Substrate Specificity of R3 Receptor-like Protein-tyrosine Phosphatase Subfamily toward Receptor Protein-tyrosine Kinases*

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**Significance:** Our understanding of the physiological relevance between receptor-like protein-tyrosine phosphatase (RPTP) and receptor protein-tyrosine kinase (RPTK) is limited.

**Results:** Multiple RPTKs were identified as substrates for the R3 RPTP subfamily.

**Conclusion:** Members of the R3 subfamily show a similar but distinct specificity toward RPTKs.

**Significance:** This study sheds light on physiological roles of the R3 RPTP subfamily.

Receptor-like protein-tyrosine phosphatases (RPTPs) are involved in various aspects of cellular functions, such as proliferation, differentiation, survival, migration, and metabolism. A small number of RPTPs have been reported to regulate activities of some cellular proteins including receptor protein-tyrosine kinases (RPTKs). However, our understanding about the roles of individual RPTPs in the regulation of RPTKs is still limited. The R3 RPTP subfamily reportedly plays pivotal roles in the development of several tissues including the vascular and nervous systems. Here, we examined enzyme-substrate relationships between the four R3 RPTP subfamily members and 21 RPTK members selected from 14 RPTK subfamilies by using a mammalian two-hybrid system with substrate-trapping RPTP mutants. Among the 84 RPTP-RPTK combinations conceivable, we detected 30 positive interactions: 25 of the enzyme-substrate relationships were novel. We randomly chose several RPTKs assumed to be substrates for R3 RPTPs, and validated the results of this screen by in vitro dephosphorylation assays, and by cell-based assays involving overexpression and knockdown experiments. Because their functional relationships were verified without exception, it is probable that the RPTKs identified as potential substrates are actually physiological substrates for the R3 RPTPs. Interestingly, some RPTKs were recognized as substrates by all R3 members, but others were recognized by only one or a few members. The enzyme-substrate relationships identified in the present study will shed light on physiological roles of the R3 RPTP subfamily.

Cellular tyrosine phosphorylation levels are governed by the opposing activities of protein-tyrosine kinases (PTKs)3 and protein-tyrosine phosphatases (PTPs). Some 90 PTK genes exist in the human genome, 58 of which encode receptor PTKs (RPTKs), and 32 non-receptor PTKs (1). RPTPs consist of an extracellular region with a ligand-binding domain, a single transmembrane domain, and a cytoplasmic region containing a tyrosine kinase domain. Ligand binding to the extracellular region of RPTPs induces dimerization of RPTK and leads to intermolecular autophosphorylation of the cytoplasmic region (2). Autophosphorylation of one or two tyrosine residues in the catalytic core of RPTKs is critical for activation of the tyrosine kinase activity, and other tyrosine phosphorylated sites in the cytoplasmic region serve as binding sites for specific downstream signaling molecules. The resultant RPTK-signaling molecule complex initiates a specific signal transduction.

The human genome contains 107 genes for PTPs (3), 38 of which encode classical tyrosine-specific PTPs: 20 transmembrane receptor-like PTPs (RPTPs) and 18 intracellular non-receptor PTPs. RPTPs consist of an extracellular domain, a single transmembrane domain, and a cytoplasmic portion with one or two tyrosine phosphatase domains. RPTPs are classified into eight subfamilies (R1/R6, R2a, R2b, R3, R4, R5, R7, and R8), based on the sequence homology of their extracellular and PTP domains (4). Although the extracellular region of RPTPs is highly variable between subfamilies, members of a subfamily share similar structures. Therefore, it has been supposed that subfamily members share similar physiological functions, although they may play different roles in some situations.

The R3 subfamily has a single PTP domain intracellularly. They share similar structures with fibronectin type III-like repeats in the extracellular region (4). The R3 subfamily includes Ptpro, Ptprb, Ptprr, and Ptpq. The members play important roles in developmental and physiological functions in several tissues including the vascular and nervous systems (5). However, there is little information about the substrates or substrate specificity of the R3 subfamily.

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§ The abbreviations used are: PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; RPTK, receptor protein-tyrosine kinase; RPTP, receptor-like protein-tyrosine phosphatase; TFP, teal fluorescence protein; ICR, intracellular region; HUVEC, human umbilical vein endothelial cell; AD, activation domain; BD, binding domain; GDNF, glial cell-derived neurotrophic factor; GFRA1, GDNF family receptor α1.
Substrate Specificity of R3 RPTP Subfamily Toward RPTKs

Some RPTPs appear to be involved in the regulation of RPTKs through dephosphorylation as substrates (inhibition of RPTK activation). For example, we have shown that Ptpro plays an important role in axonal navigation through the regulation of Eph receptors (6). In addition, Ptprrz dephosphorylates ErbB4 and TrkA as physiological substrates (7, 8). However, our understanding of the functional relationship between RPTKs and RPTPs is still limited.

