Journal of Cellular Biochemistry 86:79–89 (2002)

Purification and Characterization of Protein Tyrosine Phosphatase PTP-MEG2

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Abstract PTP-MEG2 is an intracellular protein tyrosine phosphatase with a putative lipid-binding domain at the N-terminus. The present study reports expression, purification, and characterization of the full-length form of the enzyme plus a truncated form containing the catalytic domain alone. Full-length PTP-MEG2 was expressed with an adenovirus system and purified from cytosolic extracts of human 293 cells infected with the recombinant adenovirus. The purification scheme included chromatographic separation of cytosolic extracts on fast flow Q-Sepharose, heparin-agarose, L-histidyldiazobenzylphosphonic acid agarose, and hydroxylapatite. The enrichment of PTP-MEG2 from the cytosol was about 120-fold. The truncated form of PTP-MEG2 was expressed in *E. coli* cells as a non-fusion protein and purified by using a chromatographic procedure similar to that used for the full-length enzyme. The purified full-length and truncated enzymes showed single polypeptide bands on SDS–polyacrylamide gel electrophoresis under reducing conditions and behaved as monomers on gel exclusion chromatography. With *para*-nitrophenylphosphate and phosphotyrosine as substrates, both forms of the enzyme exhibited classical Michaelis-Menten kinetics. Their responses to pH, ionic strength, metal ions, and protein phosphatase inhibitors are similar to those observed with other characterized tyrosine phosphatases. Compared with full-length PTP-MEG2, the truncated ΔPTP-MEG2 displayed significantly higher V_{max} and lower K_m values, suggesting that the N-terminal putative lipid-binding domain may have an inhibitory role. The full-length and truncated forms of PTP-MEG2 were also expressed as GST fusion proteins in *E. coli* cells and purified to near homogeneity through affinity columns. However, the specific phosphatase activities of the GST fusion proteins were 10–25-fold below those obtained with the correspondent non-fusion proteins. J. Cell. Biochem. 86: 79–89, 2002.

Key words: protein expression; adenovirus; fusion protein; chromatography; enzyme kinetics

Protein tyrosine phosphatases (PTPs) catalyze the dephosphorylation of proteins on phosphotyrosyl residues and are a large and structurally diverse family of enzymes characterized by the consensus sequence of (I/V)HCXAGXXR. They have been shown to act as positive as well as negative signaling regulators in a wide range of cellular processes including proliferation, differentiation, transformation, motility, cytoskeletal organization, and immune responses [Fischer et al., 1991; Walton and Dixon, 1993; Tonks and Neel, 2001]. Some of them have been identified as potential targets for therapeutic drug screening. PTPs can be divided into transmembrane receptor-like and intracellular enzymes. The intracellular PTPs contain a single conserved phosphatase domain linked to variable sequences that modulate the activity and/or intracellular localization of the enzymes. Over 100 PTPs exist in human cells, but only a few of them have been purified and characterized. PTP-MEG2 is a widely distributed intracellular phosphatase originally cloned from human MEG-01 megakarocyte and umbilical vein

Abbreviations used: PTP, protein tyrosine phosphatase; p-NPP, *para*-nitrophenylphosphate; P-Tyr, *O*-phospho-L-tyrosine; CRALBP, cellular retinaldehyde-binding protein; GST, glutathione-S-transferase.

Grant sponsor: National Institutes of Health; Grant numbers: CA75218, HL-57393, DK-15555, 2 T32-DK07186, CA-68485; Grant sponsor: Veterans Health Administration Merit Review Grant.

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Received 8 March 2002; Accepted 11 March 2002

DOI 10.1002/jcb.10195

endothelial cell cDNA libraries [Gu et al., 1992]. The enzyme contains two domains. The catalytic domain located at the C-terminus displays 30–40% sequence identity to other known PTPs. The N-terminal non-catalytic segment has ~250 amino acids and shares 24–29% sequence identity with cellular retinaldehyde-binding protein (CRALBP), α-tocopherol transfer protein, and yeast Sec14p [Gu et al., 1992; Aravind et al., 1999]. CRALBP is a water-soluble protein found in the retina and pineal gland. It acts as a carrier protein for 11-cis-retinaldehyde or 11-cis-retinol and modulates the interactions of these retinoids with visual cycle enzymes [Crabb et al., 1988; Wolf, 1991]. α-Tocopherol transfer protein (A-TTP) of liver specifically sorts out α-tocopherol from all incoming tocopherols for incorporation into plasma lipoproteins [Sato et al., 1993]. Sec14p acts as a phosphatidylinositol transfer protein by catalyzing the transfer of phosphatidylinositol and phosphatidylcholine between membrane bilayers [Aitken et al., 1990; Bankaitis et al., 1990]. It is required for protein transport through the Golgi complex in *Saccharomyces cerevisiae*. With significant sequence homology to these lipid-binding proteins, PTP-MEG2 may also bind certain lipid molecules that may regulate its activity and/or localization.

In order to study the biochemical and regulatory properties of PTP-MEG2, we expressed the full-length form of PTP-MEG2 with an adenovirus expression system and a truncated form containing the catalytic domain in *E. coli* cells. We purified the recombinant enzymes to near homogeneity and further performed detailed kinetic characterization.

