Partial Purification and Characterization of Phosphotyrosyl-Protein Phosphatase from Ehrlich Ascites Tumor Cells†

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ABSTRACT: We have previously described a phosphotyrosyl-protein phosphatase in membrane vesicles from human epidermoid carcinoma A431 cells which is inhibited by micromolar concentrations of Zn^{2+} and is insensitive to ethylenediaminetetraacetic acid (EDTA) and NaF [Brautigan, D. L., Bornstein, P., & Gallis, B. (1981) *J. Biol. Chem. 256*, 6519–6522]. Here we present the identification and partial purification of a similar enzyme from lysates of Ehrlich ascites tumor cells. The enzyme was purified by using diethylaminoethyl-Sephadex, Zn^{2+} affinity, and Sephadex G-75 chromatography. During purification, the phosphatase was separated into at least three fractions, all of which exhibited very similar properties and an apparent molecular weight of 40 000 upon gel filtration. The enzyme dephosphorylated phosphotyrosine (P-Tyr)-containing carboxymethylated and succinylated (CM–SC) phosphorylase with an apparent K_m of 0.8 μM, as well as P-Tyr-containing casein and epidermal growth factor (EGF) receptor kinase, but did not dephosphorylate P-Ser-phosphorylase. The phosphatase was inhibited by Zn^{2+} at micromolar concentrations ($K_{0.5}$ with EGF receptor kinase = 5×10^{-6} M; with CM–SC-phosphorylase = 3.3×10^{-5} M) but not by millimolar concentrations of EDTA and NaF. No inhibition was seen with 1 mM tetramisole, a specific inhibitor of alkaline phosphatases. P-Tyr inhibited the enzyme by 50% at 0.4×10^{-3} M, while Tyr, P_i, PP_i, and *p*-nitrophenyl phosphate, an excellent substrate for alkaline phosphatases and structurally very similar to P-Tyr, exerted partial inhibition at concentrations above 10^{-3} M. The pH optimum was found to be 6.5–7, depending on the substrate used. Very little activity was seen below pH 5 and above pH 8.5. These properties clearly distinguish this enzyme from alkaline phosphatases, as well as the neutral and acidic protein phosphatases so far described, and therefore define it as a new enzyme of the phosphatase family—a phosphotyrosyl-protein phosphatase.

Phosphorylation of tyrosine residues is a recently discovered posttranslational modification of proteins (Hunter & Sefton, 1980). The level of tyrosine phosphorylation is correlated with the transformation of cells by certain RNA tumor viruses (Sefton et al., 1980; Barbacid et al., 1980). Protein kinase activity specific for tyrosine residues was first shown to be associated with the transforming protein of Rous sarcoma virus, $pp60^{src}$ [1] (Hunter & Sefton, 1980). Cells transformed by RSV, as well as some other RNA tumor viruses (Barbacid et al., 1980; Blomberg et al., 1980; Sefton et al., 1981), showed elevated levels of phosphotyrosine. When cells infected by RSV containing a temperature-sensitive mutation in the transforming gene are shifted to the restrictive temperature for transformation, 60% of the phosphate is lost from phosphotyrosine residues within 75 min (Sefton et al., 1980), suggesting that the level of phosphotyrosine in proteins reflects the relative activities of the kinase and a phosphotyrosyl-protein phosphatase. It is possible that tyrosine phosphorylation of certain proteins with regulatory functions mediates the complex alterations in the cellular metabolism which ultimately lead to the transformed phenotype.

Specific tyrosine phosphorylation has also been observed following addition of growth factors such as EGF (Carpenter et al., 1979; Cohen et al., 1980; King et al., 1980; Ushiro & Cohen, 1980) and platelet-derived growth factor (PDGF) (Ek et al., 1982) to cell membranes. In addition, in A431 human epidermoid carcinoma cells, the EGF receptor, a phosphoprotein of M_r 170 000, undergoes phosphorylation at tyrosine residues upon stimulation by the hormone (Hunter & Cooper, 1981). Recently, it has become evident that the receptor copurifies with a protein kinase specific for tyrosine residues (Cohen et al., 1982). Other mitogenic peptides, called transforming growth factors, which are secreted from normal (Roberts et al., 1981) and a variety of transformed cells (Roberts et al., 1980; DeLarco & Todaro, 1978) not only induce phosphorylation in the EGF receptor (Reynolds et al., 1981) but may also themselves be potentiated by EGF (Roberts et al., 1982). The EGF receptor kinase can also phosphorylate a synthetic tyrosine-containing peptide of $pp60^{src}$ (Pike et al., 1982) as well as antibodies against $pp60^{src}$ (Chinkers & Cohen, 1981; Kudlow et al., 1981). In RSV-transformed rat cell vesicles, tyrosine phosphorylation has been shown to be reversible, and phosphotyrosine-protein phosphatase activity has been identified in both normal and RSV-transformed rat cells (Brautigan et al., 1981; Gallis et al., 1981). A similar activity has been found in rat muscle and liver extracts (Foulkes et al., 1981).

We have taken advantage of the apparent binding of Zn^{2+} to this phosphatase to partially purify the protein by affinity chromatography and to isolate it from other phosphatases in EAT cell lysates. The properties of the phosphatase clearly distinguish it from alkaline and other phosphoprotein phosphatases: it is strongly inhibited by micromolar concentrations of Zn^{2+}, has a neutral pH optimum for activity, is unaffected by a specific inhibitor of tissue alkaline phosphatases, and exhibits specificity for phosphotyrosine residues in proteins.