We performed a large scale examination of the enzyme-substrate interaction between all RPTPs and representative RPTKs covering RPTK subfamilies by conducting mammalian two-hybrid assays with substrate-trapping mutants. In this screening, substrate-trapping mutants of the R3 RPTP subfamily showed marked interaction with multiple RPTKs. We therefore examined the relationship between the R3 subfamily and RPTKs in detail in the present study. To verify that the R3 subfamily indeed regulates these RPTKs as substrates, we examined effects of overexpression and knockdown of RPTPs in cells. Importantly, the R3 proteins showed differences in specificity toward individual RPTKs.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-FLAG, anti-Myc, and anti-HA antibodies were purchased from Sigma. Anti-phosphotyrosine (4G10) and anti-phosphorylated Eph receptor antibodies were from Millipore. Anti-FGFR1 and anti-phosphorylated FGFR1 antibodies were from Cell Signaling. Anti-Ptprrb and anti-EphA4 antibodies were from Santa Cruz. Anti-Ptprj antibody was from R&D Systems. Alexa-conjugated secondary antibodies were from Invitrogen.

DNA Constructs—Mouse EphA4 and EphB2 cDNAs were cloned by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA from mouse brain. Other RPTKs were cloned by RT-PCR using total RNA from human brain, spleen, and cell lines as templates. cDNAs for RPTKs were cloned by RT-PCR using total RNA from mouse organs as templates. Substrate-trapping DA RPTP mutants, in which a conserved aspartic acid in the active center was substituted with alanine, were constructed by site-directed mutagenesis.

The intracellular regions (ICRs) of RPTKs were subcloned into the pCMV-BD (Stratagene) to produce fusion proteins comprising the Gal4 DNA-binding domain and a RPTK ICR (BD-RPTKs). The ICRs of RPTPs were subcloned into the pCMV-AD (Stratagene) to produce fusion proteins comprising the NF-κB activation domain and a RPTP ICR (AD-RPTPs). The ICRs of RPTPs were also subcloned into the vector pGEX-4T (GE Healthcare) to produce fusion proteins with glutathione S-transferase (GST).

The full-length cDNAs of human FGFR1, TYRO3, and RET were subcloned into the expression vector p3xFLAG-myc-CMV-23 (Sigma) to produce FLAG-tagged RPTK proteins (FLAG-RPTKs). The full-length cDNA of mouse EphB2 was subcloned into the expression vector pcDNA-Myc to produce Myc-tagged EphB2 protein (EphB2-Myc). The full-length RPTPs were subcloned into the expression vector pDisplay (Invitrogen) to produce HA-tagged RPTP proteins (HA-RPTPs). The DNA fragments encoding HA-tagged full-length RPTPs from pDisplay-RPTP plasmids were also subcloned into the expression vector pBabe-puro, which contains a puromycin resistance gene, for the cell proliferation assay.

Short Hairpin RNA (shRNA) Constructs—The pBasi-hU6 Pur vector (Takara Bio, Shiga, Japan), which contains a puromycin resistance gene, was used to express shRNAs to knockdown the expression of targeted genes in HUVEC-C cells. To construct shRNA vectors, the following oligonucleotide DNAs were inserted into BamHI–XbaI sites of the pBasI vector: Control scrambled shRNA, 5′-GATCCGCTGTCATAGGGTTCGTTCTAGTTATGTCTGCGATTTGTTAGGACGAGTTTTTTTA-3′ and 5′-CTAGTAAAAAACGCTTCTAGTCCTAGTACACATACCTGACACACACACACAGCGAGCTTTTTTAA-3′ and 5′-CTAGTAAAAAACGCTTCTAGTCCTAGTACACATACCTGACACACACACACACACAGCGAGCTTTTTTAA-3′.

Cell Culture and cDNA Transfection—COS7 and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium (1:1, Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS). HUVEC-C cells were culture in HCB-1310 medium (Invitrogen) supplemented with 10% FBS and 10 ng/ml of basic FGF (Wako, Osaka, Japan). PC12D cells were cultured in DMEM/F-12 medium (1:1) supplemented with 10% FBS and 5% horse serum. COS7, HEK293T, and HUVEC-C cells were transfected with Lipofectamine Plus (Invitrogen), and PC12D cells, with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol.

