Purification and Characterization of the Cytoplasmic Domain of Human Receptor-like Protein Tyrosine Phosphatase RPTPμ†

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ABSTRACT: RPTPμ is a recently described receptor-like protein tyrosine phosphatase (PTP), the ectodomain of which mediates homophilic cell–cell adhesion. The cytoplasmic part contains two homologous PTP-like domains and a juxtamembrane region that is about twice as large as in other receptor-like PTPs. The entire 80-kDa cytoplasmic part of human RPTPμ was expressed in insect Sf9 cells and its enzymatic activity was characterized after purification to electrophoretic homogeneity. In addition, the effects of deletion and point mutations were analyzed following expression in *Escherichia coli* cells. The purified cytoplasmic part of RPTPμ displays high activity toward tyrosine-phosphorylated, modified lysozyme (V_{max} 4500 nmol min^{-1} mg^{-1}) and myelin basic protein (V_{max} 8500 nmol min^{-1} mg^{-1}) but negligible activity toward tyrosine-phosphorylated angiotensin or the nonapeptide, EDNDpYINASL, that serves as a good substrate for protein tyrosine phosphatase PTP1B. This suggests that RPTPμ and PTP1B have distinct substrate specificities. Catalytic activity is independent of Ca^{2+} (up to 1 mM) but is strongly inhibited by Zn^{2+}, Mn^{2+}, vanadate, phenylarsenic oxide, and heparin. The first of the two catalytic domains is 5–10 times less active than the expressed catalytic region containing both domains. Mutation of Cys 1095 to Ser in the first catalytic domain abolishes enzymatic activity when analyzed following expression in either *E. coli* or mammalian COS cells. Deletion of the first 53 amino acids from the juxtamembrane region reduces catalytic activity about 2-fold.

Tyrosine-specific protein phosphorylation plays a central role in transmembrane signaling and the control of cell proliferation and differentiation (Yarden & Ullrich, 1988; Hunter, 1989). Tyrosine-phosphorylated proteins can be specifically dephosphorylated through the action of protein tyrosine phosphatases (PTPs).[1] Purification and sequencing of the first enzyme that could catalyze protein tyrosine dephosphorylation, termed PTP1B (Tonks et al., 1988a,b; Charbonneau et al., 1989), has led to the identification and subsequent cloning of a great number of PTPs [reviewed in Fischer et al. (1991), Charbonneau and Tonks (1992), and Pot and Dixon (1992)]. The family of PTPs can be classified into two major subgroups: (i) low molecular weight cytosolic proteins such as PTP1B and (ii) transmembrane, receptor-like PTPases, consisting of a N-terminal extracellular domain, a single transmembrane segment, and a cytoplasmic catalytic region. The ectodomains of the various receptor-PTPs are structurally very different. One widely studied subtype, CD45 or the leukocyte common antigen, has a large ectodomain unrelated to other protein sequences (Ralph et al., 1987), although recent analysis reveals the presence of at least one FNIII repeat (Bork et al., 1993). The ectodomains of a major subfamily comprising LAR (Streuli et al., 1988), RPTPμ (Gebbink et al., 1991), RPTPκ (Jiang et al., 1993), and DPTP and DLAR (Streuli et al., 1989) show similarities to cell adhesion molecules, whereas others have no sequence homology to known proteins. All receptor-like PTPs except for HPTPβ (Krueger et al., 1990) contain two tandem domains in their intracellular regions, each homologous to the low molecular weight PTPs. The second domain displays little or no activity toward artificial substrates in vitro, suggesting that this domain serves solely a regulatory function (Itoh et al., 1992; Streuli et al., 1989, 1990), although a recent report describes catalytic activity of the isolated second domain of CD45 (Tan et al., 1993).

We previously reported the cloning of a new receptor-like PTP, termed RPTPμ, from human and mouse cDNA libraries (Gebbink et al., 1991). The extracellular domain of RPTPμ contains one Ig-like and four fibronectin type III domains, reminiscent of the structure of neural cell adhesion molecules. Recently, we and others demonstrated that RPTPμ dramatically promotes cell-to-cell adhesion in a homophilic, Ca^{2+}-independent manner (Gebbink et al., 1993; Brady-Kalnay et al., 1993), indicating that RPTPμ may play a physiological role in signaling cell–cell recognition. Elucidation of the enzymatic properties and structure–activity relationship of RPTPμ will help in understanding its role in intercellular signal transduction. In the present study, we analyze the enzymatic characteristics of the intracellular part of RPTPμ as well as various mutant RPTPμ forms following expression in *Escherichia coli* and baculovirus-infected Sf9 insect cells.