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; CM–SC, carboxymethylated and succinylated; DEAE, diethylaminoethyl; EAT, Ehrlich ascites tumor; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NP-40, Nonidet P-40; PNPP, *p*-nitrophenyl phosphate; PP_i, pyrophosphate; $pp60^{src}$, phosphoprotein product of the Rous sarcoma virus transforming gene; P-Ser, phosphoserine; P-Tyr, phosphotyrosine; RSV, Rous sarcoma virus; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; Cl_3CCOOH, trichloroacetic acid; BSA, bovine serum albumin; PDGF, platelet-derived growth factor.

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Experimental Procedures

Materials

Phosphoserine, phosphothreonine, adenosine 5′-triphosphate, tetramisole, hydrolyzed, partially dephosphorylated casein, subtilisin type VIII, cacodylic acid, zinc chloride, and base-free L-histidine were from Sigma. EGF was obtained from Collaborative Research. Succinic anhydride, ninhydrin, and iminodiacetic acid–agarose were purchased from Pierce Chemical Co., *p*-nitrophenyl phosphate was from Calbiochem, and Norit A was from Pfanstiehl Laboratories. DEAE-Sephadex A-50, Sephadex G-75 Superfine, Sephacryl S-300, and protein molecular weight standards were from Pharmacia Fine Chemicals, and cellulose thin-layer plates were from Merck. Phosphotyrosine and Ehrlich ascites tumor cells were a gift from Dr. Linda Pike, Department of Pharmacology, Howard Hughes Medical Institute, University of Washington. Crystalline rabbit muscle phosphorylase *b* (Fischer & Krebs, 1955), [^{32}P]phosphorylase *a*, prepared with purified muscle phosphorylase kinase (Krebs et al., 1964), and rabbit muscle phosphorylase phosphatase prepared according to Brautigan et al. (1980) were gifts from Dr. Edmond Fischer, Curt Diltz, and Lisa Ballou, Department of Biochemistry, University of Washington.

Methods

Preparation of Membrane Vesicles from A431 Cells. Membrane vesicles were prepared by a modification (Brautigan et al., 1981) of the method of Carpenter et al. (1979).

Preparation of ^{32}P-Phosphorylated Casein, Phosphorylase, and A431 Membrane Protein. Phosphorylase was carboxymethylated and succinylated by a modification (Koide et al., 1978) of the methods of Crestfield et al. (1963) and Yaoi et al. (1964). These modifications denatured the protein and exposed the phenolic side chains of tyrosine for reaction with EGF receptor kinase. Casein and CM–SC-phosphorylase were phosphorylated with the EGF-stimulated tyrosine protein kinase (Cohen et al., 1982). Reactions contained 20 mM Hepes, pH 7.4, 2 mM $MnCl_2$, 6 mg/mL modified phosphorylase (or 16 mg/mL casein), 100 nM EGF, 100 μg of A431 membrane protein, 0.3% NP-40, and [γ-^{32}P]ATP (330 μM, 1.4×10^8 cpm) in a reaction volume of 300 μL. Reactions were incubated for 45 min at 30 °C, terminated by 10-fold dilution with water, and made 10% with trichloroacetic acid by addition of a 100% (w/w) solution. After 10 min on ice, the precipitate was collected by centrifugation for 5 min at 1500*g*. The protein pellets were washed by repeated centrifugation and suspension in 95% ethanol and then dried under a stream of N_2. Dried protein was suspended in water and dissolved by addition of dilute NaOH. The phosphorylase was then mixed with a suspension of 50 mg of Norit A and 100 mg of Hyflo Super Cel to remove nucleotides and the suspension filtered through a 0.25-μm disposable filter. The phosphorylated protein was dialyzed extensively against 20 mM Hepes, pH 7.4. Finally, the [^{32}P]phosphorylase was electrodialyzed 3 times at 75 mA for 1 h against 500 mL of 20 mM Hepes, pH 7.4, to remove radioactive nucleotides and $^{32}P_i$. This reduced the noncovalently bound radioactivity in the substrate to ≤5% of the total cpm. The phosphorylase was then frozen at −20 °C until use. This substrate will be referred to as ^{32}P-Tyr-CM–SC-phosphorylase. Phosphorylated casein was prepared similarly to phosphorylase, except that, after the ethanol wash, it was dialyzed against two changes of 20 mM Hepes, pH 7.4, and then frozen at −20 °C until use. Phosphorylated A431 membrane protein was prepared in the same manner as described for phosphorylase.

The concentration of P-Tyr in each substrate was calculated from the incorporation of ^{32}P into trichloroacetic acid precipitable protein and the specific radioactivity of the [γ-^{32}P]ATP. The specific radioactivities of substrates were 700–2100 cpm/μg for modified phosphorylase, 50000 cpm/μg for A431 membrane protein, and 2300–3500 cpm/μg for partially hydrolyzed, dephosphorylated casein.

Analysis of ^{32}P-Phosphorylated Substrates. Casein or modified phosphorylase was digested with 5% (w/w) subtilisin type VIII at 37 °C for 18 h in 0.2 M NH_4HCO_3, pH 8.5. The samples were divided in two; half was used for phosphoamino acid analysis on cellulose thin-layer plates following 1-h hydrolysis in 6 N HCl under N_2 at 110 °C (Brautigan et al., 1981), and half was used for two-dimensional phosphopeptide analysis on cellulose thin-layer plates. Samples were subjected to electrophoresis in the first dimension at pH 3.5 for 60 min at 500 V in acetic acid/pyridine/H_2O (25:2.5:472.5), dried for 10 min at 80 °C, and then chromatographed in the second dimension in 1-butanol/acetic acid/pyridine/H_2O (40:20:15:30).

Autoradiography of the plates on Kodak X-ray film (X-omat) was done for 12–24 h.

