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Hepatic protein tyrosine phosphatases in the rat

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Regulation of cell growth and metabolism by protein tyrosine phosphorylation involves dephosphorylation via the action of protein tyrosine phosphatases (PTPases). We have characterized the membrane PTPases in rat liver, monitoring their activity by measuring the dephosphorylation of *P*-Tyr-reduced, carboxyamidomethylated and maleylated lysozyme (*P*-Tyr-RCML) and *P*-Tyr-myelin basic protein (*P*-Tyr-MBP). Separation of membrane PTPases by poly (L-lysine) chromatography yielded three peaks of PTPase, termed I, II and III. PTPases I and II were most active with *P*-Tyr-RCML, whereas PTPase III showed greater activity with *P*-Tyr-MBP than with *P*-Tyr-RCML (ratio of activities 4:1). Separation of membrane proteins by gel-filtration chromatography yielded two peaks of activity. Based on substrate specificity, sensitivity to inhibitors and requirement for thiol-containing compounds, the activity peak with an M_r of ~ 400000 corresponded to PTPase III, whereas that with an M_r of approx. 40000 contained PTPases I and II. All three PTPases dephosphorylated epidermal growth factor receptors and insulin receptors, but only PTPases I and II were active with *P*-Tyr-asialoglycoprotein receptors. Although none of the above characteristics distinguished between PTPases I and II, only PTPase I reacted in a Western immunoblotting procedure with anti-peptide antibodies directed towards human placental PTPase. We conclude that the membrane fraction from rat liver contains at least three distinct PTPases.

INTRODUCTION

Protein phosphorylation is well established as a post-translational regulatory mechanism which is involved in the control of numerous and diverse cellular processes. In recent years the phosphorylation of proteins on tyrosine has assumed a role of central importance in the control of cell growth [1,2]. Although numerous protein tyrosine kinases have been characterized over the last decade, elucidation of the nature and regulation of protein tyrosine phosphatases (PTPases; protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48) has begun only recently. The purification to homogeneity of a 35000-M_r PTPase from human placenta several years ago by Tonks and colleagues [3,4] led to the first data on the primary structure of a PTPase, termed 1B. Surprisingly, the sequence of this enzyme showed similarity to that of the cytoplasmic domain of the leucocyte cell surface protein CD45 [5]. Shortly thereafter it was demonstrated that CD45 (also called Ly-5, T200 or B220; for a review, see [6]) is indeed a transmembrane PTPase [7]. Subsequent work has demonstrated at least two distinct classes of PTPases. PTPases analogous to placental PTPase 1B with an M_r of approx. 50000 have been cloned from rat brain [8], human T cells [9] and human placenta [10]. CD45, a leucocyte-specific protein, has been shown to have a relative, called LAR (leucocyte antigen related), which is also a transmembrane protein with PTPase activity in its cytoplasmic domain [11].

We have recently reported on the characterization of PTPases in rat liver, with emphasis on the effects of experimental diabetes on protein-tyrosine dephosphorylation [12]. Using phosphotyrosyl-reduced, carboxyamidomethylated and maleylated lysozyme (*P*-Tyr-RCML) and *P*-Tyr-poly(Glu_4,Tyr_1) (polyEY) as substrates, our results indicated a single class of PTPases distributed in soluble, membrane (cell surface and endosomal) and cytoskeletal fractions. Soluble PTPase activity could be generated by proteolysis of membranes. PTPase in the particulate subcellular fractions had activity against multiple substrates, including insulin receptors and epidermal growth factor (EGF) receptors. PTPase activities in the subcellular fractions also had similar sensitivities to PTPase inhibitors. Our earlier results were consistent with the presence of a single PTPase in rat liver, in contrast with the molecular cloning data now available indicating multiple PTPases. We have therefore attempted the further characterization and purification of rat liver PTPases.

MATERIALS AND METHODS

Materials

Wheat-germ-lectin–Sepharose 6MB, DEAE-Sepharose CL-6B, Ultrogel AcA 44 and MonoQ (prepacked, f.p.l.c.) were obtained from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Affi-Gel Blue was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Poly(L-lysine)–agarose, heparin–agarose, iminodiacetic acid–agarose and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. [γ-^{32}P]ATP was obtained from New England Nuclear Research Products, Boston, MA, U.S.A. Lysozyme was reduced and carboxyamidomethylated as described previously [3] for use as a phosphatase substrate. Myelin basic protein (MBP; from bovine brain), also used as phosphatase substrate, was obtained from Sigma.

Measurement of PTPase activity

PTPase activity was measured as the release of ^{32}P from substrates phosphorylated on tyrosine [13]. Assays were carried out in the presence of 1 mM-EDTA and 0.1 % (v/v) 2-mercapto-

Abbreviations used: PTPase, protein tyrosine phosphatase; *P*-Tyr-RCML, phosphotyrosyl-reduced, carboxyamidomethylated and maleylated lysozyme; *P*-Tyr-MBP, phosphotyrosyl-myelin basic protein; polyEY, poly(Glu_4,Tyr_1); PMSF, phenylmethanesulphonyl fluoride; EGF, epidermal growth factor; ASG, asialoglycoprotein; LAR protein; leucocyte antigen-related protein; IC_{50}, concentration causing 50 % inhibition.

