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Purification and characterization of a higher-molecular-mass form of protein phosphotyrosine phosphatase (PTP 1B) from placental membranes

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Purification of a major placental membrane protein phosphotyrosine phosphatase (PTP-I) through the use of a non-hydrolysable phosphotyrosine analogue affinity ligand has enabled identification of the enzyme as a single polypeptide of at least 46 kDa. This phosphatase specifically dephosphorylates phosphotyrosine-containing substrates, including the *src* peptide, the epidermal-growth-factor receptor tyrosine kinase and the non-receptor tyrosine kinase $p56^{lck}$. The $p56^{lck}$ can be dephosphorylated by PTP-I at two tyrosine residues (Tyr-394 and Tyr-505), which are differentially phosphorylated *in vitro* and *in vivo* and have been suggested to modulate kinase activity. The activity of PTP-I towards these substrates indicates a possible function of regulation of cellular tyrosine phosphorylation pathways at the level of growth-factor receptor and/or oncogene/proto-oncogene tyrosine kinases. Kinetic analyses show that PTP-I exhibits a K_m value of about 2 μM with either *src* peptide or reduced, carboxyamidomethylated and maleylated (RCM)-lysozyme as substrate, and is inhibited in a mixed competitive manner by the polyanions heparin and poly(Glu_4,Tyr_1). Sequencing of PTP-I peptides reveals almost complete identity with sequences within the *N*-terminal half of the 37 kDa non-receptor tyrosine phosphatase 1B. However, the size and amino acid composition of PTP-I are similar to that of a higher-molecular-mass form of PTP 1B predicted from cDNA cloning. These results suggest that the 37 kDa PTP 1B is a proteolysed form of PTP-I, and provide evidence that a larger form of PTP 1B exists *in vivo*, at least in association with placental membranes.

INTRODUCTION

The protein tyrosine kinase activity shared by several growth factor receptors and retroviral oncogene products is an essential feature of their ability to initiate the processes of cell growth and transformation (for reviews see refs. [1] and [2]). Cellular phosphotyrosine content reflects the relative activities of tyrosine kinases and phosphatases. Inhibition of the expression of temperature-sensitive tyrosine kinase mutants results in reversion of the transformed phenotype with an associated rapid decrease in intracellular phosphotyrosine content [3], and, conversely, large increases in phosphotyrosine content are observed in cells treated with inhibitors of phosphotyrosine phosphatases [4,5]. Although protein phosphotyrosine phosphatases (PTPases) may function in terminating the cellular processes triggered by tyrosine kinase activation, the finding that tyrosine dephosphorylation is involved in the activation of a mitotic control element (cdc2/MPF kinase) [6–8] indicates that these phosphatases can also play an active role in initiating other events. Furthermore, the general structural similarity between the recently identified receptor-like PTPases and receptor tyrosine kinases suggests that the former enzymes are themselves transducers of signalling pathways. The identification and characterization of the phosphatases involved in modulating the effects and activities of the tyrosine kinases is an essential step towards understanding the regulatory nature of tyrosine phosphorylation pathways.

Multiple PTPase activities have been identified in virtually all tissues and cells examined, and a few of these enzymes have been purified. They share a common set of responses to various effector compounds, as well as an absolute specificity for phosphotyrosine, which define this family of enzymes and distinguish them from all other types of phosphatase. Determination of the amino acid sequence of a PTPase (1B) isolated from placenta has confirmed the unique nature of this class of enzyme [9–11]. It has led to the identification of a novel group of putative receptor-linked PTPases [10–13] as well as to the cloning of apparently non-receptor PTPases [14–16]. Together these comprise a multigene family, the extent of which is unknown.

We report the purification of a major placental membrane tyrosine phosphatase which dephosphorylates both receptor and non-receptor tyrosine kinases. Analysis of this membrane-associated enzyme indicates the possibility that it corresponds to a larger form of PTP 1B predicted by cDNA cloning [16] and that the alternative 37 kDa form of the non-receptor phosphotyrosine phosphatase 1B arises through proteolysis of a larger enzyme.

MATERIALS AND METHODS

Materials

L-Histidyldiazobenzylphosphonic acid (HDBP)–agarose, wheat-germ agglutinin–agarose, sodium orthovanadate, tetramisole, levamisole, polycations, heparin, poly(GluTyr) and CNBr were purchased from Sigma Chemical Co. Sequencing-grade trypsin and endoproteinase Glu-C were from Boehringer Mannheim. The monoclonal antibody 1G2 was from Oncogene Science. The CHO-T cell line expressing insulin receptor [17] was a gift from Dr. S. Clark, Walter and Eliza Hall Institute of Medical Research. The *src* peptide was purchased from Multiple Peptide Systems.

Abbreviations used: CHO cells, Chinese-hamster ovary cells; DTT, dithiothreitol; EGF, epidermal growth factor; HDBP, L-histidyldiazobenzylphosphonic acid; RCM-lysozyme, reduced, carboxyamidomethylated and maleylated lysozyme; PBS, phosphate-buffered saline (0.14 M-NaCl/8 mM-sodium phosphate/1.5 mM-potassium phosphate buffer, pH 7.3); PMSF, phenylmethanesulphonyl fluoride; poly(GluTyr), poly(Glu_4,$NaTyr_1$); PTPase, protein phosphotyrosine phosphatase.

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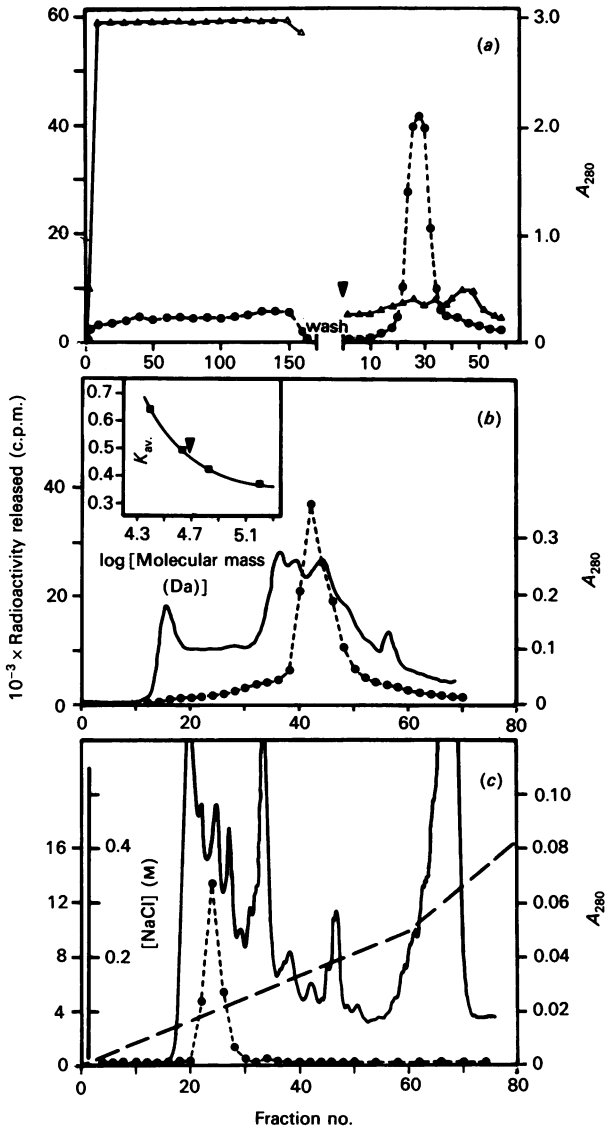