MATERIALS AND METHODS

Materials

para-Nitrophenylphosphate (p-NPP), *O*-phospho-L-tyrosine (P-Tyr), all-trans-retinal, 11-cis-retinal, α-tocopherol, crude phosphatidylinositol, and L-histidyldiazobenzylphosphonic acid-agarose were purchased from Sigma. Fast flow Q-Sepharose and heparin-agarose were obtained from Pharmacia Biotechnology, Inc. Hydroxylapatite was purchased from Bio-Rad. Human 293 cells were obtained from the American Type Culture Collection. Antiserum #144 was generated in a rabbit against a synthetic peptide conjugated to maleimide activated keyhole limpet hemocyanin (Pierce, Rockford, IL). The peptide with a sequence of NH2-CAEKEGMVSSGQNLLAVESQ-COOH was derived from the 19 amino acid residues at the C-terminus of PTP-MEG2.

Cloning of PTP-MEG2 cDNA

The entire coding region of PTP-MEG2 was amplified by polymerase chain reaction from a human bone marrow cDNA library and cloned into the pBluescript KS vector (Stratagene). The PCR primers used were 5′-GGGATG-GAGCCCGCGACCG-3′ and 5′-GGAAGGCTGG CCAACAGGTAGGAGG-3′. The sequence was confirmed by DNA sequencing, and it totally agrees with the reported sequence [Gu et al., 1992].

Generation of Recombinant Adenovirus and Infection of 293 Cells

The PTP-MEG2 insert from the pBluescript KS construct was subcloned into adenovirus transfer vector pACCMV·pLpA at the Kpn I and BamH1 sites. Recombinant adenovirus was generated by co-transfection of 293 cells with the pACCMV · pLpA construct and pJM17 adenovirus genome DNA by using Fugene 6 cell transfection reagent (Boehringer Mannheim). The resulting recombinant virus was purified by soft agar plaque assays and then amplified in 293 cells according to standard procedures [Becker et al., 1994]. Positive clones were selected based on expression of PTP-MEG2 in infected 293 cells as determined by anti-PTP-MEG2 serum #144. Human 293 cells were cultured at 37°C under 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Science Technology). Cells were grown to approximately 80–90% confluence before addition of the virus. The infected cells were cultured for another 48 h before harvesting.

Purification of PTP-MEG2

Recombinant virus-infected 293 cells were collected by centrifugation at 5,000*g* for 5 min and washed twice with phosphate buffered saline. Cells were then homogenized with a Teflon glass homogenizer in Buffer A (25 mM Tris-Cl, pH 7.5, 10 mM β-mercaptoethanol, 1 mM EDTA, and 1 mM EGTA) supplemented with a protease inhibitor mixture (1 mM benzamidine, 0.1 mM phenylmethylsulfonyl flouoride, 2 μg/ml leupeptin, 1 μM pepstatin A, and

1 μg/ml aprotinin). The homogenate was cleared by centrifugation at 45,000*g* for 1 h in a Beckman 60 Ti-rotor at 4°C. The cytosolic supernatant was loaded onto a fast flow Q-Sepharose column equilibrated with Buffer A. After washing with the same buffer, the column was eluted with a linear gradient of 0–0.6 M NaCl. Fractions with PTPase activity were pooled, diluted threefold with Buffer A, and then were further purified on a heparin-agarose column preequilibrated with Buffer A. The column was eluted with a linear gradient of 0–0.6 M NaCl in the same buffer. The active fractions were combined and diluted threefold with Buffer A. They then were applied to an L-histidyldiazobenzylphosphonic acid-agarose column equilibrated with Buffer A and were eluted with a linear gradient of 0–0.6 M NaCl. The active fractions were again pooled and directly applied to a hydroxylapatite column equilibrated with Buffer B (25 mM Tris-Cl, pH 7.5, 10 mM 2-mercaptoethanol, and 0.2 M NaCl). The column was washed with 5 bed volumes of the above buffer and then with 2 bed volumes of Buffer B supplemented with 10 mM KH_2PO_4/K_2HPO_4 (pH 7.5). The final elution was achieved with Buffer B supplemented with 20 mM KH_2PO_4/K_2HPO_4 (pH 7.5). The active fractions were combined and dialyzed overnight against Buffer A and concentrated by Buffer A containing 50% glycerol. The enzyme was stored at −20°C.

Purification of the Catalytic Domain of PTP-MEG2 (ΔPTP-MEG2) From *E. Coli* Cells

As previously described [Zhao et al., 1994], we employed the pT7-7 vector to express the catalytic domain of PTP-MEG2 as a non-fusion protein in *E. coli* cells. First, a cDNA fragment encoding the catalytic domain of PTP-MEG2 (amino acid residues 283–593) was amplified by PCR and cloned into the pT7-7 vector. The construct was then used to transform DE3-pLysS *E. coli* cells (Novegen) to give rise a recombinant ΔMEG2 protein of 315 amino acids in which the first 4 residues (MARI) were derived from the vector. Expression of the recombinant protein was induced by 50 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG) overnight at 28°C. *E. coli* cells were broken up by sonication in Buffer A plus protease inhibitors as above. Purification of ΔPTP-MEG2 followed a similar procedure developed for the full-length enzyme as described above except that the heparin-agarose step was omitted. Dialysis and storage conditions were the same as that described for full-length PTP-MEG2.

Expression and Purification of PTP-MEG2 and ΔPTP-MEG2 as GST Fusion Proteins

cDNA inserts corresponding to full-length PTP-MEG2 and truncated ΔPTP-MEG2 were inserted in-frame into the pGex-2T vector (Pharmacia). To facilitate purification, we also attached a 6xHis tag to the C-terminal end of full-length PTP-MEG2. DE3pLysS *E. coli* cells were used as the host for expression of the recombinant GST fusion proteins. The cells were cultured overnight in the presence of 50 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 28°C. GST-ΔPTP-MEG2 was purified by using a single glutathione-Sepharose column (Pharmacia), while GST-PTP-MEG2 was purified through Ni-NTA-agarose (Qiagen) and then glutathione-Sepharose. The purification procedures followed the protocols provided by the manufacturers of the columns. The purified GST fusion proteins were dialyzed against Buffer A plus 50% glycerol and stored at −20°C.