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ethanol. RCML and MBP were labelled on tyrosine residues using partially purified human placental insulin and EGF receptor tyrosine kinases, as described by Tonks *et al.* [3]. Phosphorylation of MBP was optimized by freezing and thawing five times before use. Dephosphorylation of both substrates was measured at a final *P*-Tyr-substrate concentration of 1 μM.

Preparation of rat liver membrane and cytoskeletal fractions

Unfractionated liver membranes were made from fresh livers by the method of Williams *et al.* [14]. As described previously [12], we define membrane proteins as being soluble in 1% Triton X-100, whereas cytoskeletal proteins are defined as being insoluble in detergent but soluble in 0.6 M-KCl.

Preparation of rat liver extracts for PTPase purification

Sprague–Dawley rats (Charles River Breeding Laboratories Inc., Wilmington, MA, U.S.A.) were killed by decapitation under Nembutal anaesthesia. Livers were removed immediately and placed on ice until homogenization. Pooled livers (55–70 g) were homogenized using a Waring blender with 10 ml of buffer A/g (buffer A = 50 mM-Hepes, 0.25 M-sucrose and 5 mM-EDTA, pH 7.4) containing freshly added 5 mM-benzamidine, 0.002% phenylmethanesulphonyl fluoride (PMSF), and aprotinin (3 units/ml). The homogenate was centrifuged at 1000 *g* for 15 min and the pellet was discarded. The supernatant was brought to 1% Triton X-100 and stirred for 45 min at 4 °C. Detergent-insoluble protein was removed by centrifugation (14000 *g* for 30 min) and 2-mercaptoethanol was added to the extract to a concentration of 0.4% (v/v). Protein determinations were made using the method of Bradford [15] or, for detergent-extracted samples, the bicinchoninic acid method (BCA; Pierce Chemical Co., Rockford, IL, U.S.A.). Protein assays used BSA as a standard.

Production of anti-peptide antibodies

Antibodies against a synthetic peptide (ENLTTQETREIL-HFHYTT) corresponding to residues 161–178 in the human PTPase [16] were produced in rabbits and affinity purified using the immobilized peptide (F. Pinault & D. L. Brautigan, unpublished work).

Western immunoblotting

Proteins separated by PAGE were transferred electrophoretically to nitrocellulose membranes and detected by a Western immunoblotting procedure utilizing ^{125}I-Protein A [17]. Briefly, the nitrocellulose membranes were first incubated in a quench solution, after which they were incubated overnight at room temperature in a solution of anti-peptide antibodies (5 μg of protein/ml) in quench solution. The nitrocellulose was washed and incubated with ^{125}I-Protein A in the same quench solution. Immunoreactive proteins were detected by exposure of the nitrocellulose to Kodak XAR-5 film at −70 °C in the presence of intensifying screens.

Dephosphorylation of rat liver EGF receptors, insulin receptors and asialoglycoprotein (ASG) receptors

Rat liver insulin receptors and EGF receptors were partially purified from membrane preparations using wheat-germ lectin affinity chromatography [12]. For the present studies, receptor preparations were concentrated 10-fold with an Amicon Micropartition System MPS-1 using a YMT membrane. Receptor autophosphorylation was accomplished by incubating receptor preparations (40 μl) with hormones (20 μl; 0.3 μM-insulin and -EGF, final concentrations) for 30 min at 30 °C. Receptor activation was monitored by the addition of 15 μl containing 6.75 μCi of [γ-^{32}P]ATP, 50 μM-ATP and 25 mM-$MnCl_2$. Incubation at 4 °C proceeded for 5 min and was carried out in the absence of phosphatase inhibitors. Phosphorylation was terminated by the addition of 2-mercaptoethanol (15 mM) and EDTA (6 mM).

For insulin receptors and EGF receptors, dephosphorylation was initiated by the addition of PTPase (10 μl). The reaction proceeded at 37 °C and was terminated by the addition of 5× concentration gel electrophoresis sample buffer containing 50 mM-ATP, 10% (w/v) SDS and 0.5 M-dithiothreitol and incubation for 10 min in a boiling water bath. Phosphoproteins were separated by PAGE (7.5% gels) and detected by exposure of the dried gel to X-ray film as noted above.

ASG receptors were labelled on tyrosine residues with ^{32}P in membranes prepared from HepG2 cells [18] and immunoprecipitated using antibodies against the human receptor bound to Sepharose beads [19]. A time course of dephosphorylation was performed by incubating 110 μl of suspended beads with 110 μl of PTPase at 37 °C. At various times, 40 μl portions of the mixture were transferred to a tube containing 10 μl of 5 mM-vanadate. The beads were collected by centrifugation and the bound ASG receptor was eluted by boiling in gel electrophoresis sample buffer. Electrophoresis and autoradiography proceeded as above.

