Protein phosphotyrosine phosphatase purified from the particulate fraction of human placenta dephosphorylates insulin and growth-factor receptors

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Protein phosphatase activity specific for Tyr(P) (phosphotyrosine) residues (PTP-phosphatase) was found in the cytosol and particulate fractions of human placenta. In the particulate fraction, half of the PTP-phosphatase activity could be extracted with 1% Triton X-100. The PTP-phosphatase remaining in the Triton-insoluble residue was solubilized with 0.6 M-KCl plus 1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]propane-1-sulphonate} and was purified 1850-fold by adsorption to DEAE-Sephrose, affinity chromatography on Zn²⁺-iminodiacetate-agarose, phosphocellulose adsorption, Fractogel filtration and Mono Q chromatography. The cytoskeleton-associated PTP-phosphatase was distinguished from acid, alkaline and other protein Ser(P) (phosphoserine)/Thr(P) (phosphothreonine) phosphatases by its neutral pH optimum, activity in the presence of EDTA, inhibition by Zn²⁺, vanadate, or molybdate, and low activity with either [Ser(P)]phosphorylase *a* or *p*-nitrophenyl phosphate. The PTP-phosphate displayed a *K_m* of 0.15 μM with [Tyr(P)]serum albumin as substrate, 10–100-fold lower than the *K_m* for previously described protein phosphatases. The cytoskeleton-associated PTP-phosphatase catalysed the dephosphorylation of receptors for insulin, insulin-like growth factor-1 and epidermal growth factor labelled by auto-phosphorylation. The properties of this PTP-phosphatase suggest that it plays a role in the regulation of hormone receptors and cytoskeleton proteins by reversible phosphorylation on tyrosine residues.

INTRODUCTION

Reversible phosphorylation of tyrosine residues in receptors for EGF, insulin, IGF-1 and PDGF, as well as in the products of various oncogenes (e.g. p60^{src}, p120^{abl}), appears to operate as a mechanism for regulation of the protein tyrosine kinase activity inherent in these proteins (for reviews see: Sefton & Hunter, 1984; Heldin & Westermark, 1984; Cobb & Rosen, 1984; Schlessinger, 1986). Furthermore, expression of protein tyrosine kinase activity that is capable of phosphorylating tyrosine residues in other intracellular proteins, in addition to the kinase itself, has been shown to be essential for transmission of the hormonal signal. Site-directed mutagenesis of a single lysine residue in the insulin receptor presumed to be involved in ATP binding produced mutant receptors with native binding characteristics, yet lacking protein tyrosine kinase activity. Transfected cells expressed the mutant receptor, and bound insulin, but did not produce characteristic responses to the hormone (Ebina *et al.*, 1987; Chou *et al.*, 1987).

Phosphatases specific for the Tyr(P) residues formed in response to hormone stimulation were first characterized by their activity in the presence of EDTA and inhibition with micromolar concentrations of Zn²⁺ (Brautigan *et al.*, 1981; Foulkes *et al.*, 1981), and by in-

hibition with vanadate (Swarup *et al.*, 1982). However, the question has been raised repeatedly whether acid, alkaline and protein Ser(P) phosphatases, in particular phosphatases 2A and 2B (calcineurin), account for the dephosphorylation of cellular Tyr(P) proteins (Merlevede & DiSalvo, 1987). Results from several laboratories have not ruled out a role for these enzymes in protein Tyr(P) dephosphorylation, but have shown that there is a separate class of protein phosphatases in various tissues and cell lines that are specific for Tyr(P) proteins. These specific phosphatases are likely to play a major role in dephosphorylation of cellular Tyr(P) proteins.

Recent work in our laboratory has focused on PTP-phosphatase activity in membranes (Rotenberg & Brautigan, 1987). Earlier studies showed that the amount of Tyr(P) relative to Ser(P) and Thr(P) was 200-fold higher in a particulate fraction prepared from cells transformed with Rous sarcoma virus compared with the whole cells (Gallis *et al.*, 1981). Anti-Tyr(P) antibodies were used to localize Tyr(P) proteins at focal contacts in fibroblasts and at intercellular junctions in epithelial cells infected with Abelson murine leukaemia virus (Maher *et al.*, 1985). Moreover, forms of the p60^{src} protein that were translocated intracellularly into structures resistant to extraction with Triton X-100 were found to be most effective in transformation (Hamaguchi & Hanafusa, 1987). In the present study we report the purification and

Abbreviations used: EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; PTP, protein tyrosine phosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate; WGA, wheat-germ agglutinin; Ser(P), phosphoserine; Thr(P), phosphothreonine; Tyr(P), phosphotyrosine; poly(Glu:Tyr), a synthetic polymer of glutamic acid and tyrosine in a 4:1 molar ratio; FPLC, fast protein liquid chromatography.

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properties of a PTP-phosphatase from human placenta derived from the Triton X-100-insoluble particulate fraction. We use the term 'cytoskeleton' as a working definition of the Triton X-100-insoluble residue, though we recognize that many other proteins are present.