Phosphatase Assays

PTP activity assays were performed with p-NPP and P-Tyr as substrates at room temperature as previously described [Tonks et al., 1988; Zhao et al., 1992]. The assay system contained 1.0 mM EDTA and 1.0 mM dithiothreitol. The buffers used were 20 mM HEPES-NaOH (pH 7.0) and 25 mM sodium acetate (pH 5.0). For assays performed at pH 5.0, 20% glycerol was added to stabilize the enzymes. One unit of activity is defined as 1 nmol of phosphate released per min. Throughout the purification procedure, PTP activity was analyzed by using p-NPP as a substrate at pH 5.0, and protein concentrations were determined by using the Bradford method with bovine serum albumin as a standard.

Lipid Preparation

To make retinal isomers, all-trans-retinal at a concentration of 1 mg/ml in ethanol was irradiated on ice for 2 h with a 150 W incandescent lamp 8 inches above the surface of the solution as previously described [Bridges et al., 1980; Bridges, 1990]. The resulting mixture contains 13-cis, 11-cis, 9-cis, 7-cis, and all-trans retinal. Working solutions of retinal and α-tocopherol were made in the PTP assay buffer (HEPES-

NaOH, pH 7.0) plus 1% Triton X-100 and were diluted 10-fold after adding to the assay mixtures. Phospholipids in chloroform were dried under N_2 and dissolved in required amounts of Triton X-100 to generate mixed micelles containing 15 mol% phospholipids and 85 mol% Triton X-100 in a buffer containing 20 mM HEPES-NaOH (pH 7.0) and 1% (v/v) Triton X-100 [Hannun et al., 1985; Zhao et al., 1991]. The concentration of Triton X-100 in the final assay mixtures was kept at 0.1% for all assays involving lipids. For PTP assays with P-Tyr as a substrate, reduced Triton X-100 was used in place of regular Triton X-100 to avoid interference of absorbance at 280 nm. Assays with all the forms of retinal were performed in a dark room to avoid photoisomerization.

RESULTS

Purification of PTP-MEG2

Primary structure analyses suggest that PTP-MEG2 is an intracellular protein. However, expression of the full-length enzyme in *E. coli* as a non-fusion or a fusion protein resulted in distribution of most of the protein in inclusion bodies with little PTP activity in the exclusion bodies. This made purification of active PTP-MEG2 from *E. coli* cells unfeasible. We thus employed an adenovirus expression system to express the enzyme. For this purpose, cDNA encoding the full-length PTP-MEG2 was subcloned into the transfer vector pACCMV·pLpA. Recombinant adenovirus was obtained by co-transfection of the transfer construct with the pJM17 adenovirus genome in human 293 cells. Recombinant viral clones expressing PTP-MEG2 were purified by soft agar plaque assays. Western blot analyses with PTP-MEG2 antiserum 144 revealed that infection of 293 cells with recombinant adenovirus resulted in a high level of expression of PTP-MEG2. As an intracellular PTP, the majority of PTP-MEG2 (~70% according to Western blot analyses and activity assays) was distributed in the cytosolic fraction, with ~30% found in the membrane fraction. We, therefore, used cytosolic extracts as starting materials to purify the enzyme. The purification procedure included four chromatographic steps. PTP-MEG2 was eluted as a single symmetric peak at 0.19, 0.21, and 0.20 M NaCl from fast flow Q-Sepharose, heparin-agarose, and L-histidyldiazobenzylphosphonic acid-agarose, respectively (Fig. 1). The final

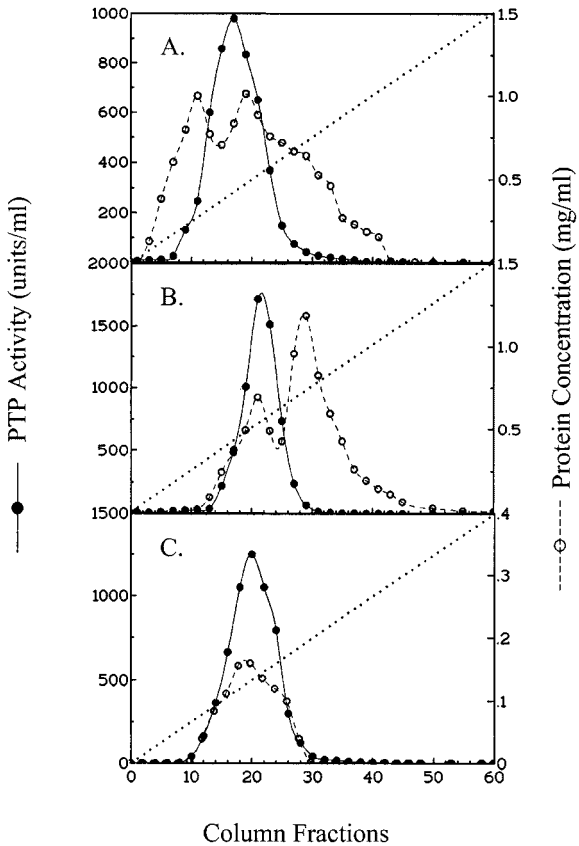

Fig. 1. Purification profile of PTP-MEG2 on chromatographic columns. PTP-MEG2 was purified from cytosolic extracts of recombinant adenovirus-infected 293 cells sequentially through fast flow Q-Sepharose (**A**), heparin-agarose (**B**), and L-histidyldiazobenzylphosphonic acid-agarose (**C**) columns. Solid line, phosphatase activity determined by using 10 mM p-NPP at pH 5.0; dashed lines, protein concentrations determined by the Bradford method; dotted lines, 0–0.6 M NaCl gradient.