Fig. 1. Chromatography of PTP-I on heparin–Separose CL-6B, Superose 12 and Mono Q

Phosphatase activity was measured with 20 μM-*src* peptide. (*a*) Chromatography on heparin–Sepharose CL-6B. After loading, the column was washed with 10 column volumes of equilibration buffer (wash). The arrowhead indicates the start of the NaCl gradient. Fractions (15 ml) were collected and 5 μl of a 5-fold-diluted portion of indicated fractions was assayed in a 50 μl reaction mixture. ●, Phosphatase activity; △, A_{280}. (*b*) Chromatography on Superose 12. Fractions (0.4 ml) were collected and 5 μl of 100-fold-diluted portion of indicated fractions was assayed in a 50 μl reaction mixture. ●, Phosphatase activity; ——, A_{280}. Inset: molecular-mass determination of phosphatase (arrowhead) from a plot of $K_{av.}$ versus log(molecular mass) of known marker proteins. Marker proteins (from high to low $K_{av.}$) are aldolase, BSA, ovalbumin and chymotrypsinogen. (*c*) Mono Q chromatography. Fractions (0.5 ml) were collected from the beginning of the NaCl gradient and 5 μl of a 100-fold-diluted portion of indicated fractions was assayed in a 50 μl reaction mixture. ●, Phosphatase activity; ——, A_{280}; — —, NaCl gradient.

Purification of PTPase

A partially purified preparation of tyrosine phosphatase from human placental membranes was prepared as previously described [18] by successive chromatographies through heparin–Sepharose CL-6B, Superose 12 and Mono Q. The pooled phosphatase activity from the Mono Q column was diluted 10-fold in buffer E [50 mM-Mes (pH 6.0)/2 mM-EDTA/0.5 mM-DTT/0.01 % CHAPS] and applied to a column (1.6 cm × 2.5 cm) of HDBP–agarose that had been equilibrated in buffer E. The column was washed with 15 vol. of buffer E, then with 7 vol. of buffer E containing 50 mM-NaCl. Bound protein was eluted with a gradient of 0.05–0.5 M-NaCl in buffer E followed by washing with buffer E containing 0.5 M-NaCl. Active fractions were pooled, dialysed against 50 mM-Mes (pH 6.0)/50 % (v/v) glycerol/25 mM-NaCl/0.5 mM-DTT/0.01 % CHAPS and stored in small aliquots at −70 °C. This enzyme is designated PTP-I.

Phosphorylation of substrates

The *src* peptide (RRLIEDAEYAARG) was phosphorylated by EGF receptor kinase as previously described [19] to a stoichiometry of 0.02–0.05 mol of phosphate/mol of peptide. If required, phosphorylated and unphosphorylated peptides were separated using an anti-phosphotyrosine monoclonal antibody (1G2) affinity column [19]. Phosphotyrosyl- or phosphoseryl-casein and -histone were prepared as described previously [18]. Phosphoproteins were resuspended in 20 mM-Mes (pH 6.0)/10 mM-2-mercaptoethanol and dialysed against several changes of this buffer (48–72 h) until only a background level of 1–2 % free ^{32}P remained. Phosphorylase *a* was prepared as described previously [18] and dialysed against two changes of 50 mM-Mes (pH 6.0)/1 mM-EDTA/250 mM-NaCl/50 mM-2-mercaptoethanol. The resulting precipitate was pelleted by centrifugation and resuspended in dialysis buffer.

Reduced, carboxyamidomethylated and maleylated lysozyme (RCM-lysozyme) was prepared as described by Tonks *et al.* [9], and phosphorylated in a reaction mixture containing 0.48 mg of CHO-T cell membranes/ml, 6 μg of insulin/ml, 10 mM-Hepes (pH 7.2), 50 mM-NaCl, 3 % glycerol, 12 mM magnesium acetate, 4 mM-$MnCl_2$, 0.05 % Triton X-100, 100 μM-Na_3VO_4, 4 mM-ATP (2000–3000 c.p.m./pmol) and 2 mg of RCM-lysozyme/ml. The reaction was terminated and the phospho-RCM-lysozyme was dialysed against 50 mM-imidazole/HCl as described in ref. [9]. A control reaction carried out in the absence of RCM-lysozyme showed that 10–20 % of the ^{32}P incorporation was due to phosphorylation of the CHO-T cell membrane components. After subtraction of this background incorporation, the stoichiometry of phosphorylation of RCM-lysozyme was routinely found to be between 0.1 and 0.2 mol of phosphate/mol.

To obtain EGF receptor, 460 μg of A431 cell membranes were solubilized for 20 min at 0 °C with 1 % Triton X-100, and unsolubilized material was pelleted by centrifugation at 100000 *g* for 30 min. The supernatant was incubated with wheat-germ agglutinin–agarose at 4 °C for 1 h, and then the incubation mixture was poured into a small column and washed with 40 mM-Hepes (pH 7.5)/0.5 M-NaCl/0.2 mM-PMSF/aprotinin (0.1 mg/ml)/0.05 % Triton X-100/10 mM-2-mercaptoethanol. Material bound to the column was eluted with the same buffer containing 0.3 M-*N*-acetylglucosamine. Collected fractions were assayed for protein tyrosine kinase activity towards *src* peptide, and active fractions were pooled. To phosphorylate the EGF receptor, the pool was incubated with 0.2 μg of EGF/ml, 12 mM-$MgCl_2$, 2 mM-$MnCl_2$, and 20 μCi of [γ-^{32}P]ATP for 30 min at 0 °C and then applied to a column (1.0 cm × 18.3 cm) of Sephadex G-25 equilibrated with 20 mM-Mes (pH 6.0)/50 mM-NaCl/0.01 % Triton X-100/1 mM-DTT. Radioactivity in collected fractions was quantified by Čerenkov counting, and peak fractions eluted at the column void volume were pooled and used as substrate in subsequent dephosphorylation experiments.