MATERIALS AND METHODS

Restriction endonucleases and modifying enzymes were purchased from Boehringer Mannheim and New England Biolabs. Oligonucleotide primers were synthesized on a New

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[1] Abbreviations: PTP, protein tyrosine phosphatase; RPTP, receptor-like protein tyrosine phosphatase; FNIII, fibronectin type III; MBP, myelin basic protein; RCML, reduced, carboxamidomethylated, and maleylated lysozyme; BSA, bovine serum albumin; Sf9 cells, *Spodoptera Frugiperda* cells; FPLC, fast performance liquid chromatography; GST, glutathione S-transferase; PMSF, phenylmethane sulfonyl fluoride; PAO, phenylarsinic oxide; KLH, keyhole lympet hemocyanin; Ac-NPV, *Autographa californica* nuclear polyhedrosis virus; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IPTG, isopropyl β-thiogalactopyranoside; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; DTT, 1,4-dithiothreitol; ECl, enhanced chemoluminescence; NP-40, Nonidet P40; pNPP, *p*-nitrophenyl phosphate.

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Brunswick Biosearch DNA synthesizer. pNPP, PAO, spermine, and heparin were from Sigma.

Construction of Expression Vectors. Standard DNA manipulations and cloning procedures were carried out according to Sambrook et al. (1989). *Escherichia coli* strain DH5α was used for transformation of plasmids. Bacterial expression vectors pRP261, 265, and 269 [derivatives of pGEX1, 2, and 3 (Glutagene), kindly provided by K. Vink] containing multiple cloning sites 3′ from the glutathione S-transferase coding region were used for cloning human RPTPμ cDNA fragments. The 1200-bp *Eco*RI (bp 2077–3565) insert from clone mc12 (Gebbink et al., 1991) was subcloned into pRP261, resulting in pB1. A *Bam*HI fragment (bp 3867–4865) was released from pMT2TR (Gebbink et al., 1991) and subcloned into pRP269, resulting in pB2. pB4 was generated by subcloning a *Bgl*II–*Hin*dIII fragment (bp 2449–4865) from pMT2TR in pRP265. pB5 was made by introducing the *Kpn*I–*Hin*dIII (bp 2293–4865) fragment from pMT2TR in pRP265. Generation of a single point mutation in codon 1095 (changing a cysteine to a serine) has been described previously (Gebbink et al., 1993). pB5m containing this point mutation was generated by replacing a *Bgl*II–*Hin*dIII fragment (2449–4865) from hFLm (Gebbink et al., 1993). Expression of fusion proteins B1, B2, B4, B5, and B5m was induced by IPTG as described (Streuli et al., 1989). For expression of recombinant protein in Sf9 cells a baculovirus transfer vector, pVLTR (based on pVL1392, Invitrogen), containing a truncated RPTPμ, cDNA, coding for an N-terminal methionine followed by a valine and the complete intracellular part of RPTPμ, was made by replacing a *Bgl*II fragment (polylinker-bp 2449) from pVLhFL (Gebbink et al., 1993) by a *Bam*HI–*Bgl*II (2449) fragment from pMT2TR. Expression vectors pMT2hFL and pMT2hFLm, encoding full-length RPTPμ or point-mutated RPTPμ, were generated by subcloning *Kpn*I–*Xba*I fragments from Bluescript vectors hFL and hFLm in pMT2m (Gebbink et al., 1991).

Antibodies to RPTPμ. The anti-C-terminal polyclonal serum 37 raised in rabbits has been described (Gebbink et al., 1991). Antibody 34 was raised by immunizing rabbits with a synthetic peptide corresponding to residues 722–736 of the human RPTPμ ectodomain coupled to KLH. Mouse monoclonal antibody M3G4 directed against the extracellular fibronectin type III repeats 3 and 4 has been described (Gebbink et al., 1993).

Preparation and Purification of Recombinant Baculovirus. All procedures were carried out according to standard protocols (Summers & Smith, 1987). Sf9 cells (Invitrogen) were grown in supplemented Grace's insect medium (Life Technologies, Inc.) containing 10% fetal calf serum. Recombinant baculovirus was produced by cotransfecting Sf9 cells with 2 μg of Ac-NPV DNA (kindly provided by A. Flint, Cold Spring Harbor Laboratory) and 20 μg of pVLTR, using the standard calcium phosphate precipitation technique (Summers & Smith, 1987). Recombinant virus (Ac-pVLTR) was plaque-purified by visual screening, and expression of RPTPμTR was verified by immunoblotting.