hydroxylapatite column was eluted with 20 mM KH_2PO_4/K_2HPO_4 (pH 7.5). It not only gave rise to essentially homogenous enzyme samples but also concentrated the sample. This four-step chromatographic procedure produced a ~120-fold purification with a yield of ~12% as summarized in Table I. From eight 150 mm plates of cells, about 70 μg of purified PTP-MEG2 was obtained.

Purification of ΔPTP-MEG2

The catalytic domain of PTP-MEG2, namely, ΔPTP-MEG2, was expressed in *E. Coli* cells by using the pT7-7 vector as employed for the catalytic domain of SHP-1 and SHP-2 described in our previous studies [Zhao et al., 1994; Liang et al., 1997]. The recombinant ΔPTP-MEG2 was mostly distributed in the exclusion body of

TABLE I. Purification of Recombinant PTP-MEG2*

Fractions	Protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification (fold)
Cytosolic extract	67	270	18,090	100	1
Q-Sepharose	12	950	11,400	63	3.5
Heparin-agarose	4.2	2,600	10,920	60	9.6
HDBP-agarose	0.65	8,300	5,395	30	31
Hydroxylapatite	0.07	32,300	2,261	12	120

*PTP activity was analyzed with 10 mM p-NPP at pH 5.0. HDBP denotes L-histidyldiazobenzylphosphonic acid.

E. Coli cell extracts. To purify the truncated enzyme, we employed a procedure similar to that used for full-length PTP-MEG2 except that the heparin-agarose column was omitted. The purification profiles on fast flow Q-Sepharose and L-histidyldiazobenzylphosphonic acid-agarose columns are shown in Figure 2. Compared with full-length PTP-MEG2, ΔPTP-MEG2 was eluted at an equivalent concentration of NaCl from the former column but at a higher salt concentration (0.35 M) from the latter column. The final hydroxylapatite column yielded homogenous enzyme in a relatively small volume. From 500 ml of *E. coli* cells, about 0.4 mg purified enzyme was obtained as summarized in Table II. It should be noted that the initial fast flow Q-Sepharose step gave rise to a seven-fold increase in the total PTP activity. This reflects the inhibition of PTP activity in the crude cell extracts, which also has been observed with SHP-2 [Zhao et al., 1994].

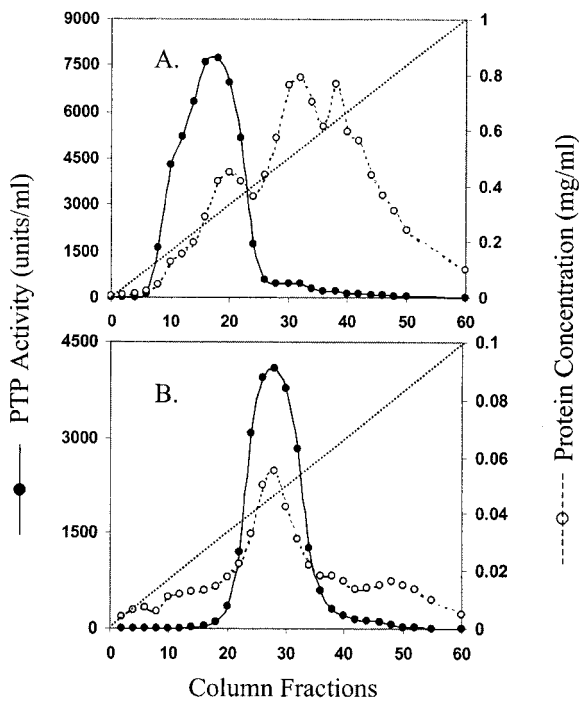

Fig. 2. Purification profile of ΔPTP-MEG2 on chromatographic columns. ΔPTP-MEG2 was purified from cell extracts of *E. coli* cells sequentially through fast flow Q-Sepharose (**A**) and L-histidyldiazobenzylphosphonic acid-agarose (**B**) columns. Solid line, phosphatase activity determined by using p-NPP at pH 5.0; dashed lines, protein concentrations determined by the Bradford method; dotted lines, 0–0.6 M NaCl gradient.

Purified PTP-MEG2 and ΔPTP-MEG2 Behave as Monomers on Gel Filtration Columns

Figure 3 shows the separation of full length PTP-MEG2 and ΔPTP-MEG2 from each purification step on SDS gels. The proteins were detected by Coomassie Blue staining or by Western blotting with PTP-MEG2 antiserum #144 after transfer to polyvinylidene difluoride (PVDF) membranes. Purified full-length PTP-MEG2 ran as a single band at 68 kDa while ΔPTP-MEG2 showed a homogeneous protein of ~37 kDa. The molecular sizes of the recombinant proteins on SDS gels matched those predicted from the primary structures. Most PTPs are monomeric. However, it has also been reported that some enzymes (e.g., RPTPα) exist as dimers [Jiang et al., 1999], and dimerization is believed to be an important inhibitory mechanism for regulation of PTPs [Desai et al., 1993; Jiang et al., 1999]. We employed the gel filtration method to examine the behavior of PTP-MEG2 and ΔPTP-MEG2. Figure 4 illustrates the profile of the purified full-length PTP-MEG2 on a Superose 12-gel filtration column (Pharmacia) under 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.1 M NaCl. Both PTP activity and protein concentration peaked at fraction number 25, which corresponds to a molecular mass of ~67,000 Da according to a linear regression analysis of standard protein markers. This value agrees with that obtained by SDS–PAGE (68,000 Da) and the value predicated from the primary sequence (68,019 Da). ΔPTP-MEG2