Assay for Phosphotyrosyl-Protein Phosphatase Activity. Phosphatase activity was measured by using ^{32}P-Tyr-CM–SC-phosphorylase or A431 membrane protein in 50-μL reactions containing 0.25–4 μM ^{32}P-labeled substrate, 0.1 mg/mL bovine serum albumin, 20 mM Hepes, pH 7.4, and 5 mM EDTA. Following addition of the phosphatase, the reactions were incubated for 5 min at 30 °C and terminated by addition of 100 μL of 25 mg/mL bovine serum albumin and 800 μL of 10% trichloroacetic acid. The reaction mixture was incubated on ice for 10 min and centrifuged for 5 min in a Beckman microfuge. A 500-μL aliquot of supernatant was mixed with 10 mL of 10% BBS-3 scintillation fluid and counted in a liquid scintillation counter. Total ^{32}P in the substrate was determined by directly counting a 10-μL aliquot, and background radioactivity was determined by incubating substrate under reaction conditions without enzyme. With phosphorylase and EGF receptor kinase, release of protein-bound ^{32}P was linear with respect to time and to enzyme concentration when less than, or equal to, 20% of the total $^{32}P_i$ was released. Units of activity are expressed in terms of picomoles of $^{32}P_i$ released per minute per milligram of protein.

Phosphotyrosyl-protein phosphatase activity was measured with casein as substrate under the same reaction conditions. The reaction was terminated by transferring a 10-μL aliquot onto a 1.5-cm square of phosphocellulose P81 paper, which was submerged in ice-cold 10% trichloroacetic acid containing 0.01 M sodium pyrophosphate and washed as previously described (Brautigan et al., 1981).

Release of $^{32}P_i$ from protein substrates was also measured by 2-methyl-2-propanol/benzene extraction of the phosphomolybdate complex (Martin & Doty, 1949; Antoniw & Cohen, 1976).

Phosphorylase phosphatase activity was measured by release of $^{32}P_i$ from rabbit skeletal muscle phosphorylase *a* according to Brautigan et al. (1980).

Determination of pH Optimum for Phosphatase Activity. The buffer system for determination of the pH optimum consisted of Tris base and cacodylic acid. The buffer was adjusted to a given pH by addition of either NaOH or HCl at 25 °C. The final buffer concentration for each reaction was 20 mM Tris/20 mM cacodylate. Reactions were performed at 30 °C without prior incubation of enzyme or substrate in the buffer system.

Preparation of Crude Phosphotyrosyl-Protein Phosphatase. Ehrlich ascites tumor cells were passaged every 12 days by injection of 0.25 mL of ascites fluid into 8-week-old Balb/c mice. The mice were sacrificed by cervical dislocation, and the ascites fluid was removed from the peritoneal cavity with a syringe. Ascites fluid was centrifuged for 5 min at 1500*g*, the supernatant discarded, and the cell pellet frozen in a dry ice/ethanol bath. Cell pellets were then stored at −70 °C until use.

The phosphatase was prepared from 20–50 mL of packed cells which were lysed by addition of 3–5 volumes of buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 0.5% NP-40. The cells were homogenized by 20 strokes in a Dounce homogenizer, and the lysate was centrifuged at 20000*g* for 10 min in a Sorvall SS-34 rotor. The supernatant was poured through several layers of cheesecloth to remove fat and other insoluble matter.

Purification of Phosphotyrosyl-Protein Phosphatase from EAT Cell Lysates. All chromatographic steps were performed at 4 °C and in rapid succession to avoid loss of enzymatic activity. The cell lysate was passed through a column of DEAE-Sephadex A50 (80-mL bed volume) that had been equilibrated with 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.02% Brij 35, and 10% glycerol (buffer A). The column was washed with buffer A until no protein was detected in the effluent; elution employed a linear gradient established between 150 mL each of 50 and 400 mM NaCl in buffer A followed by buffer A containing 1 M NaCl.

Fractions containing phosphatase activity were pooled and loaded onto a column (5 mL bed volume) of iminodiacetic acid–agarose which had been converted to the Zn^{2+}-chelate form according to the method of Kurecki et al. (1979) and equilibrated in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.02% Brij 35, and 10% glycerol (buffer B). The column was washed with 10 volumes of buffer B alone and eluted stepwise, first with buffer B containing 20 mM histidine and then with buffer B containing 60 mM histidine. A final elution used 50 mM EDTA, pH 7.0, containing 0.5 M NaCl. Fractions with the highest phosphatase activity from the elution with 20 mM histidine were directly loaded onto a column of Sephadex G-75 Superfine (1.5 × 110 cm), equilibrated in buffer B without glycerol.

Chromatography on Sephacryl S-300 (2.5 × 110 cm) was performed in buffer B. Fractions containing phosphatase activity were pooled and stored at −20 °C in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.02% Brij 35, 1 mM DTT, and 50% glycerol. Fractions containing less than 0.5 mg/mL protein were made 2 mg/mL with bovine serum albumin to minimize loss of enzyme activity during storage. The protein content of all the phosphotyrosyl-protein phosphatase fractions was determined according to Lowry et al. (1951).

Results

Characterization of the ^{32}P-Phosphorylated Substrates. Rabbit skeletal muscle phosphorylase, denatured by reduction, carboxymethylation, and extensive succinylation (CM–SC-phosphorylase), and partially hydrolyzed casein were phosphorylated with the specific tyrosine-protein kinase of A431 cell membrane vesicles and [γ-^{32}P]ATP. The phosphoamino acid composition of these proteins was analyzed by partial acid hydrolysis and two-dimensional thin-layer electrophoresis followed by autoradiography. These experiments revealed that more than 95% of the [^{32}P]phosphate incorporated into these proteins was covalently bound to tyrosine residues (Figure 1B,C). The same result was found for the autophosphorylated EGF receptor kinase in A431 cell membranes (Brautigan et al., 1981). Thus, each of these three proteins was suitable for use as a substrate to detect phosphotyrosyl phosphatase activity.