Mammalian Two-hybrid Assay—COS7 cells grown on 96-well microplates were co-transfected with pBD-RPTK (80 ng) and a pAD-RPTP (80 ng), together with the reporter plas...
mid pFR-Luc (300 ng; Stratagene), which contains five Gal4-binding sites upstream of a TATA-box and the firefly (Photinus pyralis) luciferase gene. The pRL-TK plasmid (20 ng; Promega), which expresses sea pansy (Renilla reniformis) luciferase from a constant promoter, was also cotransfected to normalize the transfection efficiency. Luciferase assays were performed at 24 h after transfection with a dual luciferase reporter assay system (Promega), according to the manufacturer’s directions.

In Vitro Dephosphorylation Assay—For in vitro dephosphorylation, we first prepared autophosphorylated RPTK proteins as substrates. A FLAG-RPTK (or Eph-Myc) was transfected into HEK293T cells. After 24 h, cells grown on a 35-mm culture dish were lysed with RIPA buffer, and the lysates were subjected to immunoprecipitation with various antibodies bound to Protein G-Sepharose (BD Healthcare). Protein G beads were then washed once and resuspended in 100 µl of 10 mM Tris-HCl, pH 7.0, containing 5 mM DTT, 5 mM EDTA, and 100 µg/ml of bovine serum albumin (PTP buffer). For the dephosphorylation assay, 10 ng of GST–RPTPs or GST alone was reacted with 10 µl of RPTK solutions at 30 °C for 30 min. The samples were separated by SDS-PAGE, followed by immunoblotting with specific primary antibodies and peroxidase-linked secondary antibodies, and visualized by chemiluminescence using ECL Reagent.

Coexpression Assay of RPTK and RPTP—A FLAG-RPTK (or Eph-Myc) was cotransfected with a HA–RPTP into HEK293T cells grown on a 35-mm culture dish. After 24 h, cells were lysed in RIPA buffer, which consists of 20 mM Hepes, pH 7.0, 120 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.05% SDS, 50 mM Na3VO4, and a protease inhibitor mixture (1 µg/ml of peptatin A, 10 µg/ml of leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation, and subjected to immunoprecipitation with various antibodies bound to Protein G-Sepharose (BD Healthcare). Immunoprecipitates were treated with SDS sample buffer and subjected to SDS-PAGE. Proteins were transferred onto Immobilon-P membranes (Millipore), reacted with specific primary antibodies and peroxidase-linked secondary antibodies (BD Healthcare), and visualized by chemiluminescence using ECL Reagent.

Down-regulation of RPTPs in HUVEC-C Cells—The pBasi shRNA expression plasmids were introduced into cells using Lipofectamine Plus according to the manufacturer’s protocol. To select plasmid-containing cells, 12 h after transfection, puromycin was added to the culture medium at 2 µg/ml. After 24 h incubation, cells were re-plated at 5 × 104 cells per 35-mm dish in the medium containing 0.5% FBS. After another 12-h incubation, cells were treated with basic FGF (20 ng/ml, Wako), or ephrin-A2-Fc proteins (10 µg/ml) (6) for 10 min. Then, cells were analyzed by Western blotting similar to the RPTK/RPTP coexpression assays.

Cell Growth Assay—NIH3T3 cells stably expressing TYRO3 (NIH-TYRO3) were generated by transfection with the full-length TYRO3 expression plasmid and selection in medium containing 100 µg/ml of G418. Expression of TYRO3 in the individual isolated cells was confirmed by Western blotting and immunocytochemistry using an anti-FLAG antibody. NIH-TYRO3 cells were then transfected with the pBabe-RPTP expression plasmids. After selection with 1 µg/ml of puromycin for 24 h, cells (2 × 103/ml) were seeded onto 96-well plates in 100 µl/well of DMEM/F-12 medium containing 0.1% FBS, 50 µg/ml of G418, and 0.5 µg/ml of puromycin. After 12 h of incubation, the culture medium was changed to DMEM/F-12 medium containing 100 ng/ml of Gas6 (R & D Systems), 0.1% FBS, 50 µg/ml of G418, and 0.5 µg/ml of puromycin, and further cultured for 48, 96, and 144 h, with the medium changed every 48 h. At the indicated time points, cell proliferation was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) according to the manufacturer’s instructions.