TABLE II. Purification of Recombinant ΔPTP-MEG2*

Fractions	Protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification (fold)
E. coli extract	46	280	12,900	100	—
Q-Sepharose	5.2	18,000	93,600	726	64
HDBP-agarose	0.51	78,000	39,800	308	4
Hydroxylapatite	0.39	130,000	50,700	393	7

*PTP activity was analyzed with 10 mM p-NPP at pH 5.0. HDBP denotes L-histidyldiazobenzylphosphonic acid.

displayed activity and protein peaks at fraction number 27 (data not shown). This gives rise to a molecular mass of ~40 kDa, a value in agreement with those obtained from SDS gels (37 kDa) and the primary structure (36,175 Da). Together, the data indicate that PTP-MEG2 and ΔPTP-MEG2 exists as monomers.

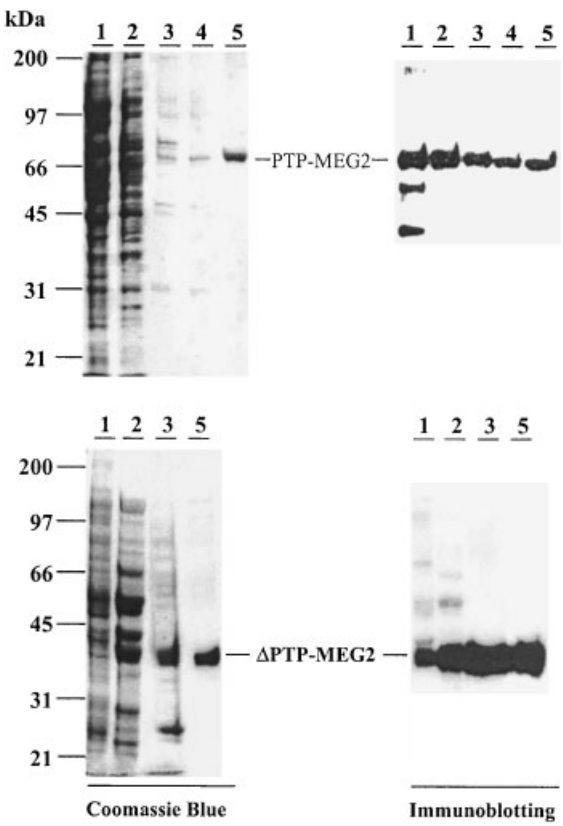

Fig. 3. Separation of PTP-MEG2 and ΔPTP-MEG2 samples on SDS gels. Protein samples from each purification step was separated by 10% SDS gels and then stained with Coomasie Blue (**left panels**) or transferred to polyvinylidene difluoride (PVDF) membranes for Western blot analyses with PTP-MEG2 antiserum #144 (**right panels**). **Lane 1**, crude cytosolic extract; **lane 2**, pooled fractions from fast flow Q-Sepharose; **lane 3**, pooled fractions from heparin-agarose; **lane 4**, pooled fractions from L-histidyldiazobenzylphosphonic acid-agarose; **lane 5**, pooled fractions from hydroxylapatite.

Dependence of PTP-MEG2 Activity on pH and Ionic Strength

Most of the characterized PTPs and dual specificity protein phosphatases display an optimal pH of around five with low-molecule-weight compounds as substrates [Tonks et al., 1988; Zhang et al., 1992; Zhao et al., 1992, 1993, 1994, 2000; Denu and Dixon, 1995]. This also applies to PTP-MEG2, which displayed sharp pH dependence curves (Fig. 5A). PTP-MEG2 displayed pH optima of 5.0 and 4.5 with p-NPP and P-Tyr as substrates, respectively. At pH 7.0, the activity was 30–40% of that obtained under the optimal pH. ΔPTP-MEG2 showed maximum activity at pH 5.0 with both substrates. The activity toward P-Tyr remained at a considerably high level at neutral pH. Changes in ionic strength also had a major effect on the activity on PTP-MEG2 (Fig. 5B).

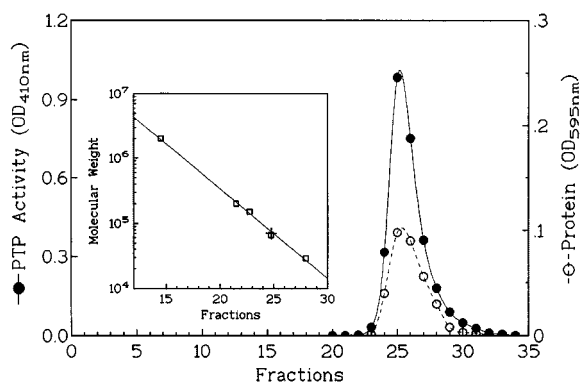

Fig. 4. Calibration of purified PTP-MEG2 with standard markers on a Superose-12 gel filtration column. Purified PTP-MEG2 from hydroxylapatite column was loaded onto a Superose 12 column (Pharmacia). PTP activities (solid line) and protein concentrations (dashed line) were determined as described in Figure 1. The inset represents a linear regression analysis of the molecular size markers (carbonic anhydrase, 29 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; β-amylase, 200 kDa; blue dextran, 2,000 kDa). PTP-MEG2 appeared at fraction 25 (indicated by "+") corresponding to a molecular size of 67 kDa.