EXPERIMENTAL

Materials

Hepes, glycerol, NaCl, imidazole, MnCl_2 , K_2HPO_4 , sucrose, KCl, ZnCl_2 , sodium orthovanadate and trichloroacetic acid were obtained from Fisher Scientific. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from DuPont-New England Nuclear, MgCl_2 from Mallinckrodt, and scintillation-grade Triton X-100 from Research Products International. Phosphocellulose P-11 was from Whatman, Fractogel TSK HW-55S from EM Reagents, and Mono Q FPLC column, Sephacryl S400, Sephadex G-25 and DEAE-Sephacryl CL-6B were from Pharmacia Fine Chemicals. WGA-agarose was purchased from EY Laboratories. Other chemicals were purchased from Sigma. LSTRA cells were given by Dr. R. Geahlen, Purdue University, and Caesarean-section placentas were obtained from Women and Infants Hospital, Providence, RI, U.S.A.

Preparation of $[\text{Tyr}(^{32}\text{P})]\text{albumin}$

Reduced and alkylated bovine serum albumin, prepared as described by Shriner & Brautigan (1984), was ^{32}P -labelled with a tyrosine kinase ($\text{p56}^{\text{lc}}\text{k}$) found over-expressed in the plasma membranes of a virally transformed lymphocytic cell line called LSTRA. The phosphorylation reaction, of 1.0 ml total volume, was made by mixing 0.1 ml each of 500 mM-Hepes, pH 7.0, 20 mM- MnCl_2 /100 mM- MgCl_2 and 5 mM-sodium orthovanadate, and 0.2 ml each of distilled deionized water and LSTRA-cell-membrane fraction (8.5 mg of protein/ml). Lastly were added 0.1 ml of 1 mM-ATP, pH 7.0, containing 75 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and 0.2 ml of modified albumin (200 mg/ml), pH 7.0. The reaction was terminated after incubation for 48 h at 30 °C by addition of 150 μl of 100% trichloroacetic acid. The mixture was chilled on ice for 15 min and the precipitate collected by centrifugation (13000 g) in a micro-centrifuge (Fisher Model 235-C) for 5 min. The pellet was washed twice with ice-cold 15% trichloroacetic acid and then dissolved in 1 ml of 1 M- K_2HPO_4 , pH 10. Acid precipitation was repeated, and the pellet was dissolved in 0.5 ml of 1 M- K_2HPO_4 and desalted on 15 ml of Sephadex G-25 (fine grade) equilibrated in 25 mM-Hepes/1 mM-EDTA/5 mM- NaN_3 .

Assay for protein Tyr(P) phosphatase activity

Protein phosphatase activity specific for Tyr(P) residues was measured by the release of acid-soluble ^{32}P from $[\text{Tyr}(^{32}\text{P})]\text{bovine serum albumin}$ as described by Shriner & Brautigan (1984). Briefly, 25 μl of substrate containing 25 pmol of $\text{Tyr}(^{32}\text{P})$ (0.01–0.05 mol of ^{32}P /mol of albumin) was incubated for 10 min at 30 °C with 5 μl of water and 20 μl of sample diluted in assay buffer (25 mM-Hepes, pH 7.0, 1 mM-EDTA, 50 mM-2-mercaptoethanol and 1 mg of bovine serum albumin/ml). To assess inhibition of PTP-phosphatase, the 5 μl of water was replaced with 5 μl of a stock solution of various compounds. The reaction was stopped at 10 min by addition of 50 μl of serum albumin (25 mg/ml) plus

0.9 ml of 15% trichloroacetic acid, and the precipitate was pelleted for 5 min (13000 g) in a micro-centrifuge. A 0.5 ml portion of the supernatant was added to 5 ml of scintillation fluid and counted for radioactivity in a Beckman LS 1701 liquid-scintillation counter. Radioactivity in blank samples was determined by incubating substrate under the same reaction conditions without phosphatase. Total ^{32}P in the substrate was assessed by dissolving the trichloroacetic acid precipitate from the blank sample with 1 ml of 1 M- K_2HPO_4 , pH 10, and counting a 0.5 ml sample for radioactivity in 5 ml of scintillation fluid. Release of $[\text{P}^{32}]\text{P}_i$ by PTP-phosphatase under these conditions was linear with respect to time and enzyme concentration up to about 30% of the total. One unit of PTP-phosphatase activity releases 1 nmol of ^{32}P /min under these reaction conditions.

