Characterization of a Human Recombinant Receptor-linked Protein Tyrosine Phosphatase*

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From the ‡Department of Biochemistry SJ-70, University of Washington, Seattle, Washington 98195, §Rhône-Poulenc-Rorer Biotechnology, Inc., King of Prussia, Pennsylvania 19406, and the **Department of Pharmacology, New York University Medical Center, New York, New York 10016

The receptor-linked tyrosine phosphatase RPTPa from human brain (Kaplan, R., Morse, B., Huebner, K., Croce, C., Howk, R., Ravera, M., Ricca, G., Jaye, M., and Schlessinger, J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7000–7004) was expressed in insect cells following infection with recombinant baculovirus. Two major forms of the enzyme, with molecular sizes of 98 kDa and 114 kDa, were detected by immunoblot analysis. This heterogeneity could be ascribed to N-linked glycosylation on the basis of two lines of evidence; namely, blockage of glycosylation with tunicamycin in vivo and removal of carbohydrates by endoglycosidase F in vitro. The 114-kDa form was purified to homogeneity by chromatography on Superose 12 and Mono Q. Compared to the low M, placenta and T-cell tyrosine phosphatases, RPTPa displayed a low optimum pH of 6 and a high K_m in the micromolar range toward two artificial substrates (tyrosyl-phosphorylated myelin basic protein and modified lysozyme, respectively). Most effectors had a different and often an opposite influence on phosphatase activity depending on the nature of the substrate and the pH at which the assays were performed. Determination of K_m and V_max values for RPTPa suggests that the enzyme could exist in low and high substrate affinity states.

The leukocyte common antigen CD45 (for review see Ref. 1) is the first transmembrane molecule found to possess intrinsic protein tyrosine phosphatase (PTP) activity (2). This protein has two homologous, intracellular domains each structurally related to a tyrosine phosphatase isolated from human placenta (PTP1B) (3). Since then, a number of receptor-linked PTPs have been identified mostly by low stringency hybridization. All except one have two highly conserved PTP domains (4). The role of the second domain is still unclear; it might serve a regulatory rather than a catalytic function (5). On the other hand, there are considerable differences in the size and structure of the extracellular segments of these receptors suggesting that they might play different parts in the regulation of cellular processes (reviewed in Refs. 4 and 6). Expression of CD45 is restricted to hematopoietic cells with the exception of mature erythrocytes and their immediate progenitors (1). Its external segment is heavily O- and N-glycosylated and contains cysteine-rich regions. Several “leucocyte common antigen-related” transmembrane molecules (LAR (6), DLAR, and DPTP (7)) have structural similarities with the neural cell adhesion molecules in that they contain type III fibronectin and IgG-like domains, while HPTPβ has only type III fibronectin repeats (4). Their structural features suggest that these molecules might be involved in cell-cell interaction. By contrast, the short external segments of RPTPa (123 residues (4, 9, 10, 11)) and HPTPt (27 residues (4)) show no homology to the other receptor-linked PTPs or to any other transmembrane molecules so far identified.

RPTPa has been cloned from human (4, 10) and murine (9, 11) cDNA libraries. The enzyme contains eight putative sites for N-linked glycosylation plus many seryl and threonyl side chains that could be O-glycosylated. Northern blot analysis indicates that RPTPa is present in many tissues and cell lines suggesting that it might have a more general function in signal transduction.

This report describes enzymatic properties of purified RPTPa following its expression in baculovirus-infected insect cells.

EXPERIMENTAL PROCEDURES

Materials—Restriction and modifying enzymes were purchased from Stratagene (La Jolla, CA), Bethesda Research Laboratories, and Boehringer Mannheim. Sequenase DNA Sequencing Kit was from United States Biochemical Corp. (Cleveland, OH). Grace's Antheraea medium (12) from Gibco Laboratories, and Fungibact antibiotic mix from Irvine Scientific (Santa Ana, CA). Yeastolate and lactalbumin hydrolysate were from Difco Laboratories, and radiolabeled nucleotides were from Du Pont-New England Nuclear. Molecular weight standards and conjugated goat anti-rabbit IgG alkaline phosphatase were from Bio-Rad. Tunicamycin, β-N-acetylglucosaminidase, and endoglucosidase F, neuraminidase, and preplated marker proteins for SDS-PAGE were supplied by Sigma. Calmodulin was a gift from Dr. D. Malencik, Oregon State University, Corvallis, OR. Oligonucleotides were synthesized at the Howard Hughes Medical Institute, DNA Synthesis Facility, University of Washington, Seattle, WA. Sf9 cells and the plasmid pVL 941 were gifts from Dr. Max Summers, Texas A & M University.