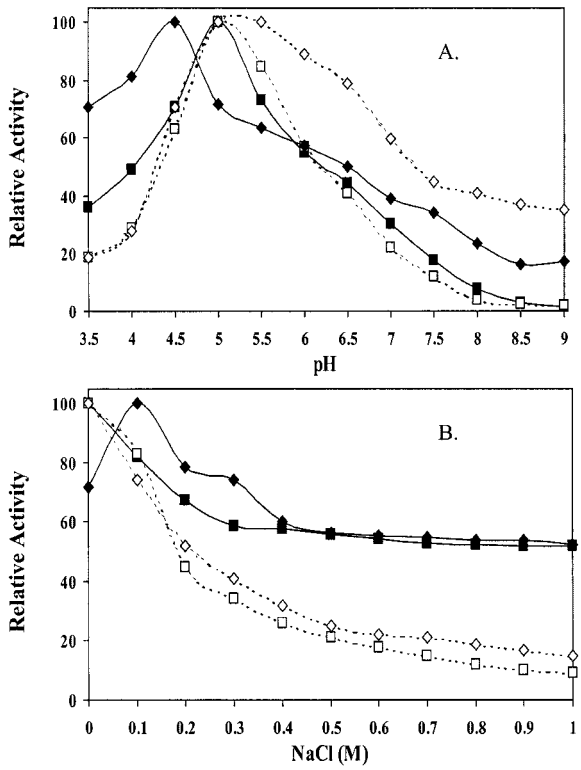

Fig. 5. Effects of pH and ionic strength on the activity of PTP-MEG2 and ΔPTP-MEG2. PTP-MEG2 (solid lines and closed symbols) and ΔPTP-MEG2 (dashed lines and open symbols) were analyzed with 10 mM p-NPP (square) or 1 mM P-Tyr (diamond) as substrates at different pH values in the absence of NaCl (**A**) or at pH 7 in the presence of the indicated concentrations of NaCl (**B**). Buffers used were 25 mM sodium acetate (pH 3.5–5.5), 25 mM MES-NaOH (pH 5.5–6.5), and 25 mM Tris-HCl (pH 7.0–9.0). Data represent relative activity.

While the activity with p-NPP was inhibited by addition of NaCl, a maximal activity of PTP-MEG2 with P-Tyr was observed at 0.1 M NaCl. Unlike PTP-MEG2, ΔPTP-MEG2 was only inhibited by the addition of NaCl.

Kinetics of PTP-MEG2

We characterized the kinetics of PTP-MEG2 and ΔPTP-MEG2 by using p-NPP and P-Tyr as substrates at pH 5.0 and pH 7.0 as summarized in Table III. Dependent on pH and substrates, turnover numbers (k_{cat} values) of 16–79 s^{-1} were observed. These values are comparable to those reported for SHP-1 [Zhao et al., 1993], higher than those for SHP-2 [Zhao et al., 1994], but below those of YOP [Zhang et al., 1992]. In all cases, ΔPTP-MEG2 displayed lower K_m values and higher calculated V_{max} values than full-length PTP-MEG2. Consequently, 2–10-fold higher k_{cat}/K_m values were obtained with ΔPTP-MEG2. This indicates that the truncated form of PTP-MEG2 is a more efficient enzyme than the full-length enzyme and suggests that the N-terminal putative lipid-binding domain has a regulatory role by suppressing the enzymatic activity. This is reminiscent of the SH2 domains of SHP-1 and SHP-2. Presumably, a ligand that binds the putative lipid-binding domain of PTP-MEG2 should activate the enzyme.

Effectors of PTP-MEG2

We further examined the effects of a number of potential effectors of PTP-MEG2 on its activity. The data are summarized in Table IV. A common feature of PTPs is that they are inhibited by vanadate. PTP-MEG2 is not exceptional, although its sensitivity is relatively low in comparison with other PTPs such as PTP1B, SHP-2, and TC-PTP [Tonks et al., 1988; Zander et al., 1991; Zhao et al., 1994]. As with other PTPs, metal ions Mg^{2+}, Ca^{2+}, and Mn^{2+} had no significant effects on the activity of PTP-MEG2 [Tonks et al., 1988; Zhao et al., 1992, 1993, 1994]. However, Zn^{2+}, a strong inhibitor for some PTPs [Tonks et al., 1988; Daum et al., 1991; Zander et al., 1991], had no major effects on PTP-MEG2. Since the N-terminal noncatalytic segment of PTP-MEG2 has a 24–29% sequence identity to CRALBP, α-tocopherol transfer protein, and yeast Sect14p [Gu et al., 1992; Aravind et al., 1999], we further examined

TABLE III. Enzyme Kinetic Constants of PTP-MEG2

Substrates and pH	K_m (mM)		V_{max} (U/mg)		k_{cat}/K_m (mM^{-1} s^{-1})	
	PTP-MEG2	ΔPTP-MEG2	PTP-MEG2	ΔPTP-MEG2	PTP-MEG2	ΔPTP-MEG2
p-NPP						
pH 5.0	2.4	1.1	7.0×10^4	1.4×10^5	33	77
pH 7.0	9.5	8.4	1.4×10^4	5.2×10^4	1.9	3.7
P-Tyr						
pH 5.0	3.2	0.54	4.9×10^4	1.6×10^5	17.5	180
pH 7.0	8.5	7.0	4.2×10^4	1.2×10^5	5.6	10.3