Isolation of hormone/growth-factor receptors

Insulin and IGF-1 receptors were isolated from the 'membrane' component of human placenta, prepared as described under 'Purification of PTP-phosphatase' (Scheme 1). After solubilization with 1% Triton X-100 and centrifugation at 100000 g for 1 h, the supernatant was fractionated on a 5 cm $\times$ 60 cm Sephacryl S400 gel-filtration column. Insulin-binding activity was pooled, and a portion (approx. 10%) was adsorbed to a Mono Q FPLC column. The active tetrameric insulin receptor/kinase was eluted from the column with a gradient of Na_2SO_4 as described by O'Hare & Pilch (1988). Insulin-binding activity, corresponding to the intact receptor, was pooled and concentrated by adsorption to WGA-agarose. The eluate contains, in addition to 0.3 M-N-acetylglucosamine, 10% (v/v) glycerol, and a complement of proteinase inhibitors. Receptor concentration was estimated to be 10–20 nM.

In order to examine IGF-1 receptor phosphorylation/dephosphorylation, the insulin receptor was removed by adsorption to an insulin-affinity column, a procedure which removes 85% of specific insulin-binding capacity (Tollefsen *et al.*, 1987). A portion of the insulin-binding activity from the Sephacryl S400 column was recycled over a 25 ml insulin-affinity column, and the flow-through material was concentrated by WGA-agarose affinity chromatography and re-applied to the insulin-affinity column. This combined procedure, in our hands, removes essentially all of the insulin receptor, as demonstrated by Scatchard analysis (results not shown). The flow-through material, essentially devoid of insulin receptor, was then applied and eluted from the Mono Q column, and concentrated as described for the insulin receptor.

EGF receptor was isolated from vesicles shed from human epidermoid carcinoma A431 cells. After solubilization with Triton X-100, lectin-affinity purification using WGA-agarose was done as described by Boni-Schnetzler & Pilch (1987).

Purification of PTP-phosphatase

(1) Preparation and extraction of placenta membranes. Human placentas, obtained immediately after Caesarean-section delivery, were washed extensively with cold saline buffer (5 mM-Hepes, pH 7.0, 150 mM-NaCl). The cord and amniotic sac were removed, and the remaining tissue was cut into small cubes, blotted with a towel and weighed (typically 250–325 g). Ice-cold homogenizing buffer [25 mM-Hepes, pH 7.0, 0.25 M-sucrose, 1 μg of leupeptin/ml, 1 μg of aprotinin/ml and 15 mM-2-

Scheme 1. Scheme for fractionation of placenta

The sequence of treatments, centrifugation and extractions used to prepare cytosol, membrane and cytoskeleton fractions from human placental tissue are shown in this diagram and described in the Experimental section. Abbreviations: S, supernatant; P, precipitate.

mercaptoethanol, plus 2.5% (w/v) phenylmethanesulphonyl fluoride dissolved in propan-2-ol, at 1:1000 dilution] was added to processed tissue (2 ml/g) and homogenized in a Waring blender for 3 × 1 min and with a Brinkman Polytron tissue disperser for 1 min. The homogenate was clarified by centrifugation at 12000 *g* for 30 min. Material that appeared as a tan-coloured paste on the surface of the pellets was scraped off gently with a spatula, and this material was mixed with the supernatant that was filtered through glass-wool. For each 10 vol. of supernatant 1 vol. of 1.1 M-NaCl plus 2.2 mM-MgCl₂ was added, and the particulate fraction was collected by centrifugation for 40 min at 40000 *g* (Cuatrecasas, 1972). The pooled supernatant was the cytosolic fraction (Scheme 1). The pellets were combined with 25 mM-Hepes/1% Triton X-100/15 mM-2-mercaptoethanol, and were dispersed with a glass homogenizer. After 30 min, the suspension was ultracentrifuged at 100000 *g* for 30 min at 4 °C in a Beckman type 30 rotor. The supernatant from this step was the solubilized placental membrane fraction (Scheme 1).

The Triton-insoluble pellets were pooled, homogenized in a glass homogenizer with 25 mM-Hepes, pH 7.0, containing 0.6 M-KCl, 1% CHAPS and 15 mM-2-mercaptoethanol, and ultracentrifuged at 100000 *g* for 30 min in a Beckman type 30 rotor. The solubilized protein recovered in the supernatant was used as the starting material for purification of PTP-phosphatase.

(2) DEAE-Sepharose adsorption. The KCl/CHAPS-soluble fraction was dialysed for 3 h at 4 °C against

4 litres of buffer A (25 mM-Hepes, pH 7.0, 10% glycerol, 15 mM-2-mercaptoethanol). The precipitate formed during removal of salt and detergent was pelleted by centrifuging at 10000 *g* for 15 min before the PTP-phosphatase was adsorbed for 1 h to 20 ml of DEAE-Sepharose CL-6B equilibrated with buffer A. The suspension was poured into a 2.5 cm-diam. column, the unbound protein was washed through with buffer A, and the column was eluted stepwise with 0.1 M-NaCl in buffer A and 0.5 M-NaCl in buffer A without 2-mercaptoethanol.