Buffers—Buffer A: 10 mM imidazole HCl, pH 7.2, 5% glycerol (v/v), 0.1% 2-mercaptoethanol (v/v), 1 mM pepstatin A, 2 μg/ml leupeptin.
tin, 20 kallikrein inhibitor units/ml aprotonin. Buffer AT: buffer A + 0.1% Triton X-100. Buffer E: buffer A + 2 mM EDTA + 1 mM benzamidine. Buffer M6: same as buffer AT except that imidazole was replaced by 20 mM MES-NaOH, pH 6.0. Buffer H7.5: same as buffer AT except that imidazole was replaced by 20 mM HEPES, pH 7.5.

Construction of the pVL 941-RPTPa Expression Vector—The full length cDNA of RPTPa (10) was cloned into pML2D (13) using a unique XhoI restriction site found within a multiple cloning site located between nucleotides 23 and 375 of the vector. Digested with Dral and BglII resulted in a 2.5-kilobase fragment that was ligated into the BamHI site of the pVL 941 expression vector (14) which had first been treated with Klenow fragment and deoxyribonucleotides to generate blunt ends. The construct pH 941-RPTPa was purified by repeated CsCl gradient centrifugations; correct orientation of the insert was verified by DNA sequence analysis (15).

Preparation and Purification of the Recombinant Ac-RPTPa Virus—All standard procedures were carried out according to the protocol of Summers and Smith (16). Monolayer cultures of Sf9 cells were grown in Grace’s Antheraea medium (12) containing 3.3 g/liter yeastolate, 3.3 g/liter lactalbumin hydrolysate, 10% fetal calf serum (17), 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone. A recombinant baculovirus (Ac-RPTPa) was produced by cotransfecting Sf9 cells with 1 µg of Ac-NPV DNA and 2 µg pVL 941-RPTPa plasmid DNA (16, method I). The method of sequential end point dilution was used to purify the recombinant virus (16). Blots were screened by hybridization with [α-32P]ATP-labeled RPTPa cDNA. The purity of the final virus suspension was checked by hybridization with a 32P-labeled oligonucleotide consisting of nucleotides 37-66 of the Ac-NPV polyhedrin gene (14). The pVL 941 vector lacks this sequence; therefore, a failure of hybridization of Sf9 cell extracts with this oligonucleotide indicates that these cells had been infected with the recombinant virus only.

Purification of RPTPa from Sf9 Cells Infected with Ac-RPTPa—Three 150-cm2 flasks containing 3-4 X 107 cells each were infected with recombinant virus at a high (>3) multiplicity of infection and incubated at 27 °C. Cells were harvested 36 h postinfection by a 5-min centrifugation at 3,000 X g. The supernatant containing the virus was kept at 4 °C for further incubation. All of the following steps were carried out at or below 4 °C. The cells were homogenized in 10 ml of buffer E containing 0.6 M KCl in a Dounce homogenizer. The homogenate was centrifuged for 45 min at 100,000 X g (50 Ti rotor, Beckman). The pellet was extracted again in 10 ml of buffer E and centrifuged for 45 min at 100,000 X g. The supernatant was discarded, and the pellet was solubilized with 1.2 ml of buffer E containing 1% Triton X-100 (v/v). The membrane extract (1 ml) was first cleared by centrifugation at 12,000 X g for 10 min and then filtered through a 0.45-µm filter (Acro LC13, Gelman Sciences). The extract was applied to a Superose 12 (preparation grade) column (HR 16/50, Pharmacia LKB Biotechnology Inc.), equilibrated in buffer AT containing 100 mM NaCl. The chromatography was performed at a flow rate of 1 ml/min. Five fractions were collected and assayed. Tyrosine phosphatase-containing fractions were pooled, dialyzed against buffer AT for 1 h, and then applied to an FPLC Mono Q column (HR 5/5, Pharmacia). The column was subsequently washed with buffer AT and buffer AT containing 50 mM NaCl. Tyrosine phosphatase activity was eluted with a 20-mi linear salt gradient (50-350 mM NaCl in buffer AT, flow rate: 0.5 ml/min, fraction size: 0.5 ml). Active fractions were pooled and dialyzed for 1 h against buffer AT containing 20% glycerol (v/v). Aliquots (50 µl) of the protein solution were frozen in liquid nitrogen and stored at -70 °C.