The $p56^{lck}$ was immunoprecipitated from LSTRA cell lysates. To prepare $p56^{lck}$ phosphorylated *in vivo*, LSTRA cells were washed three times with phosphate-free RPMI (Gibco) con-

Table 1. Purification of phosphatase PTP-I from the Triton X-100 extract of placental membranes

A total of 838 g of placental tissue was used in this preparation. Phosphatase activity was measured towards 40 μM-*src* peptide.

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/ml)	Purification (fold)	Yield (%)
1. Solubilized membrane extract	624	2371	3547	1.5	–	100
2. Heparin–Sepharose CL-6B	135	28.4	1096	38.6	26	31
3. Superose 12	14.5	4.6	547	119	79	15
4. Mono Q	3.5	0.44	395	897	598	11
5. HDBP–agarose	16.2	0.026	81.5	3133	2089	2.3

taining 10 μM-2-mercaptoethanol, 200 μM-Na_3VO_4 and 5% (v/v) dialysed foetal calf serum and then incubated at 37 °C for 4 h in the same medium containing 1 mCi of [^{32}P]orthophosphate (New England Nuclear)/ml. The cells were then pelleted and washed three times in cold PBS containing 1 mM-DTT and 200 μM-Na_3VO_4 and resuspended (2×10^7 cells/ml) in lysis buffer (PBS/0.5% Triton X-100/1 mM-PMSF/0.4% aprotinin) containing 200 μM-Na_3VO_4. After a 20 min incubation on ice, 0.1 vol. of a 50% suspension of Protein A–Sepharose in PBS was added and the mixture was microfuged for 15 min at 4 °C. Anti-p56^{lck} antibody [20] was added to the supernatant and mixed for 16 h at 4 °C. Then 0.2 vol. of Protein A–Sepharose was added and mixing was continued for 1 h. The immunocomplexes were washed three times with lysis buffer. Those prepared from ^{32}P-labelled cells were resuspended for use in 25 mM-Mes (pH 6.0). Those prepared from unlabelled cells were phosphorylated at 30 °C for 5 min in a reaction mixture containing 50 mM-Hepes (pH 7.3), 10 mM-$MgCl_2$, 10 mM-$MnCl_2$, 1 mM-PMSF, 0.4% aprotinin and 10 μCi of [γ-^{32}P]ATP (5000 Ci/mmol). Reactions were stopped with 20 mM-EDTA and the immunocomplexes were washed three times with PBS/1 mM-PMSF/0.4% aprotinin/10 mM-EDTA and resuspended for use in 25 mM-Mes (pH 6.0).

Assay of phosphatase activity

Phosphatase activity was assayed at 30 °C in reaction mixtures containing phosphorylated substrates, 50 mM-Mes (pH 6.0), 100 mM- or 25 mM-NaCl, 2 mM-EDTA, 1 mg of BSA/ml, 0.5 mM-DTT and 0.01% CHAPS. In the case of peptide substrates, the reaction was stopped by a 10-fold excess volume of 5% (w/v) trichloroacetic acid containing 100 mM-potassium phosphate, and the ^{32}P released was quantified as described by Chan *et al.* [21]. In the case of protein substrates, the reactions were either stopped by the addition of Laemmli sample buffer and subjected to SDS/PAGE, or stopped by the addition of cold 20% trichloroacetic acid and BSA added as a carrier. After incubation on ice for 10 min, the reaction mixtures were centrifuged to pellet precipitated proteins, and released ^{32}P in the supernatants was quantified by scintillation counting. One unit is defined as the amount of PTP-I that releases 1 nmol of phosphate/min.

Phosphopeptide analysis of the p56^{lck}

After treatment with or without phosphatase, reaction mixtures containing p56^{lck} were stopped by the addition of Laemmli sample buffer and subjected to SDS/10% PAGE. Gels were frozen and briefly autoradiographed to identify the position of p56^{lck}. The corresponding regions of the gel were excised and p56^{lck} was electroeluted from the gel pieces. Electroeluted material was precipitated with cold 10% trichloroacetic acid with BSA as a carrier. Pellets were washed with cold ethanol and residual ethanol was evaporated. They were then resuspended in 70% formic acid containing 50 mg of CNBr/ml, and digestion was allowed to proceed at room temperature in the dark for 1 h. Water was added to each digest, which was then freeze-dried, washed twice with water, resuspended in Laemmli sample buffer and analysed by SDS/18% PAGE and autoradiography [22].

Preparation of PTP-I peptides and amino acid analysis

Affinity-purified PTP-I (2×30 μg) in 50 mM-Mes (pH 6.0)/25 mM-NaCl/0.5 mM-DTT/0.01% CHAPS/10% glycerol was applied to a Delta-pak C18 column (2 mm × 150 mm, 30 nm particle size) and eluted with a gradient of 3.75–75% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Eluted fractions were immediately assayed for activity towards *src* peptide, and active fractions pooled and dried under vacuum. The protein was pyridylethylated [23] and desalted on a BU-300 C4 column (2.1 mm × 100 mm) eluted with a linear gradient of 4.5–90% acetonitrile in 0.1% trifluoroacetic acid. Digestions were carried out at an enzyme/substrate ratio of 1:25 (w/w) at 37 °C for 18 h in 100 mM-NH_4HCO_3 with trypsin, or at 25 °C for 18 h in 100 mM-NH_4HCO_3/5% acetonitrile with endoproteinase Glu-C. Peptides were separated using a BU-300 C4 column (2.1 mm × 100 mm) with a stepwise elution of 4.5–90% acetonitrile in 0.1% trifluoroacetic acid. Peptides were sequenced by S. Kielland (University of Victoria, Canada) using an Applied Biosystems (ABI) model 470A sequencer with an on-line phenylthiohydantoin analyser and data analysis module. Samples were applied to the instrument using protocols recommended by the manufacturer (Polybrene-coated glass-fibre filter, precycled once). Standard ABI chemistry programs were used. Cysteine was identified as pyridylethylcysteine. Amino acid analysis of non-pyridylethylated PTP-I was carried out by S. Kielland using an Applied Biosystems model 420 amino acid derivatizer–analyser system. Samples were hydrolysed at 165 °C for 1 h. All the reagents and programs used were those recommended by the manufacturer.

Other methods

Membranes from A431 and CHO-T cells were prepared as described by Thom *et al.* [24] except that Ca^{2+} and Mg^{2+} in the buffers were replaced with EDTA/EGTA and the sucrose-density-gradient centrifugation step was omitted. Protein concentration was estimated as described by Bradford [25], with BSA as a standard. Non-denaturing gel electrophoresis was performed as described by Davis [26] and SDS/PAGE as described by Laemmli [27]. Gels were stained for protein with either Coomassie Brilliant Blue and/or silver [28]. Gel pieces were solubilized in 3% Protosol (Dupont) in Econofluor overnight at 37 °C for subsequent scintillation counting. Autoradiographs were scanned with an LKB Ultroscan XL laser densitometer linked to an IBM PC/AT compatible operating on LKB 2400 Gelscan XL software.