(3) Zn²⁺-iminodiacetate affinity chromatography. The 0.5 M-NaCl eluate was applied directly to 40 ml of iminodiacetate-agarose previously loaded with Zn²⁺ and washed with buffer A without 2-mercaptoethanol. Unbound protein was washed through the column with the same buffer. Adsorbed proteins were eluted with a 300 ml linear gradient of increasing imidazole (0–25 mM) and 2-mercaptoethanol (0–50 mM) in 25 mM-Hepes (pH 7.0)/10% glycerol/0.05 M-NaCl. The fractions were assayed and pooled according to their PTP-phosphatase activity.

(4) Phosphocellulose chromatography and gel filtration.

The major PTP-phosphatase activity peak from Zn²⁺-affinity chromatography was applied to 10 ml of phosphocellulose (P-11) equilibrated with buffer A containing 0.05 M-NaCl (buffer B). Bound protein, including the PTP-phosphatase, was eluted stepwise with 0.2 M-NaCl in buffer A. The eluate was concentrated to 2 ml by ultrafiltration in an Amicon stirred-cell apparatus fitted with a PM 10 membrane, and was applied to a 2.5 cm × 95 cm column of Fractogel TSK HW-55S in buffer B. The column was eluted at 1 ml/min with an Eldex model A-30-S pump to give 5 ml fractions that were assayed for PTP-phosphatase.

(5) Mono Q chromatography. As a final step of purification, the peak of PTP-phosphatase from Fractogel filtration (approx. 40 ml) was loaded on a Mono Q HR 5/5 FPLC column by using a Superloop. The column was washed at 1 ml/min with 40 ml of buffer B by using a Beckman 110A h.p.l.c. pump. A 100 ml linear gradient of 0.05–0.25 M-NaCl in buffer A eluted a major and a minor peak of PTP-phosphatase in 1.5 ml fractions. The column was washed with buffer A plus 0.5 M-NaCl to elute other proteins retained on the column. The A₂₈₀ was monitored with a Beckman model 160 detector.

RESULTS

Distribution of PTP-phosphatase activity in human placenta

Measurements of protein phosphatase activity specific for Tyr(P) residues required the use of an artificial substrate. Denatured bovine serum albumin was labelled by protein Tyr kinase p56^{lck} from LSTRA cells by using [γ-³²P]ATP. The [Tyr(³²P)]albumin was used for measurement of PTP-phosphatase as previously described (Shriner & Brautigan, 1984). Human placenta was homogenized and an extract was recovered as a supernatant after centrifugation at 12000 *g* for 30 min. The particulate fraction was separated from cytosol, extracted

Table 1. Purification of PTP-phosphatase from the Triton-X-100-insoluble particulate fraction of human placenta

Step	Protein (mg)	PTP-phosphatase (units)	Yield (%)	Activity (units/mg)	Purification (fold)
1. CHAPS/KCl extract	949	3400	100	3.6	—
2. Dialysis and centrifugation	328	3710	—	11	3.1
3. DEAE-Sepharose adsorption	77	1850	54	24	6.6
4. Zn ²⁺ affinity chromatography	16	980	29	61	17
5. Phosphocellulose adsorption	2	910	27	455	126
6. Fractogel filtration	0.15	220	6.5	1470	408
7. Mono Q chromatography	0.009	60	1.8	6660	1850

with 1% Triton X-100 and then extracted with 0.6 M-KCl/1% CHAPS. By this method (Scheme 1), 60–65% of the total PTP-phosphatase was recovered in the cytosol. In the particulate fraction there was about an equal amount of PTP-phosphatase in the membrane (Triton X-100-extracted) and cytoskeleton (KCl/CHAPS-extracted) preparations. A similar distribution of PTP-phosphatase activity was found with rat liver (results not shown).

Purification of PTP-phosphatase from the placenta particulate fraction

The proteins extracted by KCl/CHAPS served as starting material for purification of a cytoskeleton-associated PTP-phosphatase. Dialysis resulted in formation of a precipitate containing two-thirds of the protein. Fortunately, the PTP-phosphatase remained soluble, with no loss, indeed a modest increase, in the yield of activity (Table 1). Adsorption to DEAE-Sepharose and step elution with 0.5 M-NaCl gave a 2-fold purification, with recovery of about half the PTP-phosphatase activity.

Inhibition by low concentrations of Zn²⁺ (Brautigan *et al.*, 1981) has led us to use metal-ion-chelate affinity chromatography for isolation and purification of PTP-phosphatases (Horlein *et al.*, 1982; Shriner & Brautigan, 1984, 1987). As shown in Fig. 1, the PTP-phosphatase was adsorbed selectively to Zn²⁺-iminodiacetate-agarose. After washing with 0.5 M-NaCl to remove non-specifically bound proteins, a gradient of imidazole and 2-mercaptoethanol eluted the PTP-phosphatase. The peak was pooled, directly adsorbed to phosphocellulose, and eluted stepwise with buffer containing 0.2 M-NaCl. This adsorption proved to be one of the most effective purification steps (Table 1). Proteins that bound to DEAE-Sepharose as anions would not be expected to bind at neutral pH to phosphocellulose. However, in the latter step binding is likely, owing to interaction of the matrix with a specific site on the PTP-phosphatase, presumably the active site which binds phosphoprotein substrates. This situation is reminiscent of the binding of a protein kinase to both DEAE-cellulose and phosphocellulose (DePaoli-Roach *et al.*, 1981). Although