Purification of Truncated RPTPμ from Cells Infected with Ac-pVLTR. Sf9 cells (5×10^8) were infected with recombinant virus at a multiplicity of 20 and incubated at 27 °C. Cells were harvested 60 h postinfection by centrifugation at 1500*g*. All of the following steps were carried out at 4 °C. The cells were washed once in PBS (PBS: 137 mM NaCl, 3 mM KCl, 8, 1 mM Na_2HPO_4, 1, 5 mM KH_2PO_4) and homogenized in 5 mL of buffer H (20 mM Tris, pH 7.6, 5 mM EDTA, 2 mM DTT, 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 1 mM PMSF) in a Dounce homogenizer. The homogenate was centrifuged for 5 min at 1000*g*. The supernatant was recentrifuged for 10 min at 80000*g*. The supernatant, or cytosol, was applied to a FPLC Mono Q column (HR 5/5, Pharmacia), equilibrated in buffer A (25 mM imidazole, pH 7.2, 1 mM EDTA, 2 mM DTT, 2 μg/mL aprotonin, 1 mM PMSF, and 0.1% Triton). Chromatography was performed at a flow rate of 0.5 mL/min. Proteins were eluted with a linear NaCl gradient (0–0.5 M) in buffer A. Peak fractions (21–29) were pooled, diluted in 10 mL of buffer B (25 mM Hepes, pH 7.2, 1 mM EDTA, 2 mM DTT, and 1 mM PMSF) and applied to a FPLC Mono S column (HR 5/5, Pharmacia) equilibrated in buffer B. Fractions were eluted with a NaCl gradient (0–1 M) in buffer B using a flow rate of 0.5 mL/min. Purified protein was stored in 50% glycerol at −70 °C.

PTP Assays. ^{32}P-Labeled tyrosine-phosphorylated MBP (Sigma), RCML (kindly provided by N. Tonks, Cold Spring Harbor Laboratory), angiotensin II (Sigma), and synthetic peptide EDNDYINASL (kindly provided by A. Flint, Cold Spring Harbor Laboratory; Flint et al., 1993) were used as substrates. These substrates were phosphorylated using insulin receptor kinase (kindly provided by A. Flint, Cold Spring Harbor Laboratory) purified from recombinant baculovirus-infected Sf9 cells. The kinase reaction was performed for 4 h in kinase buffer [50 mM Tris (pH 7.6), 4 mM $MnCl_2$, 10 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, and 0.1 mM Na_3VO_4] containing 500 μCi of [γ-^{32}P]ATP (Amersham). Free ATP was removed by TCA (50% in 25 mM NaH_2PO_4, containing 0.1% BSA) precipitation of substrate, several washes with 25% TCA in 25 mM NaH_2PO_4, and one wash with acetone. The substrates were dissolved in 25 mM imidazole, pH 7.4, and the content of phosphotyrosyl residues was determined. Phosphatase assays were performed in a 60-μL assay mixture containing 25 mM imidazole, pH 7.4, 0.05% β-mercaptoethanol, and 0.1 mg/mL BSA. Phosphatase reactions were performed at 37 °C. The reaction was terminated by the addition of 290 μL of activated charcoal solution [0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH_2PO_4, 4% (w/v) Norit A] (Streuli et al., 1989). After centrifugation for 10 min, the radioactivity in 200 μL of supernatant was measured. One unit is defined as the release of 1 nmol of phosphate/min. Protein concentration was measured with an assay kit (Bio-Rad) based on the method of Bradford (1976). Bacterial lysates for phosphatase assays were prepared and assayed from IPTG-induced cultures as described (Streuli et al., 1989). Small samples were prepared in SDS sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 2.5% glycerol, 25 mM DTT, and 0.01% bromophenol blue) for immunoblot analysis (see below).

Immunoblotting. Infected Sf9 cells were harvested by centrifugation for 5 min at 3000*g*. After being washed with PBS, cells were lysed in SDS sample buffer and boiled for 5 min. Samples containing approximately 5 μg of protein were subjected to SDS–PAGE (Laemmli, 1970). Following electrophoresis, the proteins were transferred to nitrocellulose filters. Filters were blocked with 5% milk powder (Nutricia) in TBST (TBST: 150 mM NaCl, 50 mM Tris, pH 8.0, and 0.2% Tween-20). Blots were subsequently incubated with anti-RPTPμ specific polyclonal antibodies (diluted 1:1000) for 1 h, washed 3 times with TBST, incubated with horseradish peroxidase-conjugated swine anti-rabbit antibody (Dako, Denmark), washed again, and developed using ECL (Amersham). Alternatively, an alkaline phosphatase conjugated

goat anti-rabbit antibody (Sigma) was used as described (Gebbink et al., 1991).