Antibodies Raised against RPTPa Peptides—A peptide corresponding to the C terminus of RPTPa (residues 785-802) was cross-linked to keyhole limpet hemocyanin with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (18). Three New Zealand white male rabbits were immunized three times at 21-day intervals with a total of 2.5 µg of antigen in 0.2 ml of complete Freund’s adjuvant. The first injection was intramuscularly, the second injection was intraperitoneally, and the third injection was subcutaneously. Rabbits were immunized at 14-day intervals until antibody titers decreased. A 1:100 dilution of serum was used for immunoblotting.

Phosphatase Assays—These were performed in buffers M6 and H7.5 (or AT for screening column fractions). Aliquots of 60 µl containing the substrate and, where indicated, effectors were incubated at 10 °C for 10 min. The reaction was stopped by adding 100 µl of 20% trichloroacetic acid (w/v). The suspension was spun in a microcentrifuge for 10 min, and the radioactivity of 200 µl of the supernatant was determined. As substrates, RCML and MBP were prepared and phosphorylated in the presence of [γ-32P]ATP as described previously (19, 20). One unit is defined as the release of 1 nmol of phosphate/min. Substrate concentration represents the concentration of phosphotyrosyl residues. Protein concentration was determined according to Bradford (21).

RESULTS

Cloning and Time Course of Expression—The full length cDNA of RPTPa was cloned into the pVL 941 expression vector as described under “Experimental Procedures.” Using the method of dot-blot hybridization, cells infected by recombinant virus could be detected following co-transfection of Sf9 cells with pVL 941-RPTPa and Ac-NPV DNA. Five to six rounds of serial end point dilution were required to yield pure recombinant virus. The titers of propagated recombinant virus solutions was in the range of 2.5-4 X 107 plaque-forming units per ml. In order to determine the time course of expression, cells were collected every 12 h postinfection, centrifuged, and extracted in buffer E containing 1% Triton X-100. The extracts were then assayed for activity and subjected to immunoblot analysis following SDS-PAGE (see, for instance, Fig. 1A, lane a). No phosphatase activity could be detected in the first 24 h, whereas 90% was found after 36 h and 100% 48 h postinfection. Further incubation of the cells led to a decrease in phosphatase activity. Immunoblot analysis of samples collected 36 h postinfection showed the presence of two bands of 98 kDa and 114 kDa (see Fig. 1E, lane a). A third band of approximately 85 kDa and several additional proteins of lower molecular weight appeared 48 h postinfection. The intensity of these bands increased with time of incubation suggesting that they are due to proteolysis.

Purification—The 114-kDa form of RPTPa was purified to electrophoretic homogeneity from a membrane fraction of Sf9 cells (Fig. 1A). As summarized in Table I, a typical preparation from three culture flasks (total of 1-2 X 107 cells) yielded

![Fig. 1. Purification of RPTPa from Sf9 cells infected with recombinant virus. Triton X-100 extract (5 µg of protein) and the indicated fractions of the chromatography on Superose 12 and Mono Q (8 µl each) were subjected to SDS-PAGE (26). A, the 7.5% gel was stained with Coomassie Blue; a, Triton X-100 extract; b, Superose 12 fraction 41; c, Mono Q fraction 16. B, following gel electrophoresis, the proteins were transferred to nitrocellulose (27). The blot was incubated with an antibody raised against the C terminus of RPTPa; a, Triton X-100 extract; b, Superose fraction 38; c, Superose fraction 41; d, Mono Q fraction 16; e, Mono Q fraction 19. The fractions are the same as shown in Figs. 2 and 3, respectively.