Fig. 1. Zn²⁺-affinity chromatography of PTP-phosphatase

After elution from DEAE-Sepharose CL-6B with 0.5 M-NaCl, the cytoskeleton-associated PTP-phosphatase was applied to a column of Zn²⁺-iminodiacetate-agarose. As described in the Experimental section, the column was washed at point A until the A_{280} fell to baseline. At point B a 300 ml gradient (broken line) of increasing imidazole (0–25 mM) and 2-mercaptoethanol (0–50 mM) concentrations was started, and 4.5 ml fractions were collected. At point C, EDTA (0.1 M) in buffer A was applied to elute the bound Zn²⁺ with remaining proteins. The PTP-phosphatase activity (●—●) and the A_{280} (—) are shown.

Fig. 2. Molecular size of PTP-phosphatase from gel filtration

After Zn^{2+} -affinity chromatography, the PTP-phosphatase was adsorbed to phosphocellulose P-11, eluted, concentrated to 2 ml by ultrafiltration, and applied to 500 ml of Fractogel TSK-55S as described in the Experimental section. The PTP-phosphatase was eluted as a single peak of activity ($\bullet$), with a K_{av} of 0.53. Elution positions of protein standards are indicated: A, bovine serum albumin (M_r 67000; K_{av} 0.47); B, ovalbumin (M_r 43000; K_{av} 0.52); C, carbonic anhydrase (M_r 29000; K_{av} 0.64). The void volume (V_0) for this column was 173 ml, and the total volume (V_T) was 380 ml. The PTP-phosphatase was estimated to have M_r 40000.

these matrices have opposite charges, one resembles the substrate, ATP, and presumably interacts with the enzyme active site.

The PTP-phosphatase was eluted as a single peak from Fractogel TSK SW-55S at a volume near that for ovalbumin, corresponding to M_r = 40000 (Fig. 2). The

peak was pooled, adsorbed to Mono Q, and eluted with a gradient of increasing NaCl concentration. One major and one minor peak of PTP-phosphatase were resolved (Fig. 3). A sample (300 μ l) of the peak tube of PTP-phosphatase eluted from Mono Q (Fig. 3) was subjected to SDS/polyacrylamide-gel electrophoresis. After silver staining the only band observed was at M_r approx. 45000 (results not shown), consistent with the behaviour of the PTP-phosphatase as a monomer during gel filtration (Fig. 2). Overall, PTP-phosphatase was purified 1850-fold from the KCl/CHAPS extract of the particulate fraction from human placenta, in about 2% yield. Calculated from the tissue extract (11 550 mg of protein), this is equivalent to a 22500-fold purification of the cytoskeleton-associated PTP-phosphatase. On the basis of the final specific activity, compared with other homogeneous preparations (Tonks *et al.*, 1988a), one can estimate that 15–20% of the protein at the final step was PTP-phosphatase.

Properties of the placental cytoskeleton-associated PTP-phosphatase

Certain properties distinguish the class of specific protein Tyr(P) phosphatases from other known phosphatases. The PTP-phosphatase exhibited a neutral pH optimum. Relative activity at pH 7, 5 and 9 was 100, 13 and 50 respectively. The PTP-phosphatase activity was inhibited by over 98% in the usual assay by inclusion of 0.1 mM- Zn^{2+} , 0.5 mM-orthovanadate or 1.0 mM-molybdate, and was unaffected by the presence of 1.0 mM-EDTA. The phosphatase also was specific for Tyr(P) residues in proteins. Use of 1 μ M-[Tyr(P)]serum albumin, 10 μ M-Ser(P) phosphorylase and 10 mM-magnesium *p*-nitrophenyl phosphate as substrates (concns. in proportion 1:10:10000) resulted in hydrolysis of 6600, 310 and

Fig. 3. Mono Q chromatography of PTP-phosphatases

As a final step in purification, PTP-phosphatase was applied to a Mono Q HR 5/5 FPLC column and eluted into 1.5 ml fractions as described in the Experimental section. At point A a gradient of 0.05–0.25 M-NaCl was applied, and at point B buffer containing 0.5 M-NaCl was applied. The PTP-phosphatase activity ($\bullet$ — $\bullet$) revealed a major peak at 0.145 M-NaCl corresponding to single peaks of A_{280} (—) and a minor peak at 0.160 M-NaCl.