Analysis of Neurite Outgrowth—PC12D cells were seeded in 35-mm dishes precoated with 1 mg/ml of collagen-I (Nitta gelatin, Osaka, Japan) at a density 5 × 104 cells/dish. After 24 h, HA–RPTP plasmids (2 µg) were transfected together with a plasmid that expresses a fused protein construct comprising the full fluorescent protein (TFP) and PDGFR transmembrane domain (TFP-TM, 1 µg) to visualize the morphology of RPTP-transfected cells. After 24 h, the medium was replaced with a fresh batch containing basic FGF (50 ng/ml), or both GDNF (25 ng/ml, R&D Systems Inc.) and GFRα1 (300 ng/ml, R&D Systems Inc.). At 24 h after the addition of FGF, or 48 h after addition of GDNF/GFRα1, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. Cells were blocked with 3% bovine serum albumin for 30 min, and then reacted with an anti-HA antibody and an Alexa 488-conjugated secondary antibody. Cells positive for both HA and TFP were blocked with 3% bovine serum albumin for 30 min, and then reacted with an anti-HA antibody and an Alexa 488-conjugated secondary antibody. Cells positive for both HA and TFP were analyzed with a BX51TRF microscope (Olympus) equipped with a DP-70 CCD camera (Olympus). Neurite lengths were measured from 200 cells selected at random in three dishes. In colonies, only peripheral cells were examined. Processes more than twice as long as their soma diameters were estimated as long neurites. Data were analyzed with the Mann-Whitney U test.

RESULTS

Identification of Interactions between R3 RPTPs and RPTKs by Mammalian Two-hybrid Assays—To examine the enzyme-substrate interaction between RPTPs and RPTKs, we took advantage of mammalian two-hybrid assays with substrate-trapping mutants of PTPs (6). The respective DA mutant in which the general aspartic acid (D) residue in the PTP domain is converted to alanine (A) retains the ability to recognize and bind substrates stably although the phosphatase activity is lost (Fig. 1A) (9). In the present study, we first examined the molecular interaction between the ICR of the R3 members (Ptprb, Ptprh, Ptpj, and Ptpro) with the DA mutation and the ICR of RPTKs in COS7 cells. We excluded Ptprq in the R3 members, because Ptprq has been shown to be a lipid phosphatase (10). As for RPTKs, we selected 21 RPTKs from 14 subfamilies (Fig. 1B). Of note, RPTKs are autophosphorylated (activated) without ligand binding when highly expressed in mammalian cells, which is a prerequisite for our two-hybrid screen.

From 84 combinations of the four R3 members and 21 RPTKs, 30 positive interactions were detected (Fig. 1C). Importantly, these positive interactions were not detected when the
Among the positive interactions, some have been already reported, indicating the effectiveness of the mammalian two-hybrid assay: enzyme-substrate relationships between Ptpro and EphA4 or EphB2 (6), Ptprb and MET (11), Ptpjr and MET (12), and Ptprj and RET (13). The others, 25 enzyme-substrate relationships, were new. The finding that the substrate candidates encompass 10 RPTK subfamilies suggests that the R3 RPTP subfamily is involved in the regulation of a variety of RPTKs. Interestingly, the R3 RPTP subfamily members showed similarities in specificity toward some RPTKs and differences toward others. For example, whereas ICRs of all of the R3 subfamily show interaction with ICRs of EphA4, EphB2, and TYRO3, only one member of the subfamily recognized FGFR1, PDGFRα, VEGFR1, VEGFR2, or ROS (Fig. 1C).

Dephosphorylation of EphB2, TYRO3, RET, and FGFR1 by the R3 RPTP Subfamily—To validate the relationships identified, we first tested whether R3 RPTP members directly dephosphorylate RPTKs as substrates by in vitro experiments using purified proteins. We selected EphB2, TYRO3, RET, and FGFR1 as representative candidates of the substrate for the R3 RPTP subfamily. When Myc-tagged EphB2 and FLAG-tagged TYRO3, RET, and FGFR1 were expressed in full-length in HEK293T cells, highly tyrosyl-phosphorylated EphB2 (~120 kDa), TYRO3 (~120 kDa), RET (~170 kDa), and FGFR1 (~130 kDa) proteins were obtained. The ICRs of the R3 RPTPs were prepared as GST fusion proteins, and reacted with each RPTK protein in vitro. As expected, GST-Ptpro and -Ptprb efficiently dephosphorylated all of the four RPTKs (Fig. 2). On the other hand, GST-Ptpjr selectively reduced the tyrosine phosphorylation levels of RET, TYRO3, and EphB2, but not FGFR1, and GST-Ptprh reduced those of TYRO3 and EphB2, but not those of FGFR1 and RET (Fig. 2).