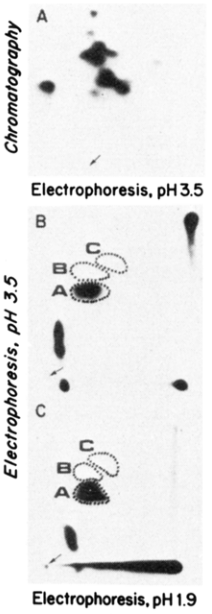

FIGURE 1: Two-dimensional peptide mapping and phosphoamino acid analysis of ^{32}P-labeled proteins. (A) ^{32}P-Labeled CM–SC-phosphorylase digested with subtilisin. The starting point is indicated (↓) in the lower lefthand corner. About 20000 cpm was applied. (B) Partially hydrolyzed subtilisin digest of ^{32}P-labeled CM–SC-phosphorylase. The starting point is in the lower left-hand corner. The dotted circles indicate reference phosphoamino acids detected by ninhydrin staining (A, P-Tyr; B, P-Thr; C, P-Ser). About 13000 cpm was applied. (C) Partially hydrolyzed ^{32}P-labeled partially hydrolyzed casein. Conditions as in (B). About 25000 cpm was applied.

The distribution of ^{32}P-labeled tyrosine in the proteins was analyzed by enzymatic digestion and two-dimensional peptide mapping by using electrophoresis and chromatography on thin-layer plates. For CM–SC-phosphorylase, more than ten individual peptides containing [^{32}P]phosphate could be resolved in this system (Figure 1A). A similar result was found for casein (not shown). The presence of several ^{32}P-labeled peptides indicated that the EGF receptor kinase had phosphorylated each of the proteins at more than one tyrosine residue.

Preparation of Phosphotyrosyl-Protein Phosphatase and Dephosphorylation of ^{32}P-Tyr-CM–SC-Phosphorylase. Supernatants of EAT cell lysates were tested for phosphatase activity by using ^{32}P-Tyr-CM–SC-phosphorylase as substrate. Dephosphorylation was found to be linear with time for release of up to 20% of the $^{32}P_i$ from substrate. All subsequent experiments used a range of substrate concentrations of between 0.25 and 4 μM P-Tyr and an amount of enzyme sufficiently low to yield linear dephosphorylation. For determination of whether the enzymatic activities measured the release of P_i or the formation of Cl_3CCOOH-soluble peptides through the action of contaminating proteases, an aliquot of each Cl_3CCOOH supernatant from the reactions was extracted with 2-methyl-2-propanol/benzene (1:1). Analyses of phosphate release by 2-methyl-2-propanol/benzene extraction closely paralleled the results of Cl_3CCOOH precipitation. Thus, contaminating protease activity did not account for the activity measured under the conditions of our assay.

Purification of Phosphotyrosyl-Protein Phosphatase Activity. Lysates from 20 mL of packed cells were made 10% with glycerol, 0.02% with Brij 35, and 50 mM with NaCl and