Fig. 4. Dephosphorylation of hormone and growth-factor receptors by PTP-phosphatase

(a) EGF receptor (EGF R; 20.2 pmol/lane) prepared from A431 cells was incubated with EGF (0.5 μ M), Mg^{2+} (10 mM) and Mn^{2+} (4 mM) for 10 min at 22 $^{\circ}$ C, and then for 20 min at 4 $^{\circ}$ C. After [γ - 32 P]ATP was added to 45 μ M, the receptor was allowed to autophosphorylate for 10 min at 4 $^{\circ}$ C. EDTA was then added (final concn. 20 mM) to stop autophosphorylation. Incubation of these samples for up to 30 min resulted in no change in 32 P labelling, indicating the lack of any phosphatase activity in the receptor preparation. Either buffer (–) or 0.5 unit of PTP-phosphatase (+) was added, and after 10 min at 22 $^{\circ}$ C the dephosphorylation reaction was terminated by addition of electrophoresis sample buffer containing 30 mM-dithiothreitol. The samples were subjected to electrophoresis on a 3–10% gradient polyacrylamide gel. The autoradiogram of the processed gel is shown. The EGF receptor migrated as the intact M_r -170000 form. (b) Human placental insulin receptor (INS R) and IGF-1 receptor (IGF-1 R) were prepared. Autophosphorylation was carried out as described in (a) above, except that insulin or IGF-1 was present at 0.2 μ M, preincubation was carried out for 30 min at 22 $^{\circ}$ C, and autophosphorylation was terminated with EDTA after 30 min at 22 $^{\circ}$ C. Buffer (–) or 0.50 unit of PTP-phosphatase (+) was added for 15 min (INS-R) or 30 min (IGF-1 R) at 22 $^{\circ}$ C before electrophoresis performed as above. The β -subunit (M_r 95000) is marked for both insulin and IGF-1 receptor.

450 pmol/min per ml respectively. In addition, dephosphorylation of [Tyr(P)]serum albumin by PTP-phosphatase displayed saturation kinetics, with a linear Lineweaver–Burk plot that extrapolated to a K_m value of 0.15 μ M for Tyr(P). This was unexpected, for the Michaelis constants for other protein phosphatase reactions are 10–100-fold higher. Taken together, these properties confirm that this enzyme is a Tyr(P)-specific protein phosphatase, distinct from mammalian acid, alkaline and protein Ser(P)/Thr(P) phosphatases.

Dephosphorylation of insulin and growth-factor receptors

The receptors for EGF, insulin and IGF-1 all are protein tyrosine kinases that catalyse phosphorylation of multiple tyrosine residues in the receptors themselves, on the intracellular face of the plasma membrane. EGF receptors were isolated from human epidermoid carcinoma A431 cells, and insulin and IGF-1 receptors from human placental membranes. Incubation in the presence of [γ - 32 P]ATP and the appropriate hormone gave phosphorylation of the EGF receptor and the β -subunits of the insulin and IGF-1 receptors (Fig. 4). The phosphorylation reactions were terminated by chelation of bivalent cations by excess EDTA. Parallel samples were incubated for up to 10–15 min with or without addition of purified PTP-phosphatase, which catalysed the dephosphorylation of each of these receptors (Fig. 4). The rat liver insulin receptor also was dephosphorylated by the human placenta PTP-phosphatase in a similar

experiment (results not shown). The 32 P-labelled bands were excised from gels of multiple experiments and counted for radioactivity to show that the rat liver and human placenta insulin receptors were 65–70% dephosphorylated, the human placenta IGF-1 receptor was 40–50% dephosphorylated, and the human A431-cell EGF receptor was 60–75% dephosphorylated. The extent of dephosphorylation did not appear to increase significantly past 5 min of incubation with the PTP-phosphatase. Thus the PTP-phosphatase catalysed the dephosphorylation of Tyr(P) residues in different receptors, despite the different primary structures surrounding the sites of phosphorylation. The approximate concentration of receptors and of the PTP-phosphatase were in the 1–10 nM range. The reaction of the phosphatase with receptors at these concentrations suggests that the reaction is relevant to physiological conditions.

DISCUSSION

A novel protein phosphatase specific for Tyr(P) has been purified from the particulate fraction of human placenta and shown to catalyse the dephosphorylation of insulin and growth-factor receptors. Evidence has accumulated from several laboratories that the dephosphorylation of Tyr(P) proteins is catalysed by a specific class of protein phosphatases, distinct from the mammalian acid and alkaline phosphatases as well as from other protein phosphatases reactive with Ser(P)

and Thr(P) (see, e.g.: Brautigan *et al.*, 1981; Foulkes *et al.*, 1981; Horlein *et al.*, 1982; Shriner & Brautigan, 1984; Nelson & Branton, 1984; Leis *et al.*, 1985; Clari *et al.*, 1986; Rotenberg & Brautigan, 1987). A more recent study demonstrated that in rat liver plasma membranes a PTP-phosphatase distinct from alkaline phosphatase dephosphorylates detergent-solubilized insulin receptor (Strout *et al.*, 1988). The effects of inhibitors were used to demonstrate that the membrane phosphatase reactive with insulin receptor had characteristics of the class of protein phosphatases specific for Tyr(P). Insulin-receptor dephosphorylation was insensitive to EDTA, which inactivates alkaline phosphatase without affecting PTP-phosphatase, and was blocked by micromolar concentrations of Zn^{2+} , which inhibits PTP-phosphatase but serves as an essential cofactor for alkaline phosphatase.