Transient Expression in COS Cells and Immunoprecipitation. Dishes (10 cm) containing approximately 5×10^5 COS-7 cells were transfected with 20 μg of plasmid pMT2hFL or pMT2hFLm as described (Gebbink et al., 1991). Cells were washed once with cold PBS and lysed on ice in NP-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1.5 mM EDTA, 1% NP-40, 1 mM DTT, 10% glycerol, 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 2 mM PMSF). Cell lysates (final volume 1 mL) were incubated for 2 h at 4 °C with protein A–Sepharose beads preincubated with antibody M3G4 (1 μg). Immunoprecipitates were washed 3 times with NP-40 lysis buffer and once with phosphatase assay buffer and assayed for phosphatase activity.

RESULTS AND DISCUSSION

The full-length cDNA sequences of both human and mouse RPTPμ have recently been reported (Gebbink et al., 1991). In brief, RPTPμ cDNA encodes a receptor-like PTP of 1452 amino acids (including signal peptide); the amino acid sequence of the complete intracellular part of RPTPμ (residues 765–1452) reveals two tandemly repeated domains with significant similarity to the catalytic domains of other PTPs. The entire intracellular part shows 35–45% overall identity to other members of the PTP family. Furthermore, the membrane-proximal stretch and amino acids that links the transmembrane segment to the first catalytic domain is about 70 residues longer than in most other receptor-like PTPs to date [with the exception of the closely related RPTPκ protein (Jiang et al., 1993)] and is relatively rich in Ser/Thr residues.

Expression of Recombinant RPTPμ in Sf9 cells. To explore the catalytic characteristics of RPTPμ we sought to purify the RPTPμ intracellular part from overproducing insect cells (Sf9 cells) using recombinant baculovirus, in which expression is driven by the strong viral polyhedrin promoter. Recently, we have successfully used this system to express the complete RPTPμ protein and elucidate its function as a homophilic cell adhesion molecule (Gebbink et al., 1993). Furthermore, the baculovirus expression system has proved to be useful for the enzymatic characterization of T-cell PTP (Zander et al., 1991) and RPTPα (Daum et al., 1991).

A recombinant baculovirus Ac-pVLTR was generated, which contains cDNA encoding the entire intracellular part (preceded by an artificial start codon, see Materials and Methods). To determine the time couse of expression, infected cells were harvested every 6 or 12 h postinfection and lysed, and the lysates were subjected to SDS–PAGE and immunoblot analysis. Expression of recombinant truncated RPTPμ (RPTPμTR), with a predicted M_r of 80 kDa, was already detectable at 12 h postinfection (Figure 1). Thereafter, expression levels increased significantly, but at 36 h postinfection a number of smaller protein products appeared, most likely representing proteolytic fragments. We also determined the tyrosine phosphatase activity at different times postinfection. Total activity in the cytosol of infected cells, using RCML as substrate, correlated with the increase in expression levels. At 72 h postinfection, the activity was 20–50-fold higher than in control cells infected with wild-type virus (data not shown).

Purification of the RPTPμTR from Sf9 Cells. The 80-kDa cytoplasmic region of RPTPμ was purified to apparent electrophoretic homogeneity from Ac-pVLTR-infected Sf9 cells, as illustrated in Figure 2. Cells were harvested 3 days postinfection followed by homogenization and centrifugation.

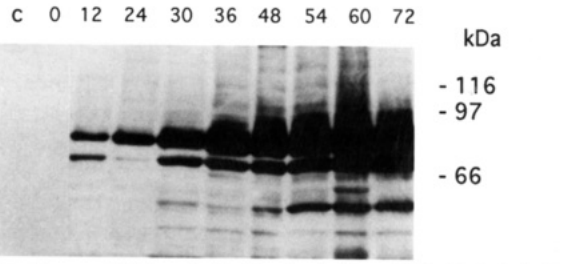

FIGURE 1: Immunoblot analysis of extracts from Sf9 cells infected with recombinant baculovirus Ac-pVLTR, encoding the intracellular domain of RPTPμ, as a function of time postinfection (in hours). Approximately 5 μg of protein was subjected to SDS–PAGE, blotted onto nitrocellulose, and probed with anti-RPTPμ peptide antibody 37. c, control extract from cells infected with recombinant virus Ac-pVLExJ (Gebbink et al., 1993), encoding a truncated RPTPμ lacking the catalytic domain.

The high-speed supernatant (cytosol) was applied to a FPLC Mono Q anion-exchange column and eluted with a linear gradient of NaCl (0–0.5 M). PTPase activity eluted as one broad peak at 0.17–0.23 M NaCl (Figure 2B). These fractions (21–29) were then pooled, analyzed by SDS–PAGE (Coomassie straining), and subjected to FPLC Mono S ion-exchange chromatography. RPTPμTR eluted as a single symmetrical peak at 0.48 M NaCl (Figure 2C). From SDS–PAGE analysis it appeared that fraction 14 contained homogeneous RPTPμTR (Figure 2A). The typical yield from 2×10^8 Sf9 cells was about 150 μg of pure phosphatase (Table I). The purified cytoplasmic domain could be stored for many months in 50% glycerol at –70 °C without significant loss of activity.