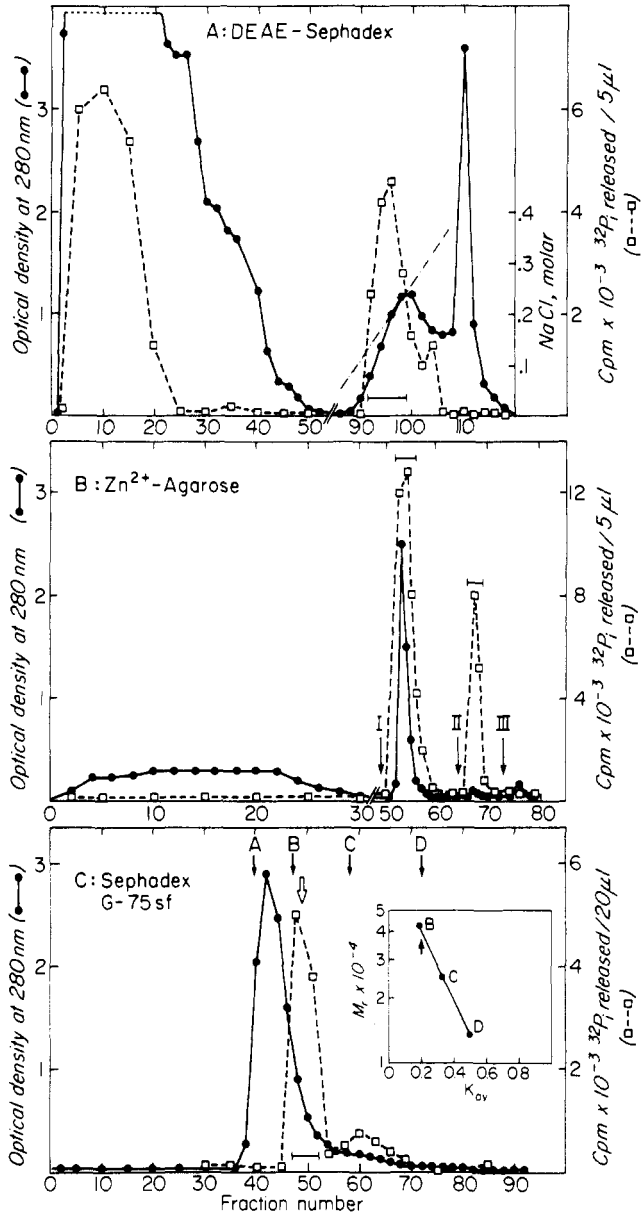

FIGURE 2: Purification of phosphotyrosyl-protein phosphatase. (A) Chromatography of crude extract from 20 mL of packed cells on DEAE-Sephadex A-50. The linear gradient from 50 to 400 mM NaCl starts with fraction 86; elution with 1 M NaCl starts with fraction 107. The bar indicates the fractions used for further purification. A 5-µL sample of the indicated fractions was used for the assay of phosphatase activity with ^{32}P-labeled Tyr–partially hydrolyzed casein (4 µM P-Tyr containing 70000 cpm) as described under Experimental Procedures. (B) Chromatography of fractions indicated by the bar in (A) on a Zn^{2+}-chelated iminodiacetic acid–agarose column. Arrows I, II, and III indicate elution of the column with 20 mM histidine, 60 mM histidine, and 50 mM EDTA/0.5 M NaCl, respectively. The bars indicate pooled fractions. Fractions 52–56 were used for further purification. A 5-µL sample of the indicated fractions was tested for phosphatase activity as in (A). (C) Chromatography of fractions 52–56 from (B) on Sephadex G-75 Superfine. For determination of molecular weights, the column was calibrated with (A) BSA (M_r 67000), (B) ovalbumin (M_r 43000), (C) chymotrypsinogen A (M_r 25000), and (D) ribonuclease A (M_r 13700). The mean elution volume of the phosphatase activity is indicated by an open arrow. The bar indicates the pooled fraction. A 20-µL sample of the indicated fractions was used for determination of phosphatase activity as in (A). Inset: log M_r vs. K_{av} plot to determine the M_r (indicated by the arrow) of the phosphatase.

loaded onto a DEAE-Sephadex A-50 column (Figure 2A). Under these conditions, most of the protein activity (80–90%) and about 60% of the total phosphatase activity were not adsorbed to the gel and were washed from the column by passage of several volumes of buffer A. The adsorbed protein was eluted with a linear gradient of NaCl between 50 and 400 mM, followed by stepwise elution with 1 M NaCl. One major peak of phosphatase activity was found, representing approximately 40% of the total activity in the lysate.

Table I: Purification of Phosphotyrosyl-Protein Phosphatase

fraction	protein[a] (mg)	phosphatase activity (units)[c]	sp act. (units/mg)	yield (%)	x-fold purification
lysate	4400	352	8	100	1
DEAE-Sephadex A-50 pool	176	153	87	43.5	11
Zn^{2+}-agarose pool	50	120	240	34	30
Sephadex G-75 pool	6	72	1200	20.4	150
Zn^{2+}-agarose pool 2	0.1[b]	29	28800	8.2	3625

[a] Protein determinations were performed according to Lowry et al. (1951). [b] It was assumed that 1 OD at 280 nm equals 1 mg/mL protein. [c] One unit of enzyme is defined as the amount that released 1 pmol of P_i min^{-1} (mg of protein)$^{-1}$ from P-Tyr protein substrate under standard conditions.

Further purification of this material was achieved by affinity chromatography on a column of Zn^{2+} chelated to iminodiacetic acid–agarose (Figure 2B). Fractions from the column of DEAE-Sephadex A-50 (as indicated by the bar in Figure 2A) were applied, and the column was extensively washed. Bound protein was then eluted stepwise with 20 mM histidine, then with 60 mM histidine, and finally with 0.05 M EDTA, pH 7.0, and 0.5 M NaCl to elute the Zn^{2+} from the column.

Approximately 75% of the protein did not bind to the Zn^{2+}–agarose column. The majority of the bound protein was subsequently eluted as a narrow peak with 20 mM histidine. Very small amounts of protein were eluted with 60 mM histidine and 0.05 M EDTA, pH 7.0, and 0.5 M NaCl, respectively. All of the phosphatase was initially bound to Zn^{2+}–agarose, as expected, because the phosphatase activity in the crude lysate showed a high degree of sensitivity to inhibition by micromolar concentrations of Zn^{2+} (not shown). However, it was surprising that the phosphatase activity could be separated into two fractions, one with moderate affinity to Zn^{2+} (Zn^{2+}–agarose pool 1) and the other with an apparently higher affinity (Zn^{2+}–agarose pool 2). These pools contained approximately 75–80% and about 25% of the activity, respectively.

The phosphatase in Zn^{2+}–agarose pool 1 was further purified by chromatography on a column of Sephadex G-75 Superfine (Figure 2C). Fractions from the Zn^{2+} affinity column (as indicated by the bar in Figure 2B) were applied. The majority of the enzymatic activity was eluted as a single peak. The bulk of the protein was separated from the phosphatase, providing substantial further purification, with approximately 80% recovery of activity. An M_r of 40000 for the major peak was estimated from comparison of the elution of the phosphatase with the elution volumes for proteins of known molecular size on the same column (see inset in Figure 2C). A minor fraction of the phosphatase activity eluted in the range of M_r 15000–20000.