The occurrence of Tyr(P) in cytoskeleton proteins and the localization of Tyr(P) in focal adhesions and intercellular junctions led us to examine this subcellular fraction for PTP-phosphatase activity. Extraction with the detergent Triton X-100 has been used on tissue-culture cells to solubilize the membrane lipid and most integral membrane proteins, leaving behind a residue enriched in the structural proteins of the cytoskeleton (Brown *et al.*, 1976; Osborn & Weber, 1977). After trying different combinations we treated this Triton X-100-insoluble residue with 0.6 M-KCl plus 1% CHAPS, a zwitterionic cholate derivative, to solubilize additional components. Surprisingly, we found that there were nearly equal amounts of PTP-phosphatase activity solubilized by Triton X-100 and by subsequent treatment of the Triton X-100-insoluble residue with KCl plus CHAPS. Purification was facilitated by the spontaneous precipitation of proteins other than the phosphatase during dialysis. We interpreted these results to indicate that there was a specific and stable association of the PTP-phosphatase with proteins in the Triton X-100-insoluble residue. This association presumably would localize the PTP-phosphatase to the membrane and cytoskeleton and be important for the physiological function of the phosphatase.

By using various artificial substrates, PTP-phosphatase activities have been found in both soluble and particulate fractions. Using carboxyamidomethylated serum albumin containing Tyr(^{32}P), we have found PTP-phosphatases in the soluble and particulate fractions of rabbit kidney (Shriner & Brautigan, 1984; Rotenberg & Brautigan, 1987), rat liver and human placenta, as presented here. We also have prepared ^{32}P -labelled synthetic polymer of glutamic acid:tyrosine (4:1) as a substrate and found the same distribution of PTP-phosphatase activity in these fractions from rat liver. Using carboxyamidomethylated and maleylated lysozyme containing Tyr(^{32}P) as substrate, Tonks *et al.* (1988a) have purified PTP-phosphatases to apparent homogeneity from the soluble and particulate fractions of human placenta. The particulate PTP-phosphatases were solubilized by extraction with Triton X-100. The Triton X-100-insoluble particulate fraction apparently was not tested for PTP-phosphatase activity. Present in both the soluble and particulate fractions were two forms, A and B. Both phosphatases, like that described here, exhibited sub-micromolar K_m values. The A and B forms had different peptide maps. The B form was activated nearly 2-fold by polycations (especially spermidine) and was inhibited by polyanions [e.g. poly(Glu:Tyr)] at 100-fold

lower concentrations than the A form. The soluble 1B form dephosphorylated placental insulin receptor (Tonks *et al.*, 1988b). We propose that our PTP-phosphatase be designated the 'C' form. Its activity was unaffected by 2 mM-spermidine (like A type; unlike B type) but was 80% inhibited by 1 μ M-poly[Glu:Tyr] (unlike A type, like B type). It is also easy to remember that the 'C' form is derived from the cytoskeleton.

The distribution of ^{32}P into multiple sites in the receptors for insulin, IGF-1 and EGF may account for the observation that the dephosphorylation reactions were incomplete, with little difference between the extent of reactions at 5, 10 or 15 min. Further investigations are needed to determine whether all the sites were partially dephosphorylated, or whether some sites were completely dephosphorylated while others were unreactive. The latter possibility is the more intriguing result, but will prove more difficult to demonstrate, because there are multiple Tyr(P) residues in a single peptide, at least in the autophosphorylated insulin receptor (Tornqvist *et al.*, 1987; White *et al.*, 1988).

One important question remains: what is the difference between the cytosolic, membrane- and cytoskeleton-associated PTP-phosphatases? When the membrane PTP-phosphatase (solubilized from the particulate fraction with 1% Triton X-100) was subjected to chromatography on Zn^{2+} -iminodiacetate-agarose and on Mono-Q FPLC, it was eluted at a position in the gradients identical with that for the cytoskeleton-associated PTP-phosphatase. These results suggest that the PTP-phosphatases are similar. Likewise, Tonks *et al.* (1988a) detected little difference between the soluble and particulate forms of the enzymes. Nonetheless, the PTP-phosphatases do partition into different subcellular preparations, and the distinction between soluble and particulate, and Triton-soluble and Triton-insoluble, are reproducible with human placenta, and in other tissues, and in other species of animals. One can surmise that there must be an association of the monomeric PTP-phosphatase catalytic subunit with other proteins in the membrane or in the cytoskeleton. If this is the case, then there might be subtle differences between the PTP-phosphatases to provide for specific interactions. There could be isoforms of the PTP-phosphatase catalytic subunit, separate genes encoding closely related but distinctly different proteins. This has proved to be the case with the catalytic subunit of protein phosphatase 2A, where only eight of 309 amino acids are substituted in the two human genes (Arino *et al.*, 1988). There could be a post-translational modification of the PTP-phosphatase catalytic subunit or its cytoskeletal binding protein. Of course, these are speculative possibilities, but we might expect that the mechanisms involved in the partition of PTP-phosphatase between different intracellular compartments (and access to its potential substrates) will be of physiological importance.