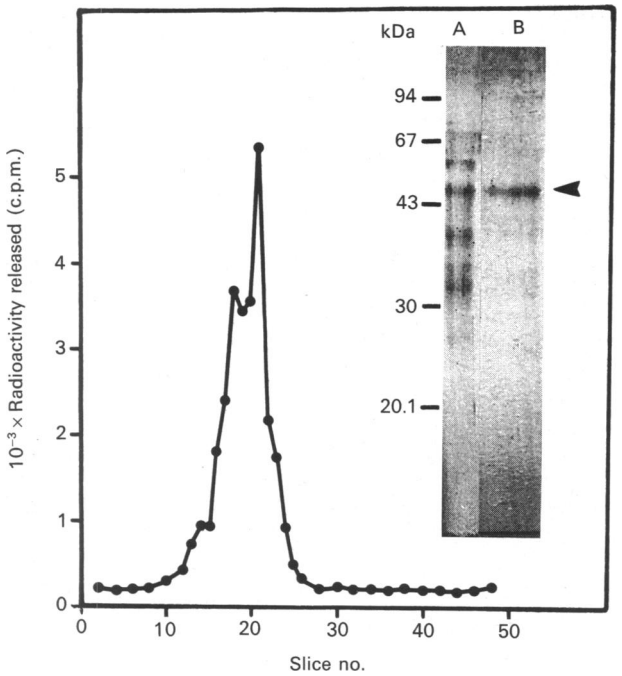

Fig. 2. Identification of PTP-I by non-denaturing PAGE

A portion of the pooled phosphatase activity obtained from Mono Q chromatography was electrophoresed at 4 °C on an 8% non-denaturing gel. The gel was cut into 2 mm slices from top to bottom and each slice was incubated at 4 °C for 8 h in 100 μl of 0.1 M-Mes (pH 6.0) / 50 mM-NaCl / 2 mM-EDTA / 0.01 % CHAPS / 0.5 mM-DTT. A portion of the buffer was then assayed for phosphatase activity (●) towards 20 μM-*src* peptide. Inset: A, a portion of the pooled phosphatase activity from Mono Q chromatography was run on a 10% SDS/polyacrylamide gel and protein visualized by silver staining. B, Gel slice number 21 obtained from non-denaturing electrophoresis was run on a 10% SDS/polyacrylamide gel and protein visualized by silver staining. The numbers along the side indicate the M_r values of protein standards.

RESULTS

Purification of a major membrane-associated PTPase

Placental membranes were prepared and solubilized with 2% Triton X-100 as previously described [18]. Assay of the placental cytosol and membrane fractions showed that the supernatant contained 61% and the membrane 39% of the total *src* peptide phosphatase activity. The solubilized membrane fraction was chromatographed on successive columns of heparin–Sepharose CL-6B, Superose-12 and Mono Q (Fig. 1). Although a significant amount of phosphatase activity was present in the breakthrough from the initial heparin–Sepharose column and could be further resolved into apparently multiple enzyme species, only one peak of phosphatase activity bound to and was eluted from the column (Fig. 1*a*). This phosphatase migrated as a single active species throughout the following chromatographic steps (Fig. 1 and see below). It is possible to estimate that, on the basis of the percentage yield of phosphatase activity after heparin–Sepharose chromatography (Table 1), this enzyme is a major species of membrane phosphatase comprising about 30% of the total extractable membrane tyrosine phosphatase activity.

When analysed by SDS/PAGE, the pool of phosphatase activity obtained from Mono Q chromatography contained at least four major and other minor protein bands (Fig. 2, inset, lane A). Before the final affinity-chromatography step, we attempted to identify the protein responsible for phosphatase activity by using native PAGE. Following electrophoresis of a portion of the Mono Q binding pool of phosphatase activity, the gel was cut into 2 mm slices and the protein(s) from these slices was eluted and assayed for phosphatase activity. A major peak of activity was observed (Fig. 2). Portions of the eluted material from slices corresponding to phosphatase activity were subjected to SDS/PAGE. The smaller side peak of phosphatase activity (slice 18) corresponded to protein bands of molecular masses 45 kDa and 52 kDa, and the major peak of phosphatase activity (slice 21) corresponded to a single protein band with an apparent

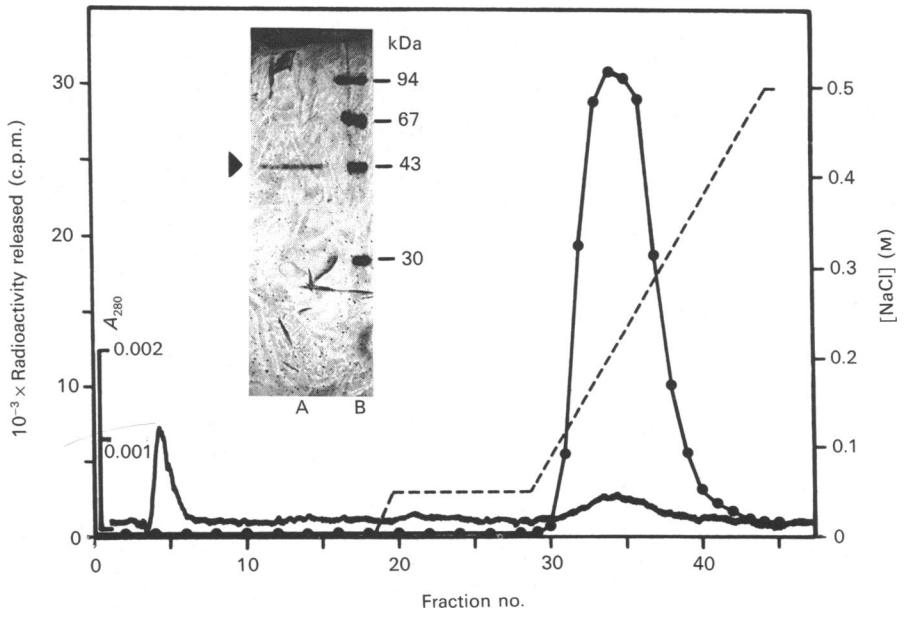

Fig. 3. Affinity purification of phosphatase PTP-I on HDBP–agarose

Fractions of 1.0 ml were collected and 5 μl of indicated fractions assayed in a 50 μl reaction mixture containing 20 μM-*src* peptide. ●, Phosphatase activity; ——, A_{280}; — —, NaCl concentration. Inset: lane A, a portion of the pooled phosphatase activity from the affinity column was subjected to SDS/PAGE and protein was located by silver staining; lane B, molecular-mass markers.