The phosphotyrosyl-protein phosphatase was purified 150-fold to a specific activity of 1200 units/mg by this three-step procedure with a yield of about 20% of the initial activity (Table I). However, approximately 60% of the phosphatase activity in the lysate did not bind to DEAE-Sephadex (see Figure 2A), and enzyme eluted from DEAE-Sephadex was

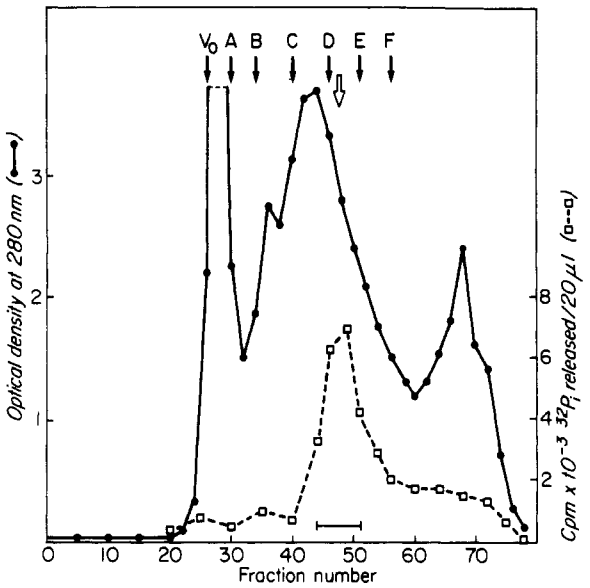

FIGURE 3: Chromatography of crude EAT cell extract on a column of Sephacryl S-300. For estimation of molecular weights, the column was calibrated with (A) catalase (M_r 232 000), (B) aldolase (M_r 158 000), (C) BSA (M_r 67 000), (D) ovalbumin (M_r 43 000), (E) chymotrypsinogen A (M_r = 25 000), and (F) ribonuclease A (M_r 13 700). The void volume (V_0) is also indicated. The main elution volume of the phosphatase activity is indicated by an open arrow. The bar indicates pooled fractions. Phosphatase activity was assayed with 20 μL of the indicated fractions as in Figure 3A.

separated into two fractions on the Zn^{2+}–agarose column. The Zn^{2+} agarose pool 2, eluted with 60 mM histidine, contained highly purified, although not homogeneous, phosphatase (see Table I).

The phosphotyrosyl-protein phosphatase did not behave as a single species during anion-exchange chromatography; more than half was not adsorbed to the gel. This raised the question of whether there was more than one type of phosphatase present in the extract. We therefore subjected the EAT cell extract to gel filtration on Sephacryl S-300 in order to determine if there were phosphatases of different molecular weights that might correspond to those that did or did not bind to DEAE-Sephadex A-50. Most of the phosphatase was eluted from the column as a single peak of activity with an average M_r of about 40 000 (Figure 3). Again, some phosphatase of lower molecular weight was detected. These results show that the majority of the phosphotyrosyl-protein phosphatase in EAT cell lysates is present as a single molecular weight species. Fractionation of the enzyme by anion-exchange chromatography may result from the presence of different but related enzymes with very similar molecular weights.

A minor proportion of the phosphatase eluted consistently at a position corresponding to a lower M_r (Figure 3 and also Figure 2C). This phosphatase may represent proteolytic fragments of the M_r 40 000 enzyme or yet another distinct enzyme.

A major problem with purified phosphotyrosyl-protein phosphatase (Zn^{2+}–agarose pool 2 and enzyme from Sephadex G-75) was that the enzymatic activity was rapidly lost during storage. Activity was usually lost completely within 2 weeks, even with the addition of a surfactant, Brij 35, and storage in 50% glycerol at −20 °C in the presence of 2 mg/mL BSA. The enzymatic activity recovered after DEAE-Sephadex chromatography and that in the Zn^{2+}–agarose pool 1, however, were stable for 6 weeks without apparent loss of activity. Therefore, these preparations were used to examine the properties of the phosphatase.

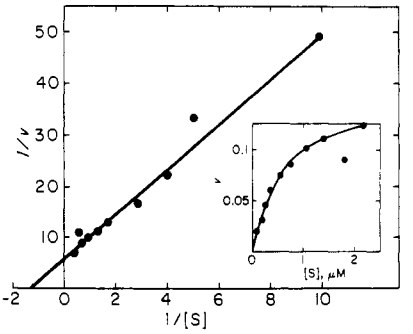

FIGURE 4: Lineweaver–Burk plot of initial velocity vs. substrate concentration. The Zn^{2+}–agarose pool 1 phosphatase was used, and ^{32}P-Tyr-CM–SC-phosphorylase was used as substrate in the indicated concentrations (see inset with original data). The assay was for 5 min, and linear dephosphorylation was obtained with all substrate concentrations. Initial velocity (v) is given in nanomoles of P_i released per minute per milligram. The K_m was calculated to be 0.8×10^{-6} M, and V_{max} was 0.17 nmol min^{-1} mg^{-1}.

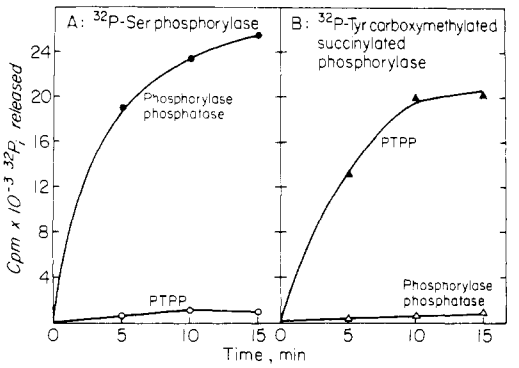

FIGURE 5: Activity of phosphotyrosyl-protein phosphatase (PTPP) and rabbit skeletal muscle phosphorylase phosphatase toward ^{32}P-Ser-phosphorylase and ^{32}P-Tyr-CM–SC-phosphorylase. (A) ^{32}P-Ser-phosphorylase (20 μM P-Ser containing 62 250 cpm; 2 mg/mL) was incubated at 30 °C with 1.7 μg of phosphorylase phosphatase (●) or with Zn^{2+}–agarose pool 1 phosphatase (○). (B) ^{32}P-Tyr-CM–SC-phosphorylase (0.25 μM P-Tyr containing 39 500 cpm) was incubated at 30 °C with 1.7 μg of phosphorylase phosphatase (△) or with Zn^{2+}–agarose pool 1 phosphatase (▲).