We next examined whether the full-length wild-type R3 RPTPs dephosphorylate the full-length RPTKs in vitro when an R3 RPTP member was coexpressed in HEK293T cells, through investigation of phosphorylation levels of RPTKs. When EphB2, TYRO3, RET, or FGFR1 were expressed in full-length in HEK293T cells, highly tyrosyl-phosphorylated EphB2, TYRO3, RET, and FGFR1 proteins were detected by immunoblot analyses (Fig. 3). Of note, coexpression of an RPTP did not affect the expression level of the RPTKs (Fig. 3). To quantitatively estimate the tyrosine phosphorylation levels of RPTKs, RPTP proteins were immunoprecipitated with antibodies against tags, and analyzed with anti-phosphotyrosine antibody (Fig. 4). As was expected, tyrosine phosphorylation levels of all four RPTKs were significantly suppressed by coexpression of Ptpro (Fig. 4A), or Ptprb (Fig. 4B). On the other hand, Ptpjr specifically suppressed tyrosine phosphorylation levels of RET, TYRO3, and EphB2, but not FGFR1 (Fig. 4C); and Ptprh suppressed phosphorylation of TYRO3 and EphB2, but not that of FGFR1 and RET (Fig. 4D). Together with the in vitro experiments above, these results were highly consistent with the results of the mammalian two-hybrid assay (Fig. 1C), indicating that the relationships identified are credible. Interestingly, the results show that there exist distinct differences in substrate specificity among the R3 RPTPs toward RPTKs in a narrow sense.
Enhancement of Tyrosine Autophosphorylation of EPH Receptors and FGFR1 by Knockdown of R3 RPTPs—To further verify that RPTKs identified as substrates are indeed physiological substrates for R3 RPTPs, we searched cell lines expressing R3 RPTPs together with their candidate substrates endogenously. Because HUVEC-C human endothelial cells expressed EPH receptors (at least EPHA2, EPHA4, and EPHB2) and FGFR1 as well as PTPRB and PTPRJ, we examined the effects of knockdown of PTPRB and PTPRJ on the phosphorylation level of EPH receptors or FGFR1 using specific shRNA in this cell line (Fig. 5A). An ~75 and 60% knockdown was attained for PTPRB and PTPRJ expression, respectively, after 48 h of transfection with shRNA expression plasmids in HUVEC-C cells, as compared with the scrambled shRNA controls (Fig. 5B).

The expression level of EPH receptors in HUVEC-C cells was similar under different experimental conditions (Fig. 5C), and no differences in the basal tyrosine phosphorylation of EPH receptors were detected by Western blotting (without ephrin-A2-Fc in Fig. 5C). When treated with ephrin-A2-Fc (10 μg/ml) for 10 min, a high level of tyrosine phosphorylation was
detected in control cells (with ephrin-A2-Fc in Fig. 5C). However, the tyrosine phosphorylation of EPH receptors was significantly suppressed in PTPRB and PTPRJ double knockdown cells compared with control cells (Fig. 5C). Here, it should be noted that single knockdown of PTPRB or PTPRJ did not affect the ephrin-A2-Fc-stimulated tyrosine phosphorylation of EPH receptors (data not shown). Thus, both PTPRB and PTPRJ recognize and dephosphorylate EPH receptors independently as physiological substrates.

We also examined the effect of knockdown of PTPRB on ligand-induced tyrosyl phosphorylation of FGFR1 by using an anti-phosphorylated FGFR1 antibody. In the absence of FGF, a low level of tyrosine phosphorylation of FGFR1 was observed in control cells (Fig. 5D). When treated with basic FGF (20 ng/ml) for 10 min, a high level of tyrosine phosphorylation was detected in control cells (Fig. 5D). In contrast to the EPH receptors, knockdown of PTPRB alone resulted in an enhancement of FGF-induced tyrosine phosphorylation of FGFR1 (Fig. 5D).

These results indicate that PTPRB negatively regulates FGFR1 signaling through dephosphorylation of FGFR1 as a physiological substrate.  