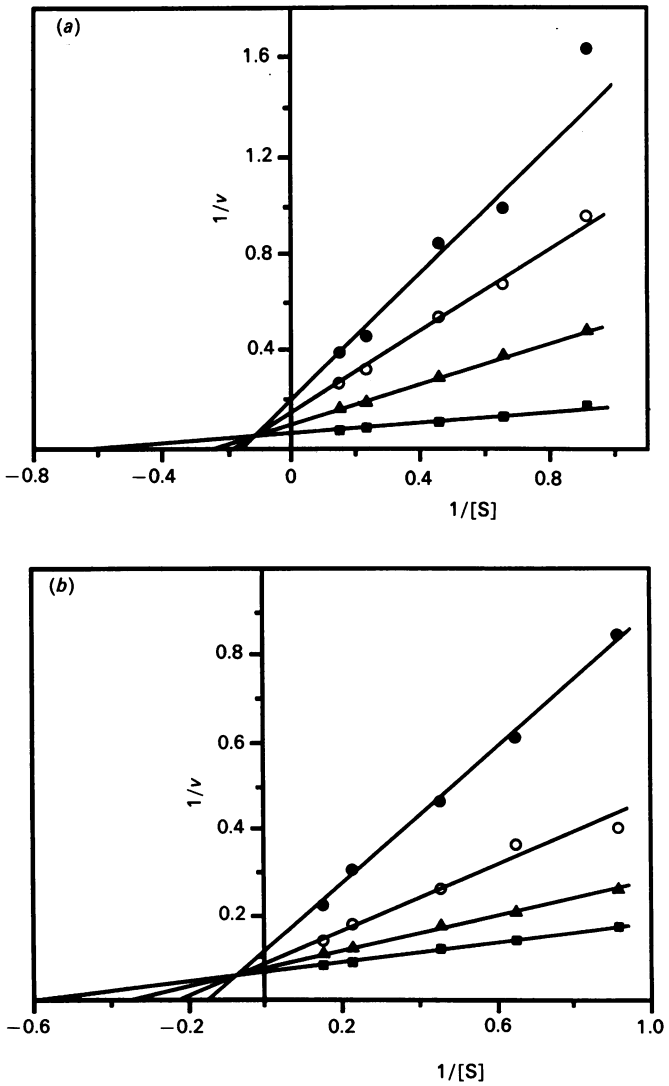

Fig. 4. Lineweaver–Burk plots of the dephosphorylation of *src* peptide by PTP-I in the presence of polyanions

(*a*) Dephosphorylation in the presence of 0 μg (■), 1 μg (▲), 2 μg (○) and 4 μg (●) of heparin/ml. (*b*) Dephosphorylation in the presence of 0 nM- (■), 100 nM- (▲), 250 nM- (○) and 500 nM- (●) poly(GluTyr). Each reaction mixture contained 0.2 unit of PTP-I/ml. Initial velocity (v) is in μmol/min per mg and substrate concentration [S] is in μM-phospho-*src* peptide and is equal to μM-phosphotyrosine.

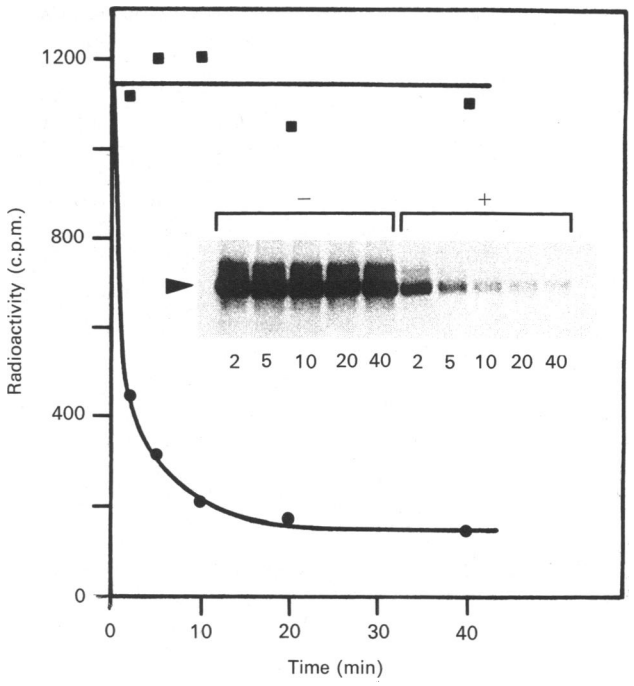

Fig. 5. Dephosphorylation of the EGF receptor by phosphatase PTP-I

Autophosphorylated EGF receptor was incubated at 30 °C with or without 0.1 unit of phosphatase in a total volume of 440 μl. At the times indicated, 80 μl of the reaction mixture was removed, added to Laemmli sample buffer and subjected to SDS/PAGE. Following autoradiography, corresponding pieces of the dried gel were rehydrated and solubilized and radioactivity was determined by scintillation counting. ●, Presence of phosphatase; ■, absence of phosphatase. Inset: autoradiograph of the electrophoresed portions of the reaction mixtures. Lanes 1–5, reaction in the absence of phosphatase (−) and lanes 6–10, reaction in the presence of phosphatase (+), at times 2, 5, 10, 20 and 40 min.

molecular mass of 45 kDa as located by silver staining (Fig. 2, inset, lane B).

The phosphatase preparation obtained from the Mono Q step was further purified by affinity chromatography with a matrix of HDBP–agarose, a non-hydrolysable analogue of phosphotyrosine. All detectable phosphatase activity reproducibly bound to this column and a single major protein with a molecular mass of 45 kDa, as estimated by SDS/PAGE, was co-eluted with phosphatase activity (Fig. 3). This protein is identical with respect to electrophoretic mobility with the protein identified by non-denaturing electrophoresis as the catalytic moiety involved in *src* peptide dephosphorylation. These results are in accord with a molecular mass of 50 kDa estimated from gel-filtration chromatography (Fig. 1*b*). The phosphatase (referred to as PTP-I) was purified about 2000-fold from the crude solubilized membrane fraction with an overall recovery of 2.3%.