RESULTS

Separation of membrane PTPases by poly(L-lysine)–agarose affinity chromatography

An extract of rat liver membranes containing 58 mg of protein was applied to a 10 ml column of poly(L-lysine)–agarose equilibrated in buffer B (see Fig. 1 legend). After washing with 20 ml of buffer B, PTPases were eluted with a linear gradient from 0 to 1 M-NaCl in the same buffer (Fig. 1). Using *P*-Tyr-RCML as substrate there were two major and two minor peaks of activity, with most of the activity eluting between 0.2 and 0.5 M-NaCl. The peak eluting at fractions 35–45 was present in varying amounts in multiple preparations. In contrast, with *P*-Tyr-MBP as substrate, the fractions up to 0.5 M-NaCl had little activity,

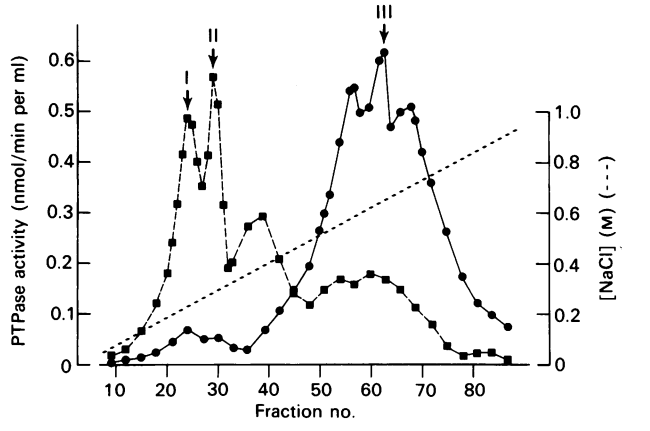

Fig. 1. Separation of rat liver membrane PTPases by poly(L-lysine)–agarose chromatography

Proteins extracted from rat liver membranes using 1% Triton X-100 were adsorbed to a 10 ml column of poly(L-lysine)–agarose equilibrated in 20 mM-Hepes (pH 7.4)/2 mM-EDTA/1 mM-benzamidine/0.002% PMSF/0.4% 2-mercaptoethanol (buffer B) and eluted with a linear gradient from 0 to 1 M-NaCl in buffer B (---). PTPase activity was measured using *P*-Tyr-MBP (●) or *P*-Tyr-RCML (■) as substrate. Single fractions designated as PTPases I, II and III were used for further analysis.

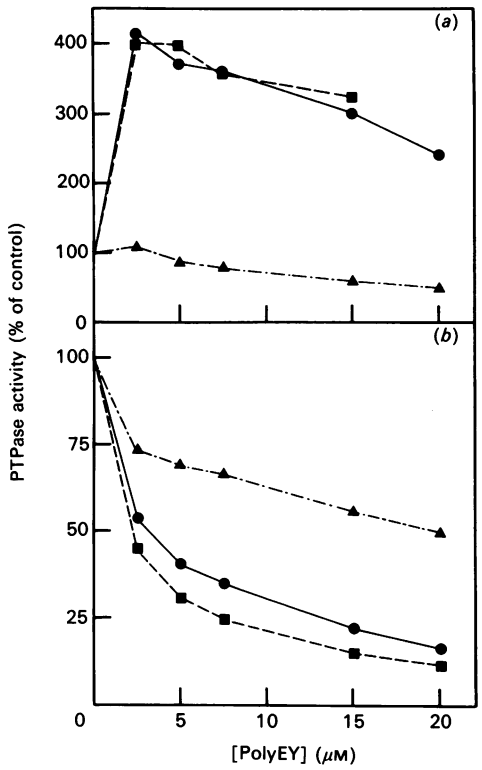

Fig. 2. Effects of polyEY on membrane PTPases

The activities of membrane PTPases separated by poly(L-lysine) chromatography were measured in the presence of various concentrations of polyEY using *P*-Tyr-MBP (*a*) or *P*-Tyr-RCML (*b*) as substrate. The three preparations used were fractions 24 (●), 29 (■) and 62 (▲) obtained from the chromatogram shown in Fig. 1.

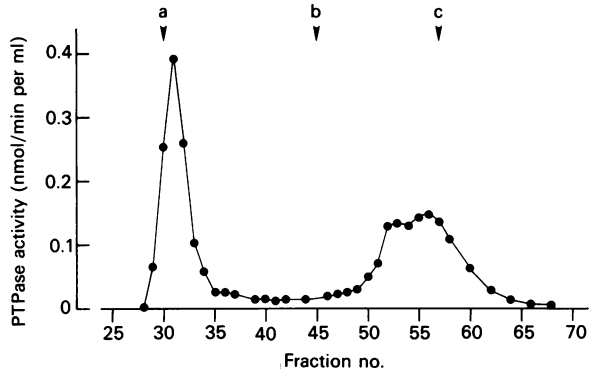

Fig. 3. Separation of rat liver membrane PTPases by f.p.l.c. gel-filtration chromatography

An extract of rat liver membranes was applied to a 15 ml SW-300 f.p.l.c. gel-filtration column equilibrated in 25 mM-Hepes (pH 7.5)/150 mM-NaCl/1 mM-EDTA/BSA (0.1 mg/ml)/0.4% 2-mercaptoethanol. Fractions (0.2 ml) were collected and assayed for PTPase activity using *P*-Tyr-RCML as substrate. Symbols at the top of the Figure denote the calibration of the column: a, void volume; b, BSA (M_r 67000); c, carbonic anhydrase (M_r 29 000).

whereas a broad peak that eluted between 0.5 and 0.8 M-NaCl had higher activity with *P*-Tyr-MBP than with *P*-Tyr-RCML. In some preparations (such as that shown in Fig. 1) three distinct peaks of activity with *P*-Tyr-MBP were recovered, whereas in other preparations a single broad peak was obtained. Sensitivity

Table 1. PTPase specific activities measured with *P*-Tyr-RCML and *P*-Tyr-MBP in rat liver membrane, cytoskeletal and cytosolic fractions

Fractions from five rat livers were analysed. Results represent the means ± S.D. * $P < 0.01$ versus activity in the same fraction measured with *P*-Tyr-RCML.