Enzymatic Characterization of RPTPμ. Since different pH optima have been described for different PTPs, we first established the pH dependence of RPTPμTR activity. RPTPμ was found to be enzymatically active toward distinct substrates MBP, RCML, angiotensin II, and EDNDpYINAS) tested over the pH range 5.5–8.5, with an optimum close to pH 7.4 (data not shown). All further assays were therefore carried out at pH 7.4.

The catalytic activity of RPTPμTR was dependent on the nature of the exogenous substrate used. RPTPμTR displayed the highest specific activity toward tyrosine-phosphorylated MBP, 2-fold less toward RCML, and very low activity toward phosphorylated angiotensin II (Table II). An unusually low level of activity was also found with another small synthetic peptide, EDNDpYINASL, derived from the sequence of PTP1B (Figure 3). Under the same experimental conditions, the nonreceptor protein tyrosine phosphatase PTP1B expressed in Sf9 cells (A.J.F. and M.F.B.G., unpublished observations) showed a high specific activity toward the latter substrate. This suggests that RPTPμ and PTP1B have distinct substrate specificities.

Table III summarizes the effects of various agents and classical inhibitors on the catalytic activity of purified RPTPμTR. As with other PTPs, RPTPμTR activity was inhibited by orthovanadate, PAO, (phenylarsenic oxide), Zn^{2+}, and Mn^{2+}. Activity was not significantly influenced by addition of 1 mM Ca^{2+} or EDTA (5 mM). However, as with the T-cell PTP (Zander et al., 1991), the enzymatic activity of RPTPμTR was markedly activated by the polycationic compound spermine. In contrast, the polyanion heparin was strongly inhibitory. Addition of unphosphorylated RCML (10 times the concentration of phosphorylated RCML) inhibited the activity also. This effect could be due to the negative charge of RCML and/or to product inhibition.

Table I: Purification of RPTPμTR from Sf9 Cells

	volume (mL)	protein (mg)	total activity[a] (units)	specific activity[a] (units/mg)	purification (*n*-fold)	yield (%)
cytosol	10	12.7	203	16	1.0	100
Mono Q	10	2.7	92	34	2.1	21.3
Mono S	1	0.15	99	663	41.1	1.2

[a] Activities were determined with MBP as substrate.