Characterization of PTP-I

PTP-I is specific for phosphotyrosine-containing substrates, being active towards phosphotyrosyl-casein and -histone but not towards phosphoseryl-casein, -histone or -phosphorylase *a*. The pH optimum of PTP-I activity towards the *src* peptide is between pH 6.0 and 6.5 with 36% and 6% of maximal activity at pH 5.0 and 8.0 respectively. Optimal activity towards *p*-nitrophenyl phosphate was observed at pH 6.4–7.0. Phosphatase activity was unaffected by EDTA or EGTA but 2 mM-Ca^{2+}, -Mn^{2+} and -Mg^{2+} were each inhibitory by 15–25%. A 60% inhibition was observed in the presence of 20 mM-Mg^{2+}. The effects of inhibitors and activators of several types of phosphatases were examined on PTP-I. Micromolar concentrations of the phosphotyrosine phosphatase inhibitors vanadate and Zn^{2+} inhibited PTP-I, with 50% inhibition at 20 μM-vanadate or 50 μM-Zn^{2+}. A partial inhibition was observed in the presence of NaF. The potent alkaline phosphatase inhibitors tetramisole and levamisole [29,30] had no effect on PTP-I activity. The activities of the type-1 and type-2 phosphoserine/phosphothreonine phosphatases can be modulated by polycations [31,32]. However, no significant inhibition or stimulation of PTP-I activity was observed in the presence of a range of concentrations (2, 20 and 100 μg/ml) of a variety of polycations (histone H1, protamine, spermine, spermidine, putrescine, poly-L-lysine and poly-L-ornithine), with the exception of poly-L-arginine, which at 100 μg/ml inhibited over 40% of phosphatase activity. Since heparin–Sepharose chromatography was used in the purification of PTP-I and since heparin has previously been reported to inhibit PTPases [33,34], we examined the effect of this polyanionic compound on PTP-I

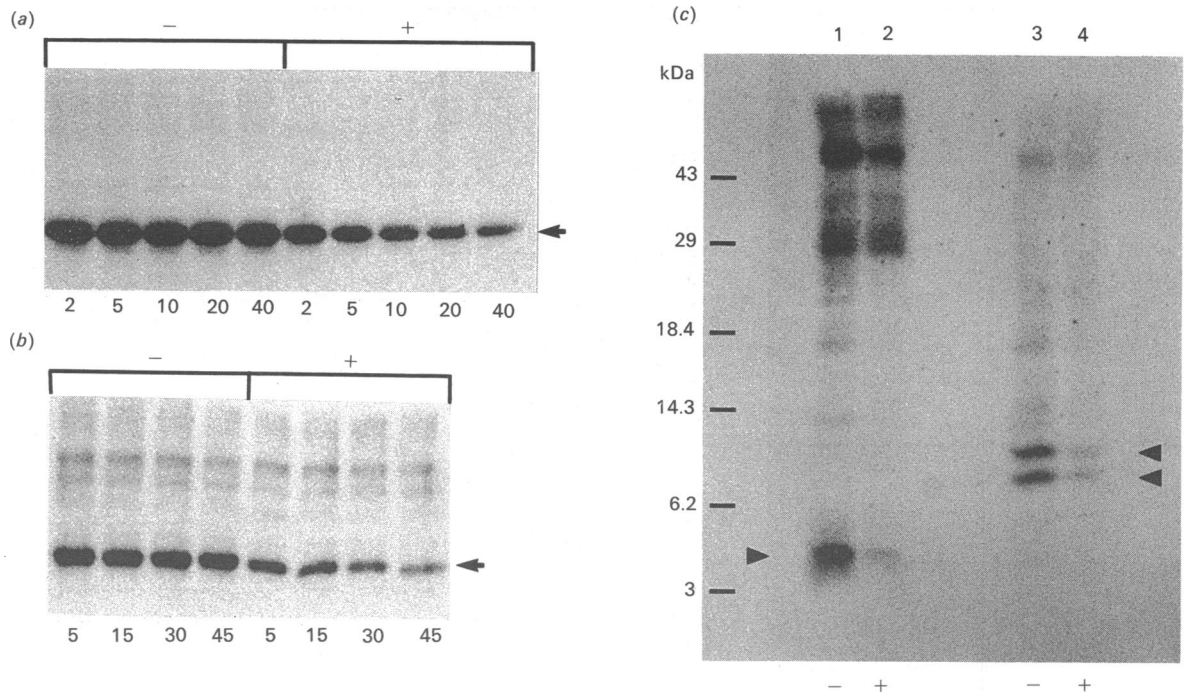

Fig. 6. Dephosphorylation of p56^{lck} and CNBr-cleavage peptide mapping

(*a*) p56^{lck}phosphorylated *in vitro* and immunoprecipitated was incubated at 30 °C in the absence (−) or presence (+) of 0.24 unit of PTP-I/ml for the times indicated. (*b*) p56^{lck} phosphorylated *in vivo* and immunoprecipitated was incubated at 30 °C in the absence (−) or presence (+) of 0.37 unit of PTP-I/ml for the times indicated. In (*a*) and (*b*) reactions were stopped with Laemmli sample buffer and subjected to 10% SDS/PAGE, followed by autoradiography of the dried gel. Arrows indicate the position of p56^{lck}. (*c*) p56^{lck} phosphorylated *in vivo* (lanes 1 and 2) or *in vitro* (lanes 3 and 4) and immunoprecipitated was incubated at 30 °C for 45 min in the absence (−) or presence (+) of 0.73 unit of PTP-I/ml or 0.24 unit of PTP-I/ml respectively. Reactions were stopped with Laemmli sample buffer, processed, and digested with CNBr as described in the Materials and methods section. The arrowheads indicate the positions of dephosphorylated peptides.

activity. Heparin was a potent inhibitor of PTP-I activity towards *src* peptide, with an IC_{50} of 2 μg/ml. Another polyanion, poly(GluTyr), inhibited *src* peptide PTP-I activity with an IC_{50} of 0.3 μM. The pH optimum, responses to the above compounds and specificity for phosphotyrosine differentiate PTP-I from acid, alkaline and phosphoserine/phosphotyrosine-specific phosphatases.

The *src* peptide was dephosphorylated by PTP-I with an apparent K_m of 1.7 μM. This is about a 10-fold higher value than that reported for some other phosphotyrosine phosphatases [33,35,36]. To determine whether the large excess of unphosphorylated peptide in the reactions mixture was affecting the phosphatase, the phospho-*src* peptide was separated from unphosphorylated peptide by using an anti-phosphotyrosine antibody coupled to Sepharose 4B. A similar apparent K_m value was determined with this purified substrate, and this was unaffected by the addition of unphosphorylated *src* peptide to the reaction mixture at a final concentration of 1 μM, 1 mM or 10 mM [10–1000-fold excess over the highest concentration (approx. 9 μM) of substrate]. A protein substrate, RCM-lysozyme, was dephosphorylated by PTP-I with an apparent K_m of 2.2 μM, indicating that these higher kinetic constants are not a result of the non-protein nature of the *src* peptide substrate. Heparin and poly(GluTyr) inhibited PTP-I activity towards the *src* peptide in a mixed competitive manner (Fig. 4); similarly, increasing concentrations of poly(GluTyr) increased the K_m and reduced the $V_{max.}$ of RCM-lysozyme dephosphorylation. Replotting the kinetic data as $1/v$ versus inhibitor concentration reveals an apparent K_i of 0.3 μg/ml for heparin and approx. 80 μg/ml for poly(GluTyr).