	PTPase activity (nmol/min per mg of protein)	
	P-Tyr-MBP	*P*-Tyr-RCML
Membrane	2.86±0.30*	0.82±0.07
Cytoskeleton	1.04±0.11	0.94±0.19
Cytosol	0.41±0.02*	0.22±0.05

to inhibitors and activators (see below) did not distinguish between the three peaks (results not shown). The three fractions with peak PTPase activity, designated I, II and III, were subjected to further analysis. The addition of Triton X-100 to the elution buffers advanced elution of PTPase III such that its position in the chromatogram overlapped those of PTPases I and II.

We have previously noted sensitivity of rat liver PTPase activity to polyEY [12]. As with rat liver extracts, PTPase activity measured with *P*-Tyr-RCML was indeed inhibited by polyEY (Fig. 2*b*). Of note is the fact that PTPases I and II were more sensitive to polyEY than was PTPase III. In contrast, with *P*-Tyr-MBP as substrate (Fig. 2*a*) PTPases I and II were activated 4-fold by as little as 2.5 μM-polyEY. However, PTPase III was not activated but was inhibited by polyEY when *P*-Tyr-MBP was used as substrate.

Proteins in membrane extracts were also separated by gel-filtration chromatography using an SW-300 pre-packed column (Waters Chromatography Division, Milford, MA, U.S.A.). PTPase activity measured with *P*-Tyr-RCML as substrate was present in two distinct activity peaks with M_r values of approx. 400000 and 40000 (Fig. 3). However, the high-M_r PTPase had almost 4-fold higher activity with *P*-Tyr-MBP (ratio of activity with MBP/RCML for fraction 31 = 3.7). The lower-M_r PTPases displayed only about one-eighth of the activity with *P*-Tyr-MBP compared with *P*-Tyr-RCML (MBP/RCML ratio = 0.11 for fraction 53 and 0.15 for fraction 57).

Charactization of the properties of the high- and low-M_r PTPases obtained by gel-filtration f.p.l.c. used *P*-Tyr-MBP as substrate. The high-M_r PTPase was inhibited by polyEY, whereas the lower-M_r PTPases were activated. Thus we conclude that PTPases I and II correspond to the lower-M_r PTPase obtained by gel-filtration f.p.l.c., whereas PTPase III corresponds to the high-M_r enzyme.

Based on substrate specificity, it seems that PTPases also differ in subcellular localization (Table 1). Activity measurements made on soluble, membrane and cytoskeletal fractions from five livers showed that the specific activity with *P*-Tyr-RCML was comparable in membrane and cytokeletal fractions. In contrast, activity with *P*-Tyr-MBP was nearly 3-fold higher in membrane fractions than in cytoskeletal fractions. In both cases, specific activities in the soluble fractions were low.

PTPase activities in unfractionated membrane extracts, measured with *P*-Tyr-RCML and *P*-Tyr-MBP, also differed in their dependence on thiol-containing reducing agents (Fig. 4). Activity measured with *P*-Tyr-RCML was stable in 0.4% 2-mercaptoethanol and 10 mM-dithiothreitol, but lower concentrations (0.1% and 1 mM respectively) were not sufficient to maintain activity. In contrast, activity measured with *P*-Tyr-MBP was stable in the presence of 0.1% 2-mercaptoethanol or

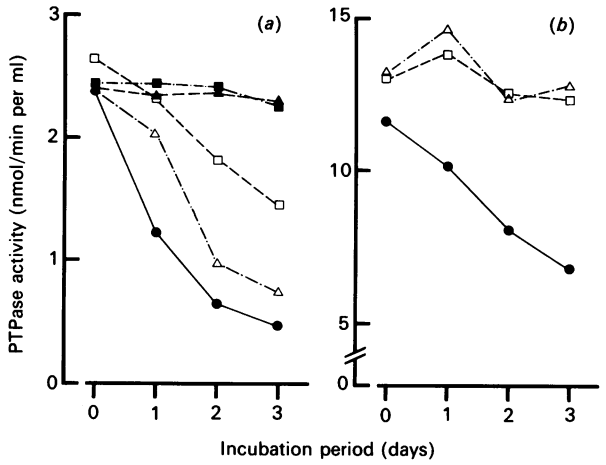

Fig. 4. Dependence of rat liver PTPases on thiol-containing compounds

PTPase activities from unfractionated membrane extracts, measured with *P*-Tyr-RCML (*a*) and *P*-Tyr-MBP (*b*), were monitored during incubation at 4 °C for 3 days in the absence of reducing agents (●) or in the presence of 15 mM-2-mercaptoethanol (□), 60 mM-2-mercaptoethanol (■), 1 mM-dithiothreitol (△) or 10 mM-dithiothreitol (▲). Data for 60 mM-2-mercaptoethanol and 10 mM-dithiothreitol are not shown for *P*-Tyr-MBP (*b*), as they were indistinguishable from activities at the lower concentrations.