Note added in proof (received 29 September 1988)

Using the purification steps described here, we have carried out large-scale preparations and identified the active form of PTP-phosphatase as a protein with M_r 38 000 on 12% polyacrylamide gels in SDS. The substrate used to monitor PTP-phosphatase activity was reduced carboxyamidomethylated and maleylated (RCAM) lysozyme phosphorylated on Tyr residues by human

placental insulin receptor. PTP-phosphatase detected during purification by using RCAM-lysozyme compared with albumin as substrate was the same.

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REFERENCES

- Arino, J., Woon, C. W., Brautigan, D. L., Miller, T. B., Jr. & Johnson, G. L. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4252–4256
- Boni-Schnetzler, M. & Pilch, P. F. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7832–7836
- Brautigan, D. L., Bornstein, P. & Gallis, B. (1981) *J. Biol. Chem.* **256**, 6519–6522
- Brown, S., Levinson, W. & Spudich, J. A. (1976) *J. Supramol. Struct.* **5**, 119–130
- Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebowitz, D., Ullrich, A. & Rosen, O. M. (1987) *J. Biol. Chem.* **262**, 1842–1847
- Clari, G., Brunati, A. M. & Moret, V. (1986) *Biochem. Biophys. Res. Commun.* **137**, 566–572
- Cobb, M. H. & Rosen, O. M. (1984) *Biochim. Biophys. Acta* **738**, 1–8
- Cuatrecasas, P. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 318–322
- DePaoli-Roach, A. A., Ahmad, Z. & Roach, P. J. (1981) *J. Biol. Chem.* **256**, 8955–8962
- Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C. S., Siddle, K., Pierce, S. B., Roth, R. A. & Rutter, W. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 704–708
- Foulkes, J. G., Howard, R. F. & Ziemiecki, A. (1981) *FEBS Lett.* **130**, 197–200
- Gallis, B., Bornstein, P. & Brautigan, D. L. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6689–6693
- Hamaguchi, M. & Hanafusa, H. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2312–2316
- Heldin, C.-H. & Westermark, B. (1984) *Cell* **37**, 9–20
- Horlein, D., Gallis, B., Brautigan, D. L. & Bornstein, P. (1982) *Biochemistry* **21**, 5577–5584
- Leis, J. F., Knowles, A. F. & Kaplan, N. O. (1985) *Arch. Biochem. Biophys.* **239**, 320–326
- Maher, P. A., Pasquale, E. B., Wang, J. Y. J. & Singer, S. J. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6576–6580
- Merlevede, W. & DiSalvo, J. (eds.) (1987) *Advances in Protein Phosphatases*, vol. 4, Leuven University Press, Leuven
- Nelson, R. L. & Branton, P. E. (1984) *Mol. Cell. Biol.* **4**, 1003–1012
- O'Hare, T. & Pilch, P. F. (1988) *Biochemistry*, in the press
- Osborn, M. & Weber, K. (1977) *Exp. Cell Res.* **106**, 339–349
- Rotenberg, S. A. & Brautigan, D. L. (1987) *Biochem. J.* **243**, 747–754
- Schlessinger, J. (1986) *J. Cell Biol.* **103**, 2067–2072
- Sefton, B. M. & Hunter, T. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **18**, 195–226
- Shriner, C. L. & Brautigan, D. L. (1984) *J. Biol. Chem.* **259**, 11383–11390
- Shriner, C. L. & Brautigan, D. L. (1987) *J. Biochem. Biophys. Methods* **14**, 273–278
- Strout, H. V., Vicario, P. P., Saperstein, R. & Slater, E. E. (1988) *Biochem. Biophys. Res. Commun.* **151**, 633–640
- Swarup, G., Cohen, S. & Garbers, D. L. (1982) *Biochem. Biophys. Res. Commun.* **107**, 1104–1109
- Tollefsen, S. E., Thompson, K. & Peterson, D. J. (1987) *J. Biol. Chem.* **262**, 16461–16469
- Tonks, N. K., Diltz, C. D. & Fischer, E. H. (1988a) *J. Biol. Chem.* **263**, 6722–6730
- Tonks, N. K., Diltz, C. D. & Fischer, E. H. (1988b) *J. Biol. Chem.* **263**, 6731–6737
- Tornqvist, H. E., Pierce, M. W., Frackelton, A. R., Nemenoff, R. A. & Avruch, J. (1987) *J. Biol. Chem.* **262**, 10212–10219
- White, M. F., Shoelson, S. E., Keutmann, H. & Kahn, C. R. (1988) *J. Biol. Chem.* **263**, 2969–2980

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