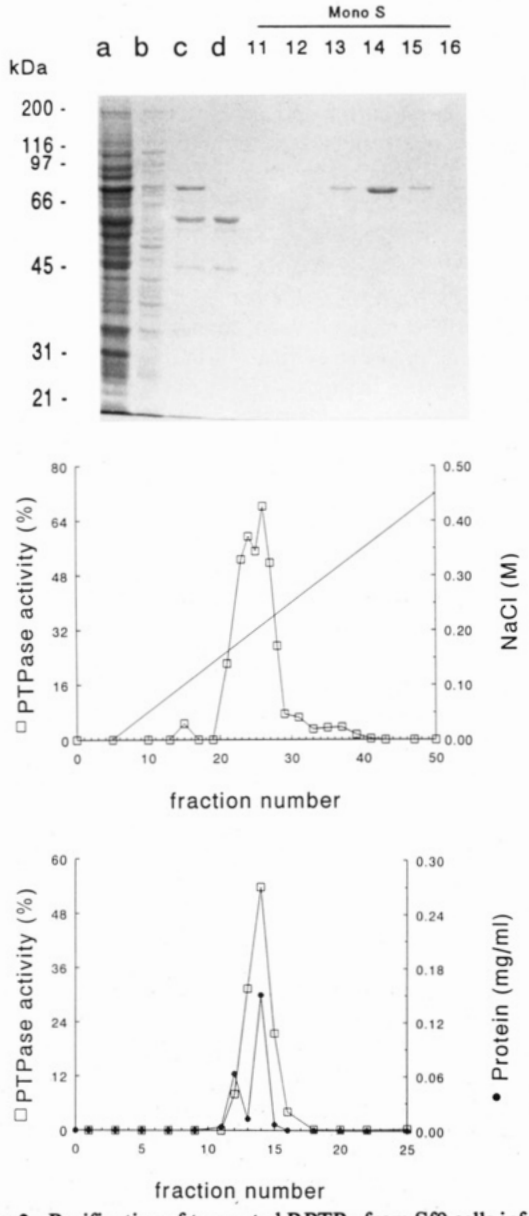

FIGURE 2: Purification of truncated RPTPμ from Sf9 cells infected with Ac-pVLTR. (A, top panel) Whole cytosolic extract and protein fractions obtained from Mono Q and Mono S chromatography were subjected to SDS–PAGE (10% gel), followed by staining with Coomassie Brilliant Blue; a cytosol; b, flowthrough from the Mono Q column; c, pooled Mono Q peak fractions; d, flowthrough from the Mono S column; lanes 11–16 refer to the corresponding Mono S fractions. (B, middle panel) Ion-exchange chromatography of RPTPμTR on Mono Q. (C, bottom panel) Ion-exchange chromatography of RPTPμTR on Mono S. Activities were determined using 2 μM RCML as substrate (incubation time, 5 min; see Materials and Methods).

Expression of RPTPμ Catalytic Domains in E. coli and Analysis of Structure–Activity Relationship. To examine the structure–function relationship of RPTPμ, we cloned various truncated and mutated forms of human RPTPμ cDNA into *E. coli* expression vectors and expressed these constructs as GST fusion proteins (Figure 4A,B). Since *E. coli* extracts lack detectable tyrosine phosphatase activity toward MBP or RCML, this expression system provides a convenient assay for structure–activity determinations, without the need of purification. Functional expression studies of PTPs in *E. coli* have been reported (Streuli et al., 1989, 1990).

The following truncated and/or mutated RPTPμ molecules were expressed: B1 (residues 692–1190), containing 51 extracellular amino acids, the transmembrane segment, and the cytoplasmic region lacking the second PTP-like domain; B2 (residues 1290–1452), only containing most of the second catalytic domain; B4 (residues 818–1452), containing the

Table II: Kinetic Parameters of RPTPμ[a]

	K_m (nM)	V_{max} (nmol min^{-1} mg^{-1})
RCML	1000	4500
MBP	1550	8500
angiotensin II	2600	5.1

[a] Parameters were determined by Lineweaver–Burk analysis. Values represent means of triplicate assays. Substrate concentrations ranged from 0.1 to 50 μM.

Table III: Effect of Various Compounds on RPTPμTR Activity[a]

effector	relative activity
none	100
1 mM Zn^{2+}	9
1 mM Mn^{2+}	33
1 mM Ca^{2+}	93
5 mM EDTA	100
100 μM vanadate	10
20 μM PAO	33
100 μM PAO	0
1 mM pNPP	76
10 mM pNPP	7
10 μM heparin	0
2 mM spermine	160
50 μM nonphosphorylated RCML	11

[a] PTP activity expressed as the percentage of released phosphate relative to control in which the effector was absent. Values represent means of triplicate assays using 5 μM RCML.

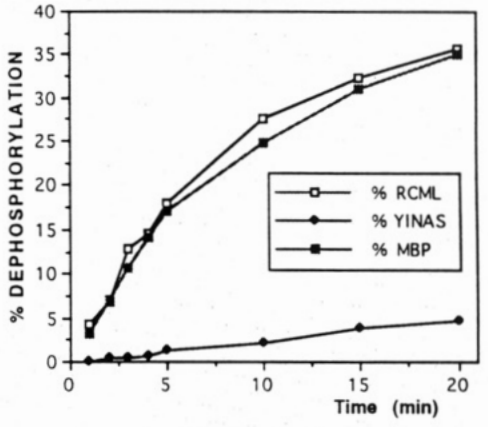

FIGURE 3: Time course of dephosphorylation of tyrosine-phosphorylated RCML, MBP, and EDNDpYINASL by purified RPTPμTR. RPTPμTR was incubated with 2 μM substrate (RCML, MBP, or EDNDpYINASL). At the indicated times samples were taken and released phosphate was counted. Dephosphorylation is depicted as percentage of released phosphate relative to input substrate.