Kinetic Properties of Phosphotyrosyl-Protein Phosphatase. Phosphatase purified through Zn^{2+}–agarose chromatography (pool 1) was used to examine the kinetics of dephosphorylation of ^{32}P-Tyr-CM–SC-phosphorylase. The phosphatase displayed saturation kinetics (Figure 4, inset) with a K_m value of 0.8×10^{-6} M, deduced from a Lineweaver–Burk plot (Figure 4). The maximal velocity (V_{max}) of the reaction was estimated to be 0.17 nmol min^{-1} mg^{-1}.

Substrate Specificity of Phosphotyrosyl-Protein Phosphatase toward Phosphoproteins. A major problem in the elucidation of the in vivo function of various protein phosphatases is their apparent broad specificity in vitro (Lee et al., 1980). Therefore, we examined the activity of phosphotyrosyl-protein phosphatase by using both ^{32}P-Ser- and ^{32}P-Tyr-phosphorylase. Phosphorylase is labeled at a single serine residue, position 14, by phosphorylase kinase and [γ-^{32}P]ATP. This ^{32}P-Ser-phosphorylase has traditionally been the primary substrate used for the study of phosphoprotein phosphatases. Whereas a preparation of skeletal muscle protein phosphatase catalyzed the dephosphorylation of ^{32}P-Ser-phosphorylase, the phosphotyrosyl-protein phosphatase was virtually unreactive with this substrate (Figure 5A). In contrast, CM–SC-phosphorylase, phosphorylated at multiple tyrosine residues, served as an excellent substrate for phosphotyrosyl-protein phosphatase but was unreactive with the skeletal muscle

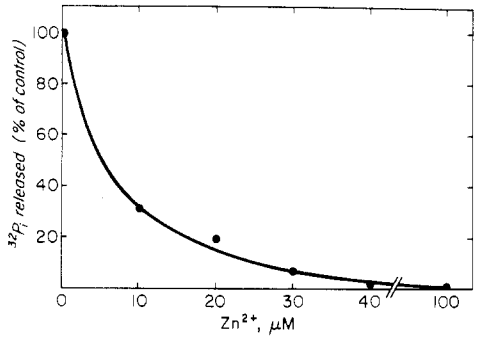

FIGURE 6: Inhibition of phosphotyrosyl-protein phosphatase by Zn^{2+}. Phosphatase, purified by DEAE-Sephadex A-50 chromatography, was preincubated for 5 min at 30 °C with the indicated concentrations of Zn^{2+}. Reactions were initiated by addition of ^{32}P-Tyr-labeled A431 membranes (0.14 μM P-Tyr containing 70000 cpm). Assay was for 10 min. In the absence of Zn^{2+}, 5900 cpm was released.

protein P-Ser phosphatase (Figure 5B).

The concentrations of P-Ser and P-Tyr in the different phosphorylase substrates were 20 and 0.25 μM, respectively. It is striking that the phosphatase does not dephosphorylate the ^{32}P-Ser-phosphorylase even at a nearly 100-fold higher substrate concentration. However, it should be pointed out that the ^{32}P-Ser-phosphorylase retained its tertiary structure whereas the ^{32}P-Tyr-CM–SC-phosphorylase was extensively modified to disrupt its tertiary structure and its net charge was drastically altered by succinylation.

Inhibition of Phosphotyrosyl-Protein Phosphatase by Micromolar Concentrations of Zn^{2+}. Dephosphorylation of the EGF receptor kinase by phosphotyrosyl-protein phosphatase in membrane vesicles from A431 human epidermoid carcinoma cells was completely inhibited by 10 μM Zn^{2+} (Brautigan et al., 1981). Since it appears that inhibition by micromolar Zn^{2+} is a property common to phosphotyrosyl-protein phosphatases, we examined the effects of Zn^{2+} on the dephosphorylation of different proteins by the EAT cell phosphatase. Membrane vesicles from A431 cells were labeled in vitro with [γ-^{32}P]ATP, previously shown to preferentially phosphorylate the EGF receptor in tyrosine residues (Ushiro & Cohen, 1980; Brautigan et al., 1981). Dephosphorylation of this ^{32}P-labeled Tyr substrate with the EAT cell phosphatase was inhibited about 50% at 5 μM Zn^{2+} and nearly completely inhibited by 40 μM Zn^{2+} (Figure 6). These results are in close agreement with previous observations (Brautigan et al., 1981; Gallis et al., 1981).

With ^{32}P-Tyr-CM–SC-phosphorylase as substrate, 50% inhibition was observed with 33 μM Zn^{2+}. This reduced sensitivity to inhibition by Zn^{2+} may be due to the chemical modification of the substrate, phosphorylase. Succinylation of the protein introduces a large number of additional carboxyl groups that will chelate Zn^{2+} and therefore lower the concentration of this ion available to interact with the phosphatase. We observed that if Zn^{2+} was added to the enzyme and the reaction initiated by mixing with ^{32}P-Tyr-CM–SC-phosphorylase substrate, sensitivity to Zn^{2+} was increased compared to reactions performed by first mixing Zn^{2+} and ^{32}P-Tyr-Cm–SC-phosphorylase, followed by addition of the phosphatase. Moreover, the dephosphorylation of ^{32}P-Tyr-casein was inhibited 80–90% with 20 μM Zn^{2+}. It should be noted that Zn^{2+}, even at concentrations as high as 100 μM, did not precipitate the substrate (data not shown). Thus, inhibition of the enzyme cannot be explained by loss of substrate.