TABLE IV. Effectors of PTP-MEG2*

Effectors	p-NPP	P-Tyr
Control	1.00	1.00
Na_3VO_4 (0.1 mM)	0.86	0.85
Na_3VO_4 (1.0 mM)	0.17	0.41
$CaCl_2$ (1 mM)	1.01	1.00
$MgCl_2$ (5 mM)	1.02	1.07
$MnCl_2$ (1 mM)	—	0.93
$ZnCl_2$ (1.0 mM)	1.26	1.10
Triton X-100 (0.1%)	1.30	1.26
All-trans-Retinal (0.1 μM)	1.05	1.10
All-trans-Retinal (40 μM)	0.97	0.92
13-cis-Retinal (0.1 μM)	0.98	1.02
13-cis-Retinal (40 μM)	0.77	0.97
Retinal mixture (8.8 μM)	1.29	1.03
Retinal mixture (35 μM)	1.03	1.03
α-tocopherol (0.1 μM)	0.96	1.05
α-tocopherol (100 μM)	1.08	0.95
Phosphotidylinositol (1 mol%)	1.10	1.20
Phosphotidylinositol (10 mol%)	0.92	1.04

*PTP activity assays were performed with 1 mM P-Tyr or 1 mM p-NPP at pH 7.0. Retinal mixture denotes the phosphoisomerized all-trans-retinal. The assays with all the lipids contained 0.1% Triton X-100 in the reaction mixtures, and all the data represent relative activity.

the effects of ligands of these proteins, namely, retinal, α-tocopherol, and phosphatidylinositol. The stock solutions of these lipid compounds were made in 1% Triton X-100, but the final concentrations in the assay mixtures were controlled at 0.1%. Triton X-100 itself had a slight stimulatory effect on PTP-MEG2 toward both p-NPP and P-Tyr. All trans-retinal, 13-cis-retinal, α-tocopherol, and phosphatidylinositol had no major effects on the activity of PTP-MEG2. If anything, they slightly decreased the activity. We further tested the effects of other retinal isomers on PTP-MEG2 activity. Since these lipids are not commercially available due to their instability, we made them by exposing all trans-retinal to light as previously reported. This procedure usually gives rise to a mixture containing about 22% 13-cis-retinal, 29% 11-cis-retinal, 11% 9-cis-retinal, 1% 7-cis-retinal, and 37% all-trans-retinal [Bridges et al., 1980; Bridges, 1990]. PTP activity assays revealed that the retinal mixtures at concentrations as high as 35 μM exhibited no significant effect on PTP-MEG2 activity. Taken together, these data suggest that despite sequence similarity, ligands of CRALBP, α-tocopherol transfer protein, and Sect14p do not act on PTP-MEG2.

Activity of PTP-MEG2 and ΔPTP-MEG2 as GST Fusion Proteins

Due to easy purification through affinity columns, proteins are often expressed as GST fusion proteins in *E. coli* cells. Initially, we tried the same strategy to express PTP-MEG2 and ΔPTP-MEG2. The GST fusion protein containing the full-length PTP-MEG2 was large insoluble with very little activity in the exclusion body of *E. coli* extracts. Consequently, separation of the soluble cell extracts on a glutathione-Sepharose column only yielded about 10% pure enzyme. To facilitate protein purification, we attached a 6xHis tag to the C-terminal end of the fusion protein. The 6xHis-tagged GST-PTP-MEG2 was purified to near homogeneity through a combination of Ni-NTA-agarose and glutathione-Sepharose columns (Fig. 6). ΔPTP-MEG2 was expressed as a GST fusion protein only and was purified to near homogeneity by using a single glutathione-Sepharose column. We further analyzed the PTP activities of the fusion proteins by using 10 mM p-NPP under the same standard condition described above. Interestingly, the activities of the GST fusion proteins were 10–26-fold below those obtained with the correspondent non-fusion proteins (Table V). Therefore, the GST part has significant effects on the activity of PTP-MEG2. This is not totally unexpected since we had observed similar phenomenon with SHP-1 and SHP-2 in our earlier studies [unpublished observation]. The low activity with GST fusion proteins may be due to dimerization of the enzyme caused by GST. However, divalent PTP-MEG2

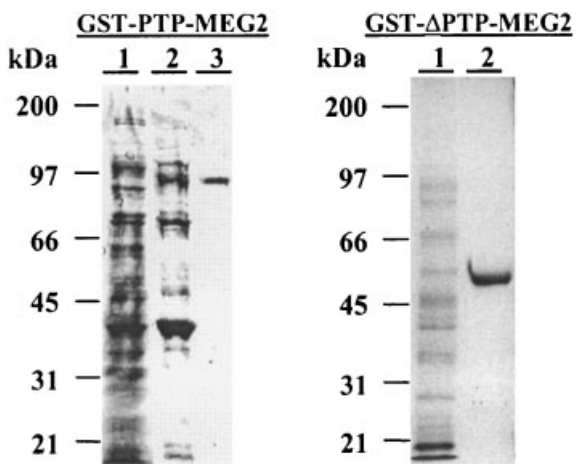

Fig. 6. Purification of PTP-MEG2 and ΔPTP-MEG2 as GST fusion proteins. PTP-MEG2 and ΔPTP-MEG2 were expressed in *E. coli* cells as a 6xHis-GST fusion protein and GST fusion protein, respectively. Recombinant proteins were purified from soluble cell extracts as described in Materials and Methods. Protein samples were separated by 10% SDS gels, and the gels were stained with Coomassie Blue. **Lane 1**, crude extracts; **lane 2**, Ni-NTA-agarose fraction of PTP-MEG2; **lane 3**, purified proteins from glutathione-Sepharose.