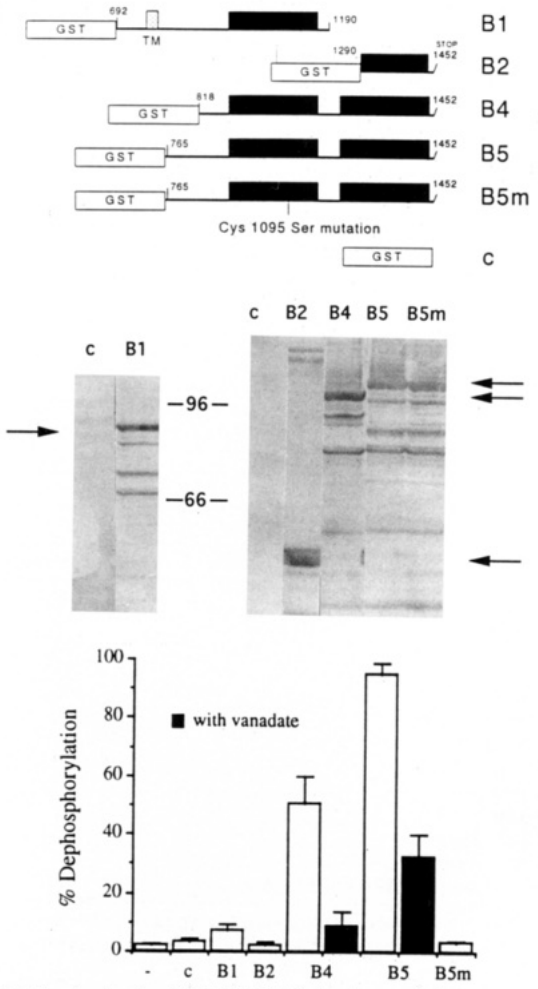

FIGURE 4: Analysis of GST–RPTPμ fusion proteins expressed in *Escherichia coli*. (A, top panel) Schematic representation of the glutathione S-transferase fusion proteins used (B1, B2, B4, B5, and B5m); c indicates control (GST, not fused); TM, transmembrane region. Homologous PTP domains are shown as black boxes. Amino acids are numbered according to Gebbink et al. (1991). (B, middle panel) Immunoblot of lysates (5 μg) from *E. coli* expressing control GST protein (c) and GST-RPTPμ fusion proteins (B1, B2, B4, B5, and B5m). Arrows indicate immunoreactive proteins of the predicted size. Proteins of smaller size probably represent proteolytic breakdown products. Left panel, control and B1 lysate detected with N-terminal antibody 34. Right panel, control, B2, B4, B5, B5m lysates detected with C-terminal antibody 37. (C, bottom panel) Activity of the GST fusion proteins as percentage of released phosphate relative to input substrate (incubation time, 15 min).

cytoplasmic region without the first 53 membrane-proximal amino acids; B5 (residues 765–1452), containing the complete intracellular region; and B5m, a point-mutated form of the complete cytoplasmic domain (B5) in which the conserved cysteine (Cys 1095) in the first catalytic domain was changed into a serine. Correct expression of these fusion proteins was verified by SDS–PAGE and immunoblotting using polyclonal anti-peptide antibodies (Figure 4B).

The level of PTP activity was determined in whole cell lysates using tyrosine-phosphorylated MBP as an exogenous substrate and measuring the amount of inorganic phosphate released. As shown in Figure 4C, marked changes in activity are observed between the different constructs. Maximal activity was detected with the construct encoding the entire intracellular part (B5). The RPTPμ protein that lacks the second PTP-like domain has strongly diminished activity (5–10-fold reduction in different experiments), whereas the expressed part of the second domain by itself lacked any detectable activity. Where tested, activity was strongly reduced by the PTP inhibitor sodium orthovanadate (1 mM). When the first 53 amino acids of the juxtamembrane region were deleted, activity dropped almost 2-fold (cf. B4 and B5), consistent with a modulatory function for this membrane-proximal part of the cytoplasmic region. Removal of the juxtamembrane region in LAR or RPTPα also negatively influenced catalytic activity (Streuli et al., 1990; Wang & Pallen, 1991).

For a number of PTPs it has been shown that a conserved cysteine in the first catalytic domain is essential for activity (Pot et al., 1991; Guan & Dixon, 1990, 1991; Streuli et al., 1989, 1990). To test whether this conserved residue is also absolutely required for activity of RPTPμ, we altered Cys 1095 to a serine residue by site-directed mutagenesis (B5m in Figure 4). Similar to other PTPs, this point mutation completely abolished catalytic activity (Figure 4C), even when tested over prolonged incubation periods and with high substrate concentrations (30 μM RCML or 100 μM MBP).

Effect of C1095 Mutation in COS Cells. To rule out that lack of activity of the second domain of RPTPμ is due to incorrect folding or modification in *E. coli*, we tested the activity of the full-length RPTPμ protein containing the C1095S mutation in mammalian cells. COS cells were transiently transfected with cDNA encoding wild-type or point-mutated RPTPμ and the enzymatic activity was tested in immune complexes obtained from these cells using RCML as substrate. As shown in Figure 5, immune complexes from COS cells expressing wild-type RPTPμ display high activity, whereas no activity is detectable in immune complexes from cells expressing point-mutated RPTPμ. Similar results were obtained when wild-type RPTPμ and point-mutated RPTPμ were expressed in Sf9 cells with the baculovirus expression system (data not shown).