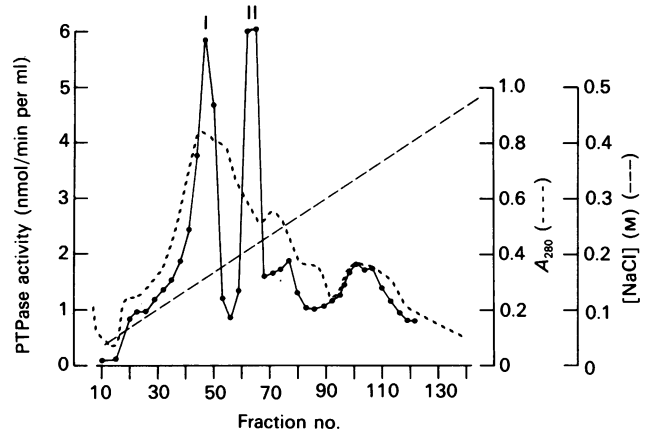

Fig. 5. Separation of rat liver PTPase I and PTPase II by poly(L-lysine)–agarose chromatography

Rat liver extract was prepared using Triton X-100 (see the Materials and methods section). PTPase activity was adsorbed to and eluted from DEAE-Sepharose, precipitated with ammonium sulphate, dialysed and adsorbed to a 10 ml column of poly(L-lysine)–agarose (see the Results section). The column was washed and eluted with a linear gradient (---) from 0 to 0.5 M-NaCl. The eluate was monitored for protein content (A_{280}; ----) and PTPase activity with *P*-Tyr-RCML as substrate (——).

1 mM-dithiothreitol. In other experiments (results not shown) PTPases I and II showed dependence on thiol-containing compounds similar to that seen for membrane activity with *P*-Tyr-RCML. Dependence of PTPase III activity on thiol-containing agents was similar to that of membrane extracts measured with *P*-Tyr-MBP.

Purification of PTPase I and PTPase II

We purified PTPases I and II further to better characterize their properties. Yields from membrane preparations were insufficient for our purpose so we began with rat liver homogenates extracted with 1% Triton X-100 (see the Materials and methods section). The addition of 0.4% 2-mercaptoethanol to the extract resulted in a significant (6–7-fold) increase in PTPase activity, consistent with the dependence on reducing agents noted above. However, 0.4% 2-mercaptoethanol did not prevent the loss of activity in the more dilute protein solutions that occurred during the latter stages of purification.

Purification of PTPase, monitored as the dephosphorylation of *P*-Tyr-RCML, proceeded as follows. The extracted homogenate was adsorbed to an equivalent volume of DEAE-Sepharose CL-6B equilibrated in buffer B and step-eluted with buffer B containing 0.5 M-NaCl. The eluate was precipitated by the addition of $(NH_4)_2SO_4$ to 70% satn. The precipitate was centrifuged and resuspended in a minimum volume of buffer B. The sample was dialysed against 10 mM-Hepes (pH 7.25)/25 mM-NaCl/0.4% 2-mercapthoethanol and adsorbed to 90 ml of poly(L-lysine)–agarose equilibrated in buffer B, and washed with 1 column vol. of the same. PTPase activity was eluted with a linear salt gradient from 0 to 0.5 M-NaCl in buffer B. In multiple preparations this generated two separate peaks of PTPase activity (Fig. 5), each of which was pooled for further purification. These PTPase activities were eluted at NaCl concentrations similar to those for peaks I and II purified from membrane extracts (see Fig. 1), and are therefore also designated as PTPases I and II. The relative activities of PTPases I and II with *P*-Tyr-MBP and *P*-Tyr-RCML, whether purified from membrane extracts or from whole liver extracts, were similar (results not shown).

Purification of liver extract PTPase I and PTPase II proceeded as follows. PTPase I and PTPase II from poly(L-lysine) chromatography were precipitated by 70% satn. with $(NH_4)_2SO_4$, centrifuged and resuspended in 50 mM-Hepes, pH 7.0, in order to eliminate reducing agent before zinc affinity chromatography.

Three and five preparations of PTPase I and PTPase II respectively were pooled and adsorbed to 20 ml of iminodiacetic acid–agarose charged with Zn^{2+} and equilibrated in 10 mM-Hepes (pH 7.0)/0.5 M-NaCl and eluted with 10 mM-Hepes (pH 7.0)/10% (v/v) glycerol/50 mM-EDTA/1 mM-dithiothreitol. PTPase I and PTPase II both bound to the column and were recovered with similar yields (Table 2).

The PTPase I and PTPase II preparations were directly adsorbed to 20 ml of Affi-Gel Blue equilibrated in buffer B, washed with 2 column vol. of the same and eluted using a linear salt gradient from 0.1 to 1.0 M-NaCl in buffer B. Both preparations were eluted as single peaks of activity that were recovered and dialysed against buffer B.

The two preparations were adsorbed to 8 ml of heparin–agarose equilibrated in buffer B for further purification. After washing with 2 column vol. of the same buffer, samples were eluted with a linear salt gradient from 0 to 1.0 M-NaCl in buffer B. Again, PTPases I and II behaved similarly eluting as single peaks in the middle of the gradient.

The overall purification for PTPase I was 400-fold, with a 3% yield (Table 2). For PTPase II, an overall purification of 195-fold was achieved, with a 1% yield (Table 2). It appears that PTPase recovery was low due to instability of the enzymes, since total protein in the preparation was lowered from 38.4 g (total protein of five pooled preparations) to 2 mg for PTPase I and from 15.9 g (three preparations) to 0.8 mg for PTPase II.

Comparison of PTPase I, II and III activities

PTPases I and II displayed similar sensitivity to the PTPase inhibitor vanadate [concn. giving 50% inhibition (IC_{50}) = 35 μM for both preparations]. PTPases I and II purified through the Affi-Gel Blue affinity chromatography step showed an IC_{50} for polyEY inhibition of 2.2 μM and 3.0 μM respectively, similar to the results shown in Fig. 2.