Dephosphorylation of tyrosine kinases

The presence of PTP-I in the particulate fraction suggests that potential substrates of this phosphatase might also have a membrane localization. The receptor and non-receptor tyrosine kinases are among the most prominent phosphotyrosine-containing membrane-associated proteins, and we examined the ability of a member of each of these classes of tyrosine kinase to serve as a PTP-I substrate.

The EGF receptor is a transmembrane protein that upon ligand binding undergoes autophosphorylation on tyrosine residues in the cytoplasmic *C*-terminal tail and exhibits enhanced tyrosine kinase activity [37]. The EGF receptor was solubilized and lectin-affinity-purified from A431 cell membranes, phosphorylated and then incubated in the presence or in the absence of PTP-I in the standard phosphatase assay buffer. A rapid receptor dephosphorylation was observed, reaching a maximum of about 80% after a 10 min incubation with PTP-I (Fig. 5).

The activity of PTP-I towards the *src* peptide suggested that it might also be active towards the protein tyrosine kinase pp60^{src} or other closely related tyrosine kinases. The p56^{lck} kinase, a member of the non-receptor type *src* kinase family, was examined as a substrate because it contains two potential sites of tyrosine phosphorylation. *In vitro*, the major site of p56^{lck} phosphorylation is Tyr-394, which is analogous to the autophosphorylation site (Tyr-416) of pp60$^{v\text{-}src}$ and to the *src* peptide. *In vivo*, the predominant phosphorylation site is Tyr-505, which lies in a region analogous to that containing the major phosphorylation site (Tyr-527) of pp60$^{c\text{-}src}$ *in vivo* [38,39]. As expected, p56^{lck} that had been autophosphorylated *in vitro* was dephosphorylated by

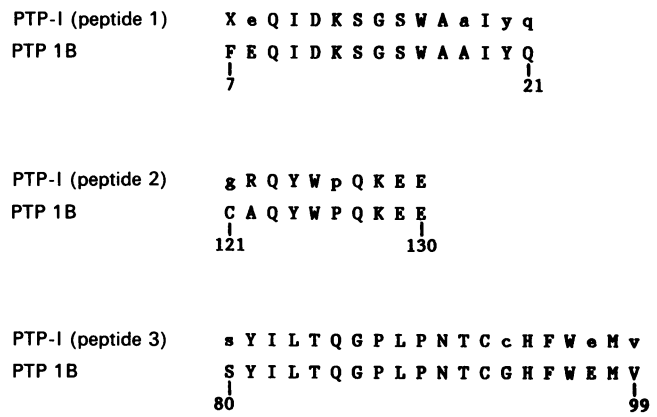

Fig. 7. Alignment of PIP-I peptide sequences with PTP 1B sequences

Residue numbering is in accordance with ref. [11]. Lower-case letters represent amino acid residues that were only tentatively identified.

Table 2. Comparison of the amino acid compositions of PTP-I and the two forms of PTP 1B

Four amino acid analyses were carried out with two different preparations of PTP-I and the results averaged. The standard error of the mean for each amino acid of PTP-I is not more than ±1. The amino acid composition of the 50 kDa form of PTP 1B is that predicted from a cDNA clone [16] whereas that of the 37 kDa form of PTP 1B is that determined from sequencing a purified protein [11]. Abbreviation: N.D., not detected.

Amino acid	Amino acid composition (mol of residue/mol)		
	PTP-I	PTP 1B (approx. 50 kDa)	PTP 1B (approx. 37 kDa)
Ala	20	23	13
Val	23	23	16
Leu	37	37	29
Ile	22	20	18
Pro	28	30	22
Phe	18	19	14
Trp	N.D.	9	6
Met	11	13	11
Gly	24	22	14
Ser	31	33	24
Thr	16	18	13
Tyr	12	13	9
Cys	N.D.	10	6
Lys	32	31	23
Arg	27	24	19
His	12	13	11
Asx	38	39	29
Glx	54	59	44

PTP-I with 80% dephosphorylation observed after a 40 min incubation with the phosphatase (Fig. 6*a*). p56^{lck} phosphorylated *in vivo* was also dephosphorylated by PTP-I (Fig. 6*b*). To ascertain that the sites dephosphorylated corresponded to either Tyr-394 or Tyr-505, p56^{lck} phosphorylated *in vivo* and *in vitro* was treated with or without PTP-I, then cleaved with CNBr, and the resulting phosphopeptides were separated and located by SDS/PAGE and autoradiography. A 4.3 kDa peptide derived from the p56^{lck} labelled *in vivo* and that contains Tyr-505 [40] was the major site of dephosphorylation (Fig 6*c*, lanes 1 and 2), whereas a 10 kDa and 8 kDa peptide doublet derived from the p56^{lck} phosphorylated *in vitro* and containing Tyr-394 [40] was extensively dephosphorylated by PTP-I (Fig. 6*c*, lanes 3 and 4).

Sequencing of PTP-I peptides and amino acid analysis

To determine whether PTP-I is related to any phosphotyrosine phosphatase of known sequence, we attempted to microsequence the purified protein. Undigested PTP-I was not sequenceable, suggesting that the *N*-terminus is blocked. Clear sequences were obtained from three PTP-I peptides. All of these sequences, with the exception of the first two residues of peptide 2 and one residue in peptide 3, are identical with sequences within the first 130 *N*-terminal amino acids of PTP 1B (Fig. 7). Whereas PTP 1B purified from placenta has a molecular mass of 37354 Da [11], a PTP 1B cDNA clone encodes a predicted protein of 49996 Da (approx. 50 kDa) containing an extra 11 kDa of *C*-terminal sequence [16]. Amino acid analysis of PTP-I shows that it has a very similar composition to this larger form of PTP 1B (Table 2) and a calculated molecular mass of 46239 Da. This is an underestimation of mass since no cysteine or tryptophan were detected during analysis (although both these residues are present in sequenced PTP-I peptides), owing to degradation of tryptophan during acid hydrolysis and to the fact that PTP-I was not pyridylethylated before analysis. If the mass of ten cysteine and nine tryptophan residues (present in the 49996 Da form of PTP 1B) are added to the calculated mass of PTP-I, the total molecular mass of PTP-I would be 48943 Da, close to the predicted molecular mass of 49996 Da.

DISCUSSION

A major phosphotyrosine phosphatase from the particulate fraction of placenta has been purified and identified as a monomeric protein of molecular mass at least 46000 Da. The last purification step utilizes a substrate analogue–affinity matrix where the ligand HDBP is structurally similar to phosphotyrosine but has a phosphatase-resistant C–P bond substituted for the hydrolysable phosphoester group of phosphotyrosine. Surprisingly, PTP-I binds to this ligand, which has essentially no higher-order structure. A parallel situation has been observed where the *src* kinase has been purified by using tyrosine–agarose affinity chromatography [41], although this kinase preferentially phosphorylates tyrosine residues *C*-terminal to predominantly acidic residues [2]. Phosphotyrosine phosphatases have been purified by using thiophosphorylated substrate-affinity- or Zn^{2+}-affinity-chromatographic steps [9,35]. Although the ligand HDBP has been used to purify alkaline phosphatases [42], the ability of PTP-I to interact with HDBP suggests that this ligand may be a useful alternative final step in the isolation of other members of the phosphotyrosine-specific phosphatase family.