Conclusions. Our studies reveal that RPTPμ, in common with other PTPs, has high basal activity toward artificial substrates. V_{max} values are on the same order of magnitude as for other PTPs tested under comparable conditions. Interestingly, RPTPμ displays hardly any activity toward small Tyr-phosphorylated peptides, such as angiotensin II and EDNDpYINASL. Also a Tyr-phosphorylated 9-amino-acid peptide library (poly 9, Schumacher et al., 1992) served as a poor substrate (M.H.G.V. and M.F.B.G., unpublished results).

From the structure–activity relationship results obtained in *E. coli*, but also in COS and Sf9 cells, it appears that changing the conserved cysteine in domain I in RPTPμ abolishes activity, as in all RPTPs tested to date. Our results demonstrate that the second conserved phosphatase domain of RPTPμ is enzymatically inactive *in vitro*, although we cannot exclude the formal possibility that inappropriate substrates have been used.

Similar findings have been reported for LAR and CD45 (Streuli et al., 1990; Pot et al., 1992). An alternative possibility is that the second domain requires activation by ligand or phosphorylation for enzymatic activity.

Given the apparent lack of activity in the second domain of LAR and CD45, it has been proposed that this domain may regulate the activity of the first domain. A modulatory role for the second domain is supported by the fact that the critical cysteine residue is not conserved in all second domains of PTPs but sometimes is substituted by aspartate [HPTPζ (Krueger et al., 1992; Levy et al., 1993), RPTPγ (Barnea et al., 1993), and DPTP99A (Harihahan et al., 1991; Tian et al., 1991; Yang et al., 1991)]. Itoh et al. (1992) suggest that the second domain of LAR has no effect on the activity of the first

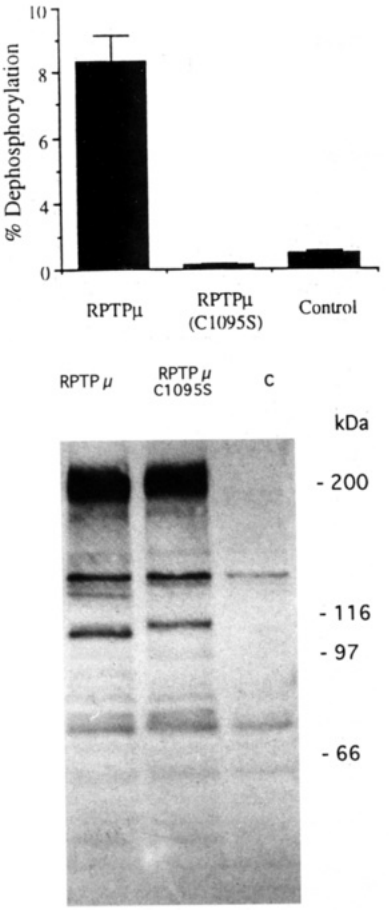

FIGURE 5: Tyrosine phosphatase activity of anti-RPTPμ immunoprecipitates from COS cells. RPTPμ was immunoprecipitated from transiently transfected COS cells using monoclonal antibody M3G4. (A, top panel) The amount of released inorganic phosphate after 10 min of incubation is expressed relative to total input substrate (MBP). (B, bottom panel) Immunoblot of immunoprecipitates confirming equal levels of wild-type RPTPμ and C1095S mutant RPTPμ. A sample ($^1/_{10}$) of the immunoprecipitate was subjected to SDS–PAGE, blotted onto nitrocellulose, and probed with carboxy-terminal antibody 37.

domain, although the second domain allowed strong stimulation of activity of polycationic polypeptides (Itoh et al., 1992). Wang and Pallen (1991) have studied the enzymatic properties of RPTPα expressed in *E. coli*. As in our studies, the presence of both domains increased the activity of the phosphatase, indicating that both domains contribute to the total activity. Catalytic activity of CD45 was dependent on the presence of both domains (Johnson et al., 1992), although another report claims that the second domain of CD45 has substantial activity toward the artificial substrates RCML and MBP (Tan et al., 1993). Further experiments should resolve these apparent discrepancies. Our own results support the view that the second domain of RPTPμ may serve a regulatory rather than an enzymatic role.

A major question, to which we have no answer yet, is whether enzyme activity is regulated by the ectodomain; in other words, whether homophilic cell–cell interaction alters enzymatic activity and thereby modulates protein tyrosine phosphorylation cascades within the cell. An alternative and perhaps more attractive possibility is that cell–cell contact causes RPTPμ to relocalize at the cell surface without changing its basal activity. Such relocalization would bring the catalytic region into proximity with specific substrates or, alternatively, disrupt association between the catalytic domain and substrates. Elucidation of these questions awaits the identification of the physiological substrates of RPTPμ.

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