Suppression of TYRO3-dependent Cell Proliferation by R3 RPTPs—We examined the functional significance of the enzyme-substrate relationship between R3 RPTPs and RPTKs in the TYRO3-dependent cell proliferation. For this assay, we generated a NIH3T3 cell line stably expressing TYRO3 (NIH3T3-TYRO3). Then NIH3T3-TYRO3 cells were transfected with an RPTP expression construct or a control empty vector. When control NIH3T3-TYRO3 cells were treated with Gas6 (100 ng/ml) for 10 min, a ligand of TYRO3, tyrosyl-phosphorylated TYRO3 proteins were clearly detected by immunoblot analysis (Fig. 6A). However, forced expression of all R3 RPTPs universally suppressed the Gas6-induced tyrosine phosphorylation of TYRO3 proteins completely.

Next we examined proliferation of these transfected cells upon stimulation with Gas6 by 3-(4,5-dimethylthiazol-2-yl)-
FIGURE 5. Enhanced tyrosine phosphorylation of RPTKs by suppression of R3 RPTPs. A, schematic representation of PTPRB and PTPRJ. The sites targeted by shRNAs are indicated. FNIII, fibronectin type III repeat domain; PTP, PTP domain. B, suppression of PTPRB or PTPRJ expression by specific shRNAs in HUVEC-C cells. HUVEC-C cells were transfected with the indicated shRNA expression plasmids. After selection with puromycin, RPTP proteins were detected with RPTP-specific antibodies by Western blotting. The right panels show a summary of the expression levels of RPTPs. The expression levels are shown relative to the control. Data are given as the mean ± S.E. (n = 4). The asterisk indicates a significant difference from the control (**, p < 0.01). C, enhancement of activation of Eph receptors by expression of PTPRB- and PTPRJ-specific shRNAs. HUVEC-C cells were transfected with the indicated shRNA expression plasmids. After selection with puromycin, cells were stimulated with a ligand for Eph receptors, ephrin-A2-Fc (10 μg/ml). Cell lysates were analyzed with antibodies to phospho-EPH and EPHA4. The right panels show a summary of the phosphorylation levels of EPH. The phosphorylation levels are shown relative to the control, which was stimulated with ephrin-A2-Fc. Data are given as the mean ± S.E. (n = 4). The asterisk indicates a significant difference between the two values (**, p < 0.01). D, enhancement of activation of FGFR1 by PTPRB-specific shRNA expression. After selection with puromycin, cells were stimulated with basic FGF (20 ng/ml). Cell lysates were analyzed with antibodies to phospho-FGFR1 and FGFR1. The right panels show a summary of the phosphorylation levels of FGFR1. The phosphorylation levels are shown relative to the control, which was stimulated with basic FGF. Data are given as the mean ± S.E. (n = 4). The asterisk indicates a significant difference between the two values (**, p < 0.01). In the experiments of C and D, an equal amount of cell lysates was loaded on each lane. The Coomassie Brilliant Blue staining profiles of lysates were all identical (data not shown).

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At 24 h after basic FGF (50 ng/ml) treatment, ~60% of mock-transfected cells extended long neurites (Fig. 8). In contrast, neurite outgrowth was considerably attenuated in Ptpro- or

FIGURE 6. Suppression of tyrosyl phosphorylation of TYRO3 and TYRO3-dependent cell proliferation by R3 RPTPs. NIH3T3 cells stably expressing TYRO3 (NIH3T3-TYRO3) were transfected with an RPTP expression plasmid or a control empty vector. After selection with puromycin, cells were stimulated with Gas6 (100 ng/ml). A, suppression of tyrosyl phosphorylation of TYRO3 by R3 RPTPs. After stimulation with Gas6 for 10 min, TYRO3 proteins were immunoprecipitated (IP) with antibodies against FLAG and analyzed with the indicated antibodies by Western blotting (WB). RPTP proteins were also immunoprecipitated and detected with an anti-HA antibody. B, suppression of TYRO3-dependent cell proliferation by R3 RPTPs. Cell proliferation of transfected cells was measured at the indicated time points using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are given as the mean ± S.E. (n = 4). The asterisk indicates a significant difference against the control value (**, p < 0.01).

Inhibition of FGFR1- and RET-dependent Neurite Outgrowth in PC12D Cells by R3 RPTP Subfamily Members—Finally, we examined the functional role of R3 RPTPs in the FGFR- and GDNF/GFRα1-induced neurite outgrowth of PC12D cells that primarily occurs via FGFR1 (14–16) and RET (17), respectively. First, we confirmed that there was no significant difference in the expression level of RPTP proteins when R3 RPTPs were individually transfected in PC12D cells (Fig. 7). When cultured in the absence of FGF or GDNF/GFRα1, none of the RPTP-transfected nor the parental PC12D cells extended long neurites (data not shown).