Effect of Inhibitors on Phosphotyrosyl-Protein Phosphatase Activity. We measured the activity of the phosphatase in the presence of a variety of inhibitors in order to determine whether this was indeed a unique enzyme distinct from the known acid, alkaline, and other protein phosphatases (Table II). As reported previously (Brautigan et al., 1981), the phosphatase was not inhibited by NaF, a widely recognized inhibitor of protein phosphatases, or by EDTA, a chelator that alone or in concert with NaF or P_i potently inactivates various alkaline and phosphoprotein phosphatases. Even though high concentrations of alkaline phosphatases have been shown to dephosphorylate P-Tyr proteins (Swarup et al., 1981), tetramisole had no effect on phosphotyrosyl-protein phosphatase activity. This compound inhibits mammalian liver and *Escherichia coli* alkaline phosphatases at a concentration of 10 μM and mammalian intestinal alkaline phosphatase at 1 mM (Van Belle, 1972). Furthermore, the phosphatase was inhibited by compounds analogous to substrates such as P_i, PP_i, PNPP, Tyr, and P-Tyr. In each case, the inhibition was incomplete and required millimolar concentrations of the reagents. It appears, in particular from the relative efficacy of Tyr and P-Tyr, that the phosphoryl group is involved in substrate recognition. The phosphatase has little, if any, activity toward PNPP as judged by the lack of formation of any visible yellow-colored product in the presence of either 5 or 10 mM PNPP under the conditions of the assay. This is taken as further evidence that the phosphatase is not an acid or alkaline-type phosphatase.

Table II: Effect of Inhibitors on Phosphotyrosyl-Protein Phosphatase Activity[a]

additions	concn (mM)	activity (%)
none		100
EDTA	5	100
NaF	25	100
P_i	10	30
PP_i	5	30
PNPP	5	42
	10	21
tetramisole	1	100
tyrosine	2.5	51
phosphotyrosine	0.4	43

[a] The substrate was ^{32}P-Tyr-CM-SC-phosphorylase at a concentration of 1.8 μM. All inhibitors were preincubated with the phosphatase for 5 min at 30 °C prior to addition of substrate. The phosphatase used for these experiments was purified by DEAE-Sephadex A-50 chromatography.

pH Optimum of Phosphotyrosyl-Protein Phosphatase. Phosphatases with pH optima for activity outside of the neutral pH range can be recovered from various mammalian tissues, and there has been some speculation concerning their potential physiological functions, based on in vitro activity with both ^{32}P-Ser- and ^{32}P-Tyr-containing proteins (Li et al., 1979; Swarup et al., 1981). However, the optimum pH for the dephosphorylation of ^{32}P-Tyr-CM–SC-phosphorylase by the EAT cell phosphatase was 6.5, tested with enzyme purified by either DEAE-Sephadex (Figure 7) or Zn^{2+}–agarose (not shown) chromatography. Because substantial alteration of the pH optimum for activity can be due to the charge on the substrate, we also used the ^{32}P-labeled Tyr EGF receptor kinase of A431 membrane vesicles as substrate and found the pH optimum for this reaction to be 7.0 (not shown). These results imply that a substantial amount of the phosphotyrosyl-protein phosphatase in EAT cells is a neutral pH phosphatase.

Discussion

Phosphotyrosine-protein phosphatase represents a new type of enzyme with properties that distinguish it from the previ-

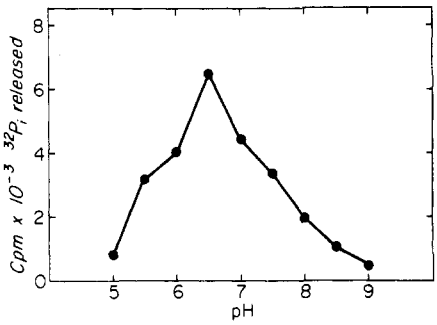

FIGURE 7: Determination of the pH optimum for dephosphorylation of ^{32}P-Tyr-CM–SC-phosphorylase (0.9 μM P-Tyr containing 59 000 cpm) in Tris/cacodylate buffers. Zn^{2+}–agarose pool 1 phosphatase was used. Reactions were for 10 min.

ously known mammalian acid, alkaline-type, and phosphoserine-protein phosphatases. The phosphatase that was partially purified from EAT cells displays many of the same characteristics we have described for the phosphatases in membrane vesicles from human epidermoid carcinoma cells and normal and RSV-transformed rat cells (Brautigan et al., 1981; Gallis et al., 1981). Most remarkable is the specific inhibition of activity by micromolar concentrations of Zn^{2+}, a property which provides the basis for the functional classification of this enzyme and its purification by using affinity chromatography on Zn^{2+} chelated to iminodiacetate–agarose. The phosphatase activity is not inhibited by EDTA or fluoride, compounds known to inhibit a variety of alkaline and other protein phosphatases. The phosphatase shows a marked specificity for P-Tyr residues with a K_m of 1 μM and does not react with P-Ser in phosphorylase *a*, even at an 80-fold higher concentration of the substrate. Compounds analogous to substrates such as P_i, PP_i, PNPP, tyrosine, and P-Tyr inhibit the phosphatase activity. Phosphatases with similar properties have also been identified in extracts of rat liver and muscle (Foulkes et al., 1981) and of EAT cells (Martensen, 1982). The phosphatase in larvae of *Drosophila melanogaster* which hydrolyzes L-Tyr-PO_4 and is inhibited by 5 mM Zn^{2+} and fluoride but not by EDTA (Fukami & Lipmann, 1982) may be a related enzyme. The distribution of phosphotyrosyl-protein phosphatases in these tissues and cell types, together with its neutral pH optimum for activity and insensitivity to tetramisole, a potent inhibitor of *E. coli* and mammalian alkaline phosphatases, makes it unlikely that alkaline