Table 2. Purification of low-M_r PTPases (I and II)

Activity was monitored as the dephosphorylation of *P*-Tyr-RCML. Data for the initial stages of purification (up to separation of PTPases I and II) represent the means of seven preparations. Data in the lower two panels are for pooled material used for further purification of either PTPase I or PTPase II (see the text). One unit is defined as hydrolysis of 1 nmol of substrate/min at 30 °C.

	Activity (Total units)	Activity (units/mg)	Purification (-fold)	Yield (%)
Extract	1922	0.29	–	100
DEAE-Sepharose	1254	0.65	2	65
Poly(L-lysine) I	206	2.38	8	11
Poly(L-lysine) II	232	3.18	11	12
PTPase I purification:				
Poly(L-lysine)	263	1.83	8	11
Zinc affinity	234	2.80	12	10
Affi-Gel Blue	151	18.9	57	6
Heparin–agarose	71	90.7	400	3
PTPase II purification:				
Poly(L-lysine)	1416	3.36	11	12
Zinc affinity	731	3.78	13	6
Affi-Gel Blue	470	22.6	77	4
Heparin–agarose	112	57.1	195	1

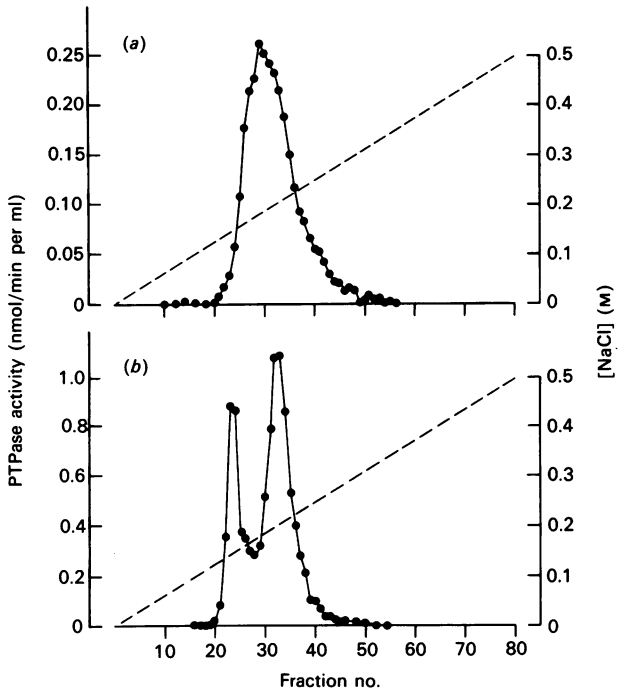

Fig. 6. Ion-exchange f.p.l.c. of PTPases I and II

PTPase I and PTPase II were purified using multiple affinity chromatography steps (see the Results section). PTPase I (*a*) and PTPase II (*b*) preparations obtained following Affi-Gel Blue chromatography were adsorbed to and eluted from a MonoQ column using a linear gradient (---) from 0 to 0.5 M-NaCl. Fractions were assayed for PTPase activity with *P*-Tyr-RCML as substrate. Nearly identical results were obtained using several PTPase I and II preparations.

As noted above, the two preparations behaved similarly during affinity chromatography with Zn^{2+}–iminodiacetic acid–agarose, Affi-Gel Blue and heparin–agarose. Gel-filtration chromatography using Ultrogel AcA 44 (results not shown) yielded an apparent M_r of 45000–55000 for both preparations during initial [poly(L-lysine)] and latter stages of purification. However, during chromatography using MonoQ, different profiles were obtained. The dialysed Affi-Gel Blue pools were adsorbed to a MonoQ column equilibrated with 20 mM-Hepes (pH 7.2)/10% glycerol/0.4% 2-mercaptoethanol and washed with 10 ml of the same buffer. Activity was eluted with a linear salt gradient from 0 to 0.5 M-NaCl in the same buffer (Fig. 6). The PTPase I preparation yielded a single peak of activity. However, PTPase II consistently yielded two peaks during several preparations. The separation by ion exchange of two forms after multiple steps of purification can be interpreted as being due to a minor difference in net charge (such as that due to phosphorylation) between two otherwise nearly identical proteins.

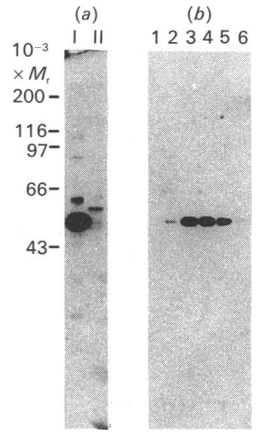

Fig. 7. Detection of PTPases by Western immunoblotting

PTPase preparations were subjected to PAGE, electrophoretic transfer to nitrocellulose membranes and Western immunoblotting using PTPase 1B anti-peptide antibodies (see the Materials and methods section). Autoradiograms of a Western immunoblot are shown. (*a*) Analysis of PTPase I and II obtained from poly(L-lysine) chromatography (see Fig. 5). (*b*) Analysis of fractions 24, 28, 30, 32, 36 and 40 (lanes 1–6) from MonoQ chromatogram of a PTPase I preparation (see Fig. 6).