At 24 h after basic FGF (50 ng/ml) treatment, ~60% of mock-transfected cells extended long neurites (Fig. 8). In contrast, neurite outgrowth was considerably attenuated in Ptpro- or
Ptprb-transfected cells: only ~30% of Ptprb- or Ptpro-transfected cells had long neurites (Fig. 8). The inhibition was dependent on the PTPase activity of Ptpro or Ptprb, because PC12D cells transfected with phosphatase-inactive DA mutants of these RPTPs normally showed neurite extension like the mock-transfected cells. Expression of either Ptprj or Ptprh had no effect on neurite outgrowth (Fig. 8). These results indicate that Ptpro and Ptprb negatively regulate ligand-in
duced FGFR1 signaling through dephosphorylation of the FGFR1 receptor.

The GDNF/GFRα1 complex is also known to induce neurite extension in PC12D cells via the RET receptor (17). Next, we examined whether GDNF/GFRα1-induced neurite extension was affected by expression of the R3 RPTPs. In mock-transfected cells, GDNF/GFRα1 treatment for 48 h induced neurite extension: ~60% of the cells extended long neurites (Fig. 9). In contrast, GDNF/GFRα1-induced neurite extension was inhibited in Ptpro-, Ptprb-, or Ptprj-transfected cells: only ~20% of cells had long neurites (Fig. 9). Expectedly, expression of Ptprh had no effect on the GDNF/GFRα1-induced neurite extension. In addition, no significant inhibition of neurite extension was observed in cells transfected with the DA mutant of Ptpro, Ptprb, or Ptprj. These results indicate that Ptpro, Ptprb, and Ptprj act as negative regulators of RET signaling.

**DISCUSSION**

Previously we identified substrates for Ptpro (with a single PTP domain) (6) and Ptprz (with tandem PTP domains) (18, 19) by using a two-hybrid system with substrate-trapping RPTP mutants. In this study, we expanded the screening to include almost the entire RPTK family to find substrates for the R3 RPTP subfamily by using mammalian cDNA clones. Our results suggest that the R3 RPTP subfamily members have similar but distinct substrate specificities toward several RPTKs: ROS and PDGFRα were recognized only by Ptprb; EGFR1, VEGFR1, and VEGFR2 only by Ptprh; FGFR1 and ERBB2 by Ptpro and Ptprb; RET, MET, and LTK by Ptpro, Ptprb and Ptprj; whereas EphA4, EphB2, and TYRO3 were recognized by all members.

The validation of the results was verified by subsequent analyses. For a RPTK to be considered a physiological substrate for a RPTP, the following criteria must be fulfilled: (i) the substrate-trap mutant of the RPTP should bind to the RPTK, (ii) the RPTP should directly dephosphorylates target RPTK proteins in vitro, (iii) coexpression of the RPTP should suppress autophosphorylation of the RPTK in cells, and (iv) enhancement of the tyrosine phosphorylation of the RPTK should be detectable by knockdown of the RPTP in cells, or (v) overexpression of the RPTP should inhibit the RPTK signal-dependent event in cells. Eph receptors, TYRO3, RET, and FGFR1, were verified to be physiological substrates for the R3 RPTPs based on these criteria. Because these RPTKs tested actually fulfilled the criteria without exception, it is conceivable that other RPTKs identified as substrate candidates (Fig. 1C) in the present study are also physiological substrates for the R3 RPTPs.

Mice with a single mutation of a RPTP gene often display a milder phenotype than expected. For example, Ptprj-null mice are viable, fertile, and show no gross abnormalities (20). The Ptpro-null mutant also has little phenotype in the formation of neural circuits (21). The substrate redundancy among the R3 subfamily revealed by our study may explain the reason; lack of one R3 member in the single mutant mice is compensated for by other members. In support of this view, enhancement of activation of EPH receptors was attained only by simultaneous repression of PTPRB and PTPRJ. Analyses of double or triple mutant mice should thus reveal the physiological roles of R3 RPTPs. We previously reported that chick Ptprj cannot dephosphorylate Eph receptors (6). This difference may be attributable to a species difference: the amino acid sequences of the ICR of mouse Ptprj and chick Ptprj have only 78% identity, whereas those of Ptpro have 93% identity. This is probably the reason why topographic retinotectal projections in chick were heavily impaired by knockdown of Ptpro alone (6).
Substrate Specificity of R3 RPTP Subfamily Toward RPTKs