| Table I | Purification of RPTP α from Sf9 cells infected with recombinant virus |
|---------|----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Fraction | Volume | Protein | Activity | Specific | Purification | Yield |
|---------|--------|---------|----------|----------|---------------|-------|
| Membrane extract | 1.0 | 6.20 | 273.0 | 44.0 | 1.0 | 100 |
| Superose 12 | 4.0 | 1.12 | 87.6 | 78.2 | 1.8 | 32.1 |
| Mono Q | 2.5 | 0.14 | 16.1 | 115.0 | 2.6 | 6.3 |

*PPT activities were determined with 0.67 µM MBP in buffer AT.
0.14 mg of pure RPTPα. The activity of the membrane fraction eluted from the Superose 12 column in a broad peak (Fig. 2) corresponding to proteins with molecular masses ranging from 160 to 1000 kDa (void volume of the column). Immunoblot analysis showed that most of the 98-kDa form eluted ahead of the 114-kDa species (compare lane b and c in Fig. 1). In order to isolate the 114-kDa protein, only the four peak fractions (40-43) were pooled and subjected to FPLC Mono Q chromatography. The PTP activity emerged in an almost symmetrical peak (Fig. 3). The remaining 98-kDa species eluted from the Mono Q column in the trailing fractions of the activity peak together with traces of proteins of lower Mₐ, which were probably generated in the course of purification (compare lane d and e in Fig. 1B). To avoid contamination by these lower Mₐ species, only five fractions (14-18) were pooled. The enzyme could be stored at -70 °C for months without significant loss of activity.

Glycosylation—It was likely that the two RPTPα forms observed resulted from a difference in carbohydrate content, since the receptor displays eight putative N-linked glycosylation sites in its external domain (10). To address this question, cells were grown in the presence or absence of tunicamycin, an inhibitor of N-linked glycosylation (22). As shown in Fig. 4A, lane c, addition of 1 μg/ml tunicamycin to the culture media led to a shift of the 114-kDa form of RPTPα to a 98-kDa species, as determined by immunoblot analysis. A 10-fold lower concentration of tunicamycin was without effect. To confirm the presence of carbohydrate in the protein, the 114-kDa form of RPTPα was incubated with various glycosidases. Only endoglycosidase F, which removes N-linked glycosyl residues (23), was able to convert the 114-kDa form into the 98-kDa species (Fig. 4B, lane c).

pH Dependence—When the assay conditions established for CD45 (pH 7.2 in the presence of 2 mM EDTA) were applied to RPTPα, only a low level of activity (5-10%) was detected. This was mainly due to the acidic optimum pH of 6 (with half-maximum activity observed at pH 5 and 6.7, respectively) exhibited by RPTPα when RCML and MBP were used as substrates. Therefore, all further characterizations were carried out at both pH 6 and 7.5 to cover the physiological range.

Substrate Specificity and Effect of Divalent Cations and EDTA—Like all other members of the protein tyrosine phosphatase family, RPTPα was specific for tyrosyl residues, displaying no activity toward MBP or histones phosphorylated by the cAMP-dependent protein kinase. Such substrates are readily dephosphorylated by serine/threonine-specific protein phosphatases (24). RPTPα showed a complex behavior since its response toward different effectors varied with pH and the nature of the substrate. As shown in Fig. 5A, at pH 6 and 7.5, however, the enzyme was unaffected or slightly inhibited by these compounds. By contrast, with MBP as substrate, all effectors were inhibitory regardless of pH (Fig. 5B). The optimum concentration of Mn⁺⁺ is far above the physiological range. Calmodulin (10 μM) in the presence of 1 mM calcium was without effect. The kinetic parameters of RPTPα determined with RCML and MBP are summarized in Table I. The low activity toward RCML observed for the enzyme at its optimum pH of 6 and in the absence of effectors can be attributed to its high Kₘ, of 80 μM; Mn⁺⁺ increases this value 7-fold. At pH 7.5 or with MBP as substrate, Mn⁺⁺ causes a 3- to 5-fold increase in Kₘ. Under all conditions, Vₘₐₓ remained essentially unaffected by Mn⁺⁺ showing variations less than 2-fold.