TABLE V. Comparison of Activity of PTP-MEG2 as Non-Fusion and GST Fusion Proteins

	pH 5.0		pH 7.0	
Enzymes	S.A. (U/mg)*	k_{cat} (s^{-1})	S.A. (U/mg)	k_{cat} (s^{-1})
PTP-MEG2	32	36	7.0	7.9
GST-PTP-MEG2	2.2	3.4	0.2	0.3
ΔPTP-MEG2	139	84	44	27
GST-ΔPTP-MEG2	5.6	5.4	1.2	1.1

*PTP activity assays were performed with 10 mM p-NPP at pH 5.0 or pH 7.0.

antibody #144 which was raised against the C-terminal peptide of PTP-MEG2 had no inhibitory effect on the activities of PTP-MEG2 and ΔPTP-MEG2. The low activity with the GST fusion proteins may be due to spatial restrains imposed by the GST portion or improper folding of the catalytic domain.

DISCUSSION

By using recombinant adenovirus, we have highly expressed PTP-MEG2 in human 293 cells. We further purified the enzyme to near homogeneity through a four-step chromatographic procedure. The purified enzyme exists as a monomer in solution and its responses to common PTP effectors are similar to those of other characterized PTPs. As a full-length enzyme, PTP-MEG2 displays a high activity towards the commonly used PTP substrates p-NPP and P-Tyr. The activity is comparable to that of SHP-1, but ~10-fold higher than that of SHP-2 [Zhao et al., 1993, 1994]. By using the *E. coli* expression system, we further purified ΔPTP-MEG2, a truncated form of PTP-MEG2 containing only the catalytic domain. Kinetic studies demonstrated that the truncated enzyme is significantly more active than the full-length enzyme as indicated by lower K_m and higher V_{max} values. This suggests that the N-terminal putative lipid-binding domain inhibits the activity of the catalytic domain. We have also expressed PTP-MEG2 and ΔPTP-MEG2 as GST fusion proteins and revealed that the GST fusion proteins had much lower activity than the non-fusion proteins. Therefore, when GST fusion proteins are used for characterization of enzymes, especially for activation and inhibition studies, the effects of GST part must be taken into consideration.

Intracellular PTPs contain various domains or segments surrounding the catalytic domain of approximately 230 amino acids. It has been well accepted that these flanking peptide segments or protein domains play regulatory and/or targeting roles. Our study demonstrated that removal of N-terminal putative lipid-binding domain significantly enhanced PTP-MEG2 activity. This further emphasizes the regulatory role of the lipid-binding domain. This is reminiscent of the inhibitory role of the N-terminal SH2 domains and the C-terminal segment of SHP-1 and SHP-2. Purified SHP-1 and SHP-2 display very low activity in comparison to other truncated forms of PTPs especially when protein substrates are used, but their activities are significantly enhanced after removal of SH2 domains or C-terminal segment [Zhao et al., 1993, 1994]. The crystal structure of SHP-2 revealed intrusion of the N-terminal SH2 domain into the catalytic center of SHP-2 [Hof et al., 1998]. This explains the inhibitory effect of the SH2 domain and suggests that the catalytic activity can be restored by structural rearrangement of the SH2 domain. Indeed, binding of the SH2 domains to tyrosine-phosphorylated peptides or proteins causes significant activation of SHP-1 and SHP-2 [Sugimoto et al., 1994; Zhao and Zhao, 2000]. Furthermore, interaction of the enzymes with anionic phospholipids results in an even more pronounced activation [Zhao et al., 1993a, 1994], which probably also involves participation of the C-terminal segment. The N-terminal putative lipid-binding domain of PTP-MEG2 shares 24–29% sequence identity with CRALBP, α-tocopherol transfers protein, and yeast SEC14P [Gu et al., 1992; Aravind et al., 1999]. These proteins specifically bind retinal, α-tocopherol, and phosphatidylinositol, respectively. We have examined the effects of these lipid molecules on PTP-MEG2 activity. However, none of them showed any significant effect. Therefore, if there are lipid molecules involved in the regulation of

PTP-MEG2, they may belong to a different type. It should be pointed out, however, that a recent study by Kruger et al. [2002] demonstrated that a GST fusion protein of PTP-MEG2 is significantly activated by phosphatidylinositol 4, 5-diphosphate and other phospho-derivatives of phosphatidylinositol. We have conducted similar studies with PTP-MEG2 purified from adenovirus-infected 293 cells, but did not see any stimulatory effects of phosphatidylinositol 4,5-diphosphate or any other phospho-derivatives of phosphatidylinositol (data not shown). It remains to be clarified whether GST has anything to do with this activation. Identification of specific activators of PTP-MEG2 is very important for understanding its function, and availability of purified native enzyme makes this highly feasible.

ACKNOWLEDGMENTS

We are grateful to Dr. David Ong for providing us all-trans retinal and 13-cis retinal and to Dr. Sidney Fleischer and Dr. J. Oliver McIntyre for their advice on lipid micelle preparation. This work was supported by grants CA75218, HL-57393 (to Z.J.Z.), DK-15555 and 2 T32-DK07186 (to S.B.K.), and CA-68485 (to Vanderbilt-Ingram Cancer Center) from the National Institutes of Health and a Veterans Health Administration Merit Review Grant (to S.B.K.).

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