaluate the transforming ability of RET-MEN2A in the presence or absence of PTPRJ. We transfected NIH3T3 cells with RET-MEN2A and increasing amounts of PTPRJ and counted transformed foci 3 weeks later. As shown in Table 1, PTPRJ dose dependently reduced the transforming activity of RET-MEN2A. As expected, PTPRJ did not affect the transforming activity of RET-MEN2B (Table 1).

***Ptprj* gene overexpression reduces colony formation by TPC1 and FB2 cells.** We used human PTC, which spontaneously harbor the RET/PTC1 oncogene, to verify the effect of PTPRJ on RET/PTC1. We transfected FB2 and TPC1 cells with PTPRJ, its C/S mutant, or the empty vector and counted the G418-selected colonies. In both cell lines, the number of colonies was smaller (at least 10-fold) after transfection with PTPRJ than after transfection with the empty vector or the phosphatase-dead mutant (Table 2).

Protein lysates from TPC1 cells transiently transfected with PTPRJ, its C/S mutant, or the empty vector were immunoblotted with anti-RET Y1062 and anti-Shc Y239/Y240 phosphorylated-specific antibodies. The PTPRJ-transfected TPC1 cells showed a significant decrease in Tyr^{1062} phosphorylation of RET/PTC1 and in Tyr^{239}/Tyr^{240} phosphorylation of Shc compared with untransfected cells (Fig. 8).

These findings indicate that PTPRJ exerts growth suppressor effects in RET/PTC1-positive human PTC cell lines.

Table 2. Colony-forming ability of TPC1 and FB2 cells transfected with PTPRJ

Experiment	Colony no.					
	TPC1			FB2		
	Vector	PTPRJ	PTPRJ-C/S	Vector	PTPRJ	PTPRJ-C/S
1	125	18	118	101	15	85
2	140	22	120	98	18	96

NOTE: The indicated cell lines were transfected with 5 μg of PTPRJ or PTPRJ-C/S DNAs. Cells were marker selected with G418, and colonies were counted 3 weeks later. Two independent experiments are reported.

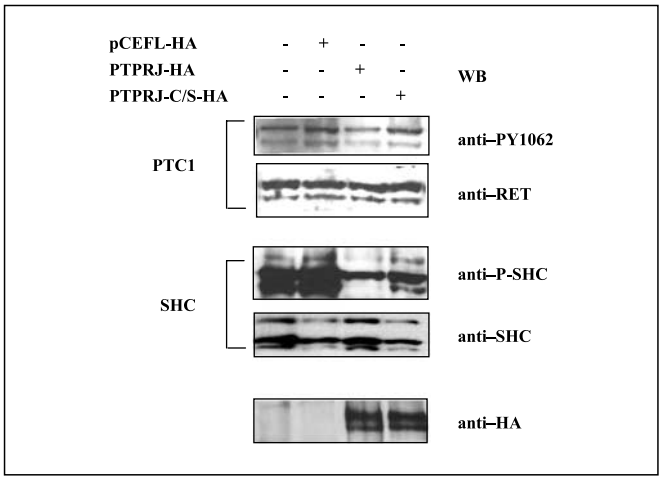

Figure 8. PTPRJ expression impairs activation of RET/PTC1 and Shc in TPC-1 cell line. Cell lysates (100 μg) prepared from TPC-1 cells or transiently transfected with 5 μg of empty vector or PTPRJ-HA or PTPRJ-C/S-HA were analyzed by immunoblotting with phosphorylated-specific RET antibody (Tyr^{1062}) and phosphorylated Shc (*P-SHC*; Tyr^{239}/Tyr^{240}) antibody. Total RET and Shc amounts. Membranes were reprobed with anti-HA antibody. Data are representative of three independent experiments.

Discussion

A key mechanism in the regulation of cell growth and differentiation is phosphorylation of proteins on tyrosine residues. This process is controlled by two families of enzymes: protein tyrosine kinases and PTPs. PTPRJ is a receptor-like PTP and a potential tumor suppressor (11–13, 17). We showed previously that PTPRJ expression is reduced in human thyroid carcinoma specimens and retrovirally transformed thyroid follicular cells in culture (2, 15, 16). However, the targets of PTPRJ in thyroid carcinomas are unknown.

PTPRJ can dephosphorylate specific receptor tyrosine kinases [i.e., HGF (9), VEGF (10), and PDGF β (6, 7)]. Different types of thyroid carcinomas feature oncogenic activation of RET, with RET/PTC rearrangements being found, on average, in 30% of PTC and RET point mutations in up to 50% of sporadic MTC and in virtually all cases of familial MTC. Thus, we investigated whether RET could be a target of PTPRJ. Here, we show that PTPRJ is able to induce RET dephosphorylation in tyrosine residues (mainly Y1062) that are crucial for RET signaling and, hence, that PTPRJ reduces Shc/ERK signaling in the RET-MEN2A and RET/PTC1 oncoproteins. In RET-MEN2A, PTPRJ-induced dephosphorylation was accompanied by physical interaction between the two proteins. Under our conditions, PTPRJ did not physically interact with RET/PTC1, which probably explains the overall reduced ability of PTPRJ to dephosphorylate RET/PTC1. PTPRJ is membrane bound; hence, delocalization of RET/PTC1 to the cytosol could account for this weak or absent association. This, notwithstanding, PTPRJ counteracted RET/PTC1-mediated proliferation as well as RET-MEN2A-mediated neoplastic transformation, thereby functioning as a bona fide RET-MEN2A and RET/PTC anti-oncogene. We mapped the molecular determinants of the RET/PTPRJ interaction on the RET and PTPRJ proteins and found that the Y1062 RET residue is necessary for the binding of RET to PTPRJ. Thus, the tyrosine residue that mediates most RET signaling downstream events also turns RET signaling off by recruiting the negative regulator PTPRJ. Because it has been shown previously that the leukocyte common antigen-related (LAR) PTP, a prototypic member of the receptor-type PTP subfamily, is able to bind to RET-MEN2A and RET-MEN2B *in vitro* (28), we retain likely that the same direct mechanism might account also for the RET-MEN2A/PTPRJ interaction.

Our observation that the RET-MEN2B mutant does not bind PTPRJ and is not dephosphorylated by PTPRJ echoes the finding of Qiao et al. (28) that, unlike RET-MEN2A, the RET-MEN2B protein resists dephosphorylation by LAR, another transmembrane PTP protein. The MEN2B (M918T) mutation targets the catalytic domain of RET and is believed to change the conformation of the RET kinase domain and alter the RET substrate specificity. Thus, it is conceivable that the M918T mutant affects RET binding to the PTP protein. MEN2B is a more aggressive disease than other MEN2 subtypes, and the MEN2B mutation is the most frequent RET mutation in sporadic cases of MTC (29). It is tempting to speculate that resistance to inhibition by PTPRJ may account for the high oncogenic activity of RET alleles carrying the MEN2B mutation.

About the biological consequences of RET-MEN2A dephosphorylation by PTPRJ, we show that PTPRJ drastically reduced the number of foci induced by RET-MEN2A in NIH3T3 cells. Similarly, PTPRJ overexpression in two thyroid carcinoma cell lines bearing the RET/PTC1 oncogene reduced cell growth.

In conclusion, PTPRJ levels can affect the activation and transforming ability of the RET/PTC and RET-MEN2A oncogenes. Consequently, loss of PTPRJ function could be related to the pathogenesis of thyroid carcinomas associated with these RET mutations.

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