The kinase p56^{lck} is a member of the *src* family of non-receptor protein tyrosine kinases that typically possess related *C*-terminal tail sequences containing a phosphorylatable tyrosine residue. The activity of the *src* kinase increases on dephosphorylation of this residue [43], and mutation of *src* and *lck* kinases at this site results in cell transformation [44,45], indicating that phosphorylation of this tyrosine residue negatively regulates kinase activity. The loss of expression of a receptor-linked phosphotyrosine phosphatase, CD45, has been correlated with increased phosphorylation of p56^{lck} at Tyr-505, and this phosphatase has been proposed to function in leucocyte growth regulation by altering the kinase activity of p56^{lck} [46]. Although the tissue distribution of PTP-I is unknown, its ability to dephosphorylate Tyr-505 of p56^{lck} indicates that the T-cell-specific p56^{lck} may also be subject to regulation by non-receptor PTPases. The high degree of sequence similarity of p56^{lck} to other *src*-related kinases suggests that the latter may also be PTP-I substrates. Transformation-competent viral analogues of several members of this kinase family have altered or lost *C*-terminal sequences and a different tyrosine (*src* 416/*lck* 394) is

the major phosphorylation site. PTP-I can dephosphorylate Tyr-394 in $p56^{lck}$ as well as the synthetic *src* peptide that corresponds to this site in the *lck* and *src* kinases. These findings, in conjunction with the ability of PTP-I to dephosphorylate the EGF receptor, indicate that PTP-I could function to regulate cellular tyrosine phosphorylation pathways at the level of growth-factor receptor or proto-oncogene/oncogene kinases.

Novel PTPases have recently been identified and cloned by virtue of their shared catalytic domains with that of placental PTPase 1B [10–16]. Other PTPases have been isolated through successive purification steps on the basis of their ability to dephosphorylate phosphotyrosine-containing substrates. PTPases purified to, or close to, homogeneity include particulate and soluble placental enzymes of 35–38 kDa [9,35], a particulate rat spleen enzyme of 36 kDa [47], and soluble enzymes of 48 kDa from bovine brain [36], 50 kDa from bovine spleen [48], and 35 and 37 kDa from rabbit kidney [49]. The relationship of these phosphatases to one another and to the PTPases of known sequence is unclear. On the basis of size, PTP-I belongs to a low-molecular-mass group (35–50 kDa) of non-receptor PTPases, as do most of the PTPases purified to date. PTP-I can be differentiated from the two kidney cytosolic PTPases, which cannot dephosphorylate *src* peptide [50], and from a 48 kDa cytosolic brain PTPase, which has little to no activity towards phosphotyrosyl-histone [34]. Furthermore, the brain enzyme is inhibited by poly-L-lysine whereas PTP-I is not.

Multiple forms of PTPases have been purified from placental membranes. Two of these, PTPases 1A and 1B, are solubilized with 1% Triton X-100 and have been shown to be structurally distinct through tryptic peptide mapping [9,33]. Another PTPase (PTP C), isolated from placental cytoskeleton and insoluble in 1% Triton X-100 but released with 0.6 M-KCl/1% CHAPS, appears to constitute a third form based on its distinct susceptibility to certain polyanions and polycations [35]. PTP-I, solubilized with 2% Triton X-100, differs in several features from the placental phosphatases 1A, 1B and C. The estimated molecular mass of PTP-I is significantly (8–12 kDa) larger, it exhibits micromolar rather than submicromolar K_m values, and it is inhibited in a mixed competitive manner, as opposed to a non-competitive inhibition, by the polyanions heparin and poly(GluTyr). Unlike PTP 1B, but like PTP 1A and C, the activity of PTP-I is unaffected by spermidine even in the absence of EDTA. In spite of these differences, evidence that PTP-I and PTP 1B are the same enzyme comes from results of initial protein sequencing of PTP-I peptides. The three PTP-I sequences obtained are nearly identical with regions in the *N*-terminal half of PTP 1B and encompass certain residues that are not conserved between PTP 1B and other PTPases. Chernoff *et al.* [16] have cloned a placental PTPase with a predicted molecular mass of approx. 50 kDa. The cDNA sequence predicts a protein identical with PTP 1B but extending further to include an extra 11 kDa *C*-terminal hydrophobic tail. The amino acid composition of PTP-I is very similar to that of this predicted PTPase. The size of PTP-I (45 kDa estimated by SDS/PAGE, 50 kDa estimated by gel filtration and at least 46 kDa estimated by amino acid analysis), amino acid composition and sequences obtained from PTP-I peptides suggest that this protein is a larger form of PTP 1B than that purified by Tonks *et al.* (37 kDa) [9,11] and possibly corresponds to the form of PTP 1B predicted by cDNA cloning (50 kDa) [16].

PTPases are known to be susceptible to proteolysis, which can effect relocalization from membrane to soluble fractions [51] and alterations in catalytic properties [47]. Chernoff *et al.* [16] have suggested that proteolytic cleavage of the hydrophobic tail of PTP 1B and consequent relocation of the phosphatase to the cytosol might represent a cellular mechanism of enzyme regulation. Our results demonstrate that membrane-associated PTP 1B does exist as a larger molecular form (PTP-I) *in vivo* and can be purified as such. Whether intracellular cleavage of the membrane-associated phosphatase occurs remains to be investigated. Although the larger form of PTP 1B identified by cDNA cloning has not been characterized biochemically, the characterization of PTP-I suggests that some differences in enzymic properties may exist between this enzyme and the smaller form of PTP 1B, particularly with respect to responses to certain polyanionic and cationic inhibitors. More significant differences resulting from the loss of the *C*-terminal tail of PTP 1B may be better observed *in vivo*. Interestingly, Guan *et al.* [15] have cloned a rat brain PTPase of predicted molecular mass approx. 50 kDa that over its first 321 residues has 97% identity with the proteolysed form of PTP 1B but possesses an extra 11 kDa *C*-terminal tail. The *C*-terminal sequence of the brain enzyme is distinct from that of the placental PTP 1B clone of Chernoff *et al.* [16]. The *C*-terminal sequence diversity exhibited by the non-receptor PTPases cloned from T cells [14], rat brain [15] and placenta [16] suggests that this region may be critical in determining individual enzyme localization, regulation and possibly substrate-specificity. Further studies of this domain may shed light on the physiological function(s) of the PTPases.

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