The findings of enzyme-substrate relationships between RPTPs and RPTKs would improve our understanding of the physiological functions of R3 RPTPs \textit{in vivo}. Here, the combinations of R3 RPTPs and substrate RPTKs are important \textit{in vivo}, because a RPTP dephosphorylates a substrate RPTK only when they are expressed in the same cell. Ptpro is preferentially expressed in the nervous system and kidney (22), and expression of Ptprb is restricted to endothelial cells (23). Ptprrj is broadly expressed in various cell types, including endothelial, epithelial, hematopoietic, and neuronal cells (24). On the other hand, expression of Ptprh is restricted to the gastrointestinal tract, and its protein product localizes to the microvilli of gastrointestinal epithelial cells (25). According to the data of Gene Expression Omnibus (GEO, NCBI), all of the RPTKs identified as substrate candidates are expected to be coexpressed with the corresponding R3 RPTPs.

Eph receptors are the largest family of RPTKs and classified into two groups, A-type (EphAs) and B-type (EphBs) (26). At least one member of the Eph receptors is likely expressed in every tissue (27). Eph receptors are implicated in a wide array of cellular responses including cell adhesion, boundary formation, and repulsion (28). Several members are also implicated in diseases such as tumorigenesis, tumor progression, and diabetes (27). Thus, the R3 members may be relevant to the development of these diseases through the control of Eph receptors. In support of this view, Ptprrj is considered to be a tumor suppressor gene in some cancers (29, 30). EphBs and their ligands, ephrin-Bs, are implicated in the formation of the blood vasculature: EphB2/B3 null and ephrin-B2 null mice die early in development with a disorganized vasculature (31). Ptprb or Ptprrj knock-in mice also show embryonic lethality due to vascular disorganization (32, 33). On the other hand, inactivation of EphB2 has been shown to accelerate tumorigenesis in the colon and rectum in heterozygous adenomatous polyposis coli (APC) mutant mice (34). Interestingly, Ptprh deficiency suppresses intestinal tumorigenesis in a heterozygous APC mutant background through an unknown mechanism (25). Here, Ptprrj presumably inhibits the tumor suppressor function of EphB2 in the intestine.

TYRO3, a member of the TAM family together with AXL and MER, is broadly expressed in the immune, nervous, reproductive, and vascular systems (35). Physiological roles of the TAM family have been investigated using a triple knock-out mouse. The most pronounced defect in the triple knock-out mouse was observed in the immune system: a severe lymphoproliferative disorder accompanied by broad spectrum autoimmunity (36). We showed that all R3 RPTP members recognize TYRO3 as a substrate. TYRO3 may be regulated by Ptprrj in the immune system, because Ptprrj is expressed in hematopoietic cells (37) and implicated in signal transduction (38). In addition, TYRO3 single mutant mice show activity-induced seizures (36). This suggests that the regulation of TYRO3 by Ptprrj and/or Ptprrj plays important roles in neuronal functions.

RET is expressed in the central and peripheral nervous system, and is known to be implicated in the maturation and survival of various neuronal types, including motoneurons, dopaminergic neurons, and peripheral sensory neurons (39). RET also plays important roles in axon growth and guidance, as well as synapse formation (39). We showed that Ptpro, Ptprrb, and Ptprrj dephosphorylate RET; Ptprrj is already known to dephosphorylate RET (13). Because Ptpro is expressed in the nervous system, it is likely involved in the regulation of RET activity in the nervous system. Consistently, abnormalities among sensory neurons were observed in Ptprrj-deficient mice (21). Moreover, Gatto \textit{et al.} (40) reported very recently that Ptpro regulates axon growth and branching of trigeminal sensory neurons through the suppression of RET signaling.

FGFR1 has been implicated in neural induction, patterning, survival, proliferation, and differentiation, and more recently, in axon pathfinding and synapse formation (41). FGFR1 was recognized by Ptpro and Ptprrb as a substrate. Therefore, it is probable that Ptpro functions in the formation of neural circuits through the regulation of not only Eph receptors (see above), but also FGFR1. FGFR1 is also implicated in vascular formation through activation of the vascular endothelial growth factor system (42). The vascular disorganization observed in Ptprrb-deficient mice (23) may be in part due to the dysregulation of FGFR1.

In conclusion, the R3 RPTPs appear to specifically play important roles through regulation of RPTKs as RPTK phosphatases. The enzyme-substrate relationship identified in our study will shed light on physiological roles of the R3 RPTP subfamily. This study is the first to demonstrate the similarities and differences in substrate specificities among a RPTP subfamily, which may be needed for drug development targeted to a specific RPTP.

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