Effect of Polycations—As shown in Fig. 6, the influence of polycations such as spermine and spermidine on RPTPα was similar to that observed with Mn⁺⁺: activity toward RCML was enhanced 12- to 14-fold at pH 6 but not at pH 7.5, while dephosphorylation of MBP was inhibited at either pH. A 10-fold higher concentration of spermidine than spermine was required for activation as seen by comparing the scales of Fig. 6, A and B. Nonphosphorylated MBP (1.5 μM) was also able to increase RCML dephosphorylation about 10-fold but only at pH 6.

Inhibition by Heparin and Zn⁺⁺—As observed with the low Mₐ, T-cell phosphatase, TC-PTP and its truncated form TCΔ11-PTP, as well as CD45 (25), heparin was inhibitory.
Fig. 5. Effect of Mn²⁺, Mg²⁺, Ca²⁺, and EDTA on activity of RPTPa. Assays were performed with 2 μM RCML (A) or 2 μM MBP (B) at pH 6 (closed symbols) and pH 7.5 (open symbols), in the presence of various concentrations of Mn²⁺-acetate (●, ○), Mg²⁺-acetate (▲, △), CaCl₂ (■, ▽), and EDTA (●, ◆). For each substrate, PTP activity is shown in relation to the value obtained at pH 6 in the absence of effectors.

Table II

| Substrate | Kᵣ | Vₘₐₓ |
|-----------|----|------|
| RCML      |    |      |
| pH 6.0    | 10 | 7810  |
| -Mn²⁺     | 83 | 1540  |
| +Mn²⁺     | 12 | 2080  |
| pH 7.5    | 33 | 4000  |
| -Mn²⁺     | 11 | 300   |
| +Mn²⁺     | 36 | 540   |

when RCML rather than MBP was used as substrate except at micromolar concentrations (Fig. 7). Likewise, RPTPa was 10 times more susceptible to Zn²⁺ inhibition in the presence of RCML, displaying IC₅₀ values of 40 and 400 μM with RCML and MBP, respectively (Fig. 8).

Discussion

Expression of the receptor-linked tyrosine phosphatase RPTPa in Sf9 cells occurred in a narrow time frame, with 90% of the protein being produced between 24 and 36 h postinfection. Approximately 90% of the phosphatase was present in a 114-kDa form with the remainder at 98 kDa, the difference being due to N-linked glycosylation. However, the size predicted on the basis of the amino acid sequence (10) is 88 kDa. This discrepancy could result from the presence of O-linked carbohydrates, since the extracellular segment exhibits a serine/threonine content of approximately 30%; such modification would not be affected by tunicamycin or endoglycosidase F. Alternatively, there could be an asymmetry in the transmembrane molecule. The murine analog of RPTPa, with 794 residues versus 802 for the human receptor and 95% sequence identity, has recently been expressed in COS cells (9). Its higher than expected molecular mass of 130 kDa could be due to a greater extent of glycosylation.

RPTPa was found only in the particulate fraction of Sf9 cell extracts. Washing this fraction with 0.6 M KCl did not
release appreciable amounts of the phosphatase but removed contaminating proteins, thereby providing a valuable purification step. Although RPTPa represents 40–50% of total protein in Triton X-100 extracts, two chromatography steps were needed to obtain the pure 114-kDa form. The circle represents the most active form of the enzyme and the square its most inactive state.

Fig. 9. Model describing the affinity states of RPTPa. The model assumes that the enzyme can exist in high affinity (circle and oval) and low affinity (square and diamond-shaped figure) states determined by pH and the effector Mn2+. Interconversions between these are described in the text. The circle represents the most active form of the enzyme and the square its most inactive state.

Addition of Mn2+ can be explained on the basis of a competitive species PTPlB and TCAl1.PTP, and is restricted to the receptor-linked phosphatases. There is no evidence that the low yield is due to the loss of an enzyme that is maximally active at pH 6 and almost inactive at pH 7.5. This model also accounts for the MBP dephosphorylation in vitro.

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