PTPase I and PTPase II preparations from poly(L-lysine) chromatography were subjected to Western immunoblotting with anti-peptide antibodies directed against human placental PTPase (Fig. 7*a*). An intense band at M_r 49000 was seen in the PTPase I but not in the PTPase II preparation. Further purification demonstrated that this 49000-M_r immunoreactive protein in the PTPase I preparation co-purified with PTPase activity, as demonstrated in a Western immunoblot (Fig. 7*b*) of the fractions from MonoQ chromatography shown in Fig. 6. It should be noted that the intensity of labelling in the immunoblot did not precisely coincide with the level of activity, raising the possibility of multiple enzymes in the PTPase I preparation.

In contrast with results obtained for PTPase I, no immunoreactive bands are detected in the Western immunoblot of the PTPase II preparation after MonoQ chromatography, even though higher amounts of PTPase activity were analysed. We take this difference in immunoreactivity as evidence of a difference in primary structure between PTPase I and PTPase II.

PTPase III obtained from poly(L-lysine) chromatography of a membrane extract was found to exhibit markedly different behaviour than PTPases I and II. PTPase III was not well adsorbed to DEAE-Sepharose (approx. 40% yield), heparin–agarose (40% yield) or Affi-Gel Blue (50% yield). Yields were unaffected by the presence or absence of Triton X-100. However,

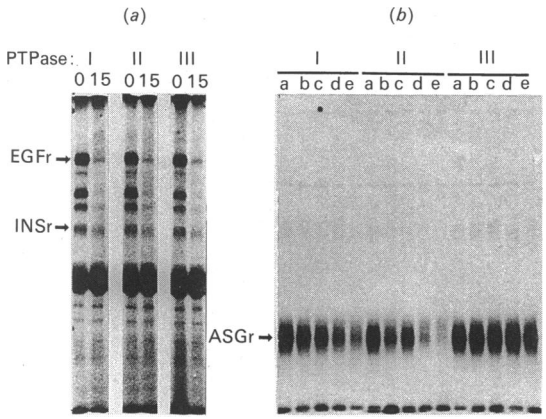

Fig. 8. Dephosphorylation of insulin receptors, EGF receptors and ASG receptors by membrane PTPases I, II and III

Membrane PTPases I, II and III obtained from poly(L-lysine) chromatography (see Fig. 1) were used to dephosphorylate hepatic receptors phosphorylated on tyrosine. (*a*) Partially purified rat liver *P*-Tyr-EGF receptors and *P*-Tyr-insulin receptors were incubated with PTPases for 0 or 15 min at 37 °C. EGF receptors (EGFr) and insulin receptor β-subunits (INSr) were identified based on M_r and dependence of phosphorylation on EGF and insulin respectively. (*b*) Immunoprecipitated *P*-Tyr-ASG receptors (ASGr; M_r 46000) were incubated with PTPases for 0 (lane a), 5 (lane b), 10 (lane c), 20 (lane d) or 30 (lane e) min at 37 °C.

like PTPase I and PTPase II, PTPase III activity was inhibited by zinc (IC_{50} approx. 120 μM) and bound strongly to Zn^{2+}–imino-diacetate–agarose.

Activity of PTPases with *P*-Tyr-receptor proteins

We studied the activity of membrane PTPases I, II and III, separated by poly(L-lysine) chromatography (see Fig. 1), with *P*-Tyr-ASG receptors, *P*-Tyr-insulin receptor and *P*-Tyr-EGF receptors (Fig. 8). Insulin receptors and EGF receptors were partially purified from human placenta membranes by detergent solubilization and wheat-germ-lectin chromatography. In the presence of [γ-^{32}P]ATP, labelling of the respective receptors was dependent on the addition of hormone. Of the ^{32}P-labelled proteins in these preparations, the insulin receptor β-subunit (M_r 95000) and the EGF receptor (M_r 170000) were major components, readily identified by their migration in polyacrylamide gels (Fig. 8). Both the insulin receptor and EGF receptor were dephosphorylated by PTPase I, II and III when comparable amounts of PTPase activity were added for 15 min at 37 °C. However, the PTPases displayed specificity when *P*-Tyr-ASG receptor was used as substrate. PTPases I and II dephosphorylated *P*-Tyr-ASG receptors, whereas PTPase III did not produce appreciable dephosphorylation within 30 min.

DISCUSSION

Based on separation during purification, substrate specificity, sensitivity to inhibitors and differential immunoreactivity, there are three distinct membrane PTPases in rat liver. PTPases I and II partially purified from rat liver extracts could be separated by poly(L-lysine)–agarose affinity chromatography. Their characteristics included the following: M_r approx. 40000 based on gel-filtration chromatography; 6–10-fold greater activity with *P*-Tyr-RCML than with *P*-Tyr-MBP; sensitivity to inhibition by polyEY (IC_{50} approx. 2.5 μM) with *P*-Tyr-RCML as substrate, in contrast with activation by 2.5 μM-polyEY with *P*-Tyr-MBP as substrate. These two PTPase activities were indistinguishable based on sensitivity to inhibitors. Their similar sensitivity to polyEY is in contrast with the placental PTPases purified by Tonks *et al.* [4], which could be distinguished on this basis. PTPases I and II behaved similarly during purification using multiple affinity chromatography steps, and both enzymes were reactive with insulin receptors, EGF receptors and ASG receptors. However, PTPase I was reactive with anti-peptide antibodies directed against a sequence in human placental PTPase 1B [10] which is conserved (16 out of 18 amino acids) in a homologous rat brain PTPase [8]. Proteins detected with these antibodies did not co-purify with PTPase II activity. The peptide against which the antibodies are directed is in the central portion of PTPase 1B, so it is unlikely that PTPase II is simply a proteolytic product of PTPase I. It is possible that the absence of immunoreactivity in the PTPase II preparation is due to post-translational modification of PTPase I, but we consider it most likely that these are two distinct proteins.

The third PTPase present in the rat liver membranes, designated PTPase III based on elution during poly-(L-lysine) chromatography, clearly differs from PTPases I and II. PTPase III had an M_r of ~ 400000 based on gel-filtration chromatography and displayed 3–4-fold greater activity with *P*-Tyr-MBP than with *P*-Tyr-RCML. A number of results show that the catalytic domain of PTPase III is different from that of PTPases I and II. Using *P*-Tyr-RCML as substrate, PTPase III was less sensitive to polyEY inhibition. Using *P*-Tyr-MBP as substrate, PTPase III was not activated by polyEY. PTPase activity with *P*-Tyr-MBP was stable in the presence of low concentrations of thiol-containing compounds. Finally, the subcellular localization of PTPase III seemed to differ from that of PTPases I and II, based on specific activities of membrane and cytoskeletal fractions with *P*-Tyr-MBP versus *P*-Tyr-RCML.

Our studies do not establish the identity of PTPase III, which is a high-M_r membrane PTPase. It seems reasonable to speculate that this enzyme may be related to the transmembrane PTPase LAR [11]. Indeed, a preliminary report describes the detection of mRNA in rat liver using the cDNA for LAR [20]. In addition, PTPase III and CD45 enzyme activities share at least one feature, i.e. high activity with *P*-Tyr-MBP [21].

Most of our experiments utilized artificial substrates to monitor and characterize the various PTPases. However, all three rat liver PTPases have activity with at least two physiological substrates, *P*-Tyr-insulin receptor β-subunit and *P*-Tyr-EGF receptor. Interestingly, only PTPases I and II were active with *P*-Tyr-ASG receptor. This receptor, unlike the EGF and insulin receptors, undergoes constitutive internalization via coated pits followed by recycling to the plasma membrane. The significance of the selective activity of multiple PTPases with physiological substrates is unknown at this time. The ability of a PTPase to dephosphorylate intrinsic membrane receptors in solution does not ensure a similar role in intact membranes and cells. Nonetheless, our studies raise the possibility of complex regulation of receptor dephosphorylation by independently regulated PTPases.

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REFERENCES

1. Hunter, T. & Cooper, J. A. (1985) Annu. Rev. Biochem. **54**, 897–930
2. Jove, R. & Hanafusa, H. (1987) Annu. Rev. Cell Biol. **3**, 31–56
3. Tonks, N. K., Diltz, C. D. & Fischer, E. H. (1988) J. Biol. Chem. **263**, 6722–6730
4. Tonks, N. K., Diltz, C. D. & Fischer, E. H. (1988) J. Biol. Chem. **263**, 6731–6737
5. Charbonneau, H., Tonks, N. K., Walsh, K. A. & Fischer, E. H. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7182–7186
6. Thomas, M. L. (1989) Annu. Rev. Immunol. **7**, 339–369
7. Tonks, N. L., Charbonneau, H., Diltz, C. D., Fischer, E. H. & Walsh, K. A. (1988) Biochemistry **27**, 8696–8701
8. Guan, K., Haun, R. S., Watson, S. J., Gehlen, R. L. & Dixon, J. E. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1501–1505
9. Cool, D. E., Tonks, N. K., Charbonneau, H., Walsh, K. A., Fischer, E. H. & Krebs, E. G. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5257–5261
10. Chernoff, J., Schievella, A. R., Jost, C. A., Erikson, R. L. & Neel, B. G. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2735–2739
11. Streuli, M., Hall, L. R., Saga, Y., Schlossman, S. F. & Saito, H. (1988) J. Exp. Med. **168**, 1523–1530
12. Gruppuso, P. A., Boylan, J. M., Posner, B. I., Faure, R. & Brautigan, D. L. (1990) J. Clin. Invest. **85**, 1754–1760
13. Shriner, C. L. & Brautigan, D. L. (1984) J. Biol. Chem. **259**, 11383–11390
14. Williams, L. W., Tremble, P. M., Lavin, M. F. & Sunday, M. S. (1984) J. Biol. Chem. **259**, 5287–5294
15. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
16. Charbonneau, H., Tonks, N. K., Kumar, S., Diltz, C. D., Harrylock, M., Cool, D. E., Krebs, E. G., Fischer, E. H. & Walsh, K. A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5252–5256
17. Brautigan, D. L., Gruppuso, P. A. & Mumby, M. (1986) J. Biol. Chem. **261**, 14924–14928
18. Fallon, R. J. (1990) J. Biol. Chem. **265**, 3401–3406
19. Fallon, R. J. & Schwartz, A. L. (1986) J. Biol. Chem. **261**, 15081–15089
20. Zhang, W. R., Kahn, C. R. & Goldstein, B. J. (1990) Program and Abstracts, The Endocrine Society 72nd Annual Meeting, p. 72
21. Tonks, N. K., Diltz, C. D. & Fischer, E. H. (1990) J. Biol. Chem. **265**, 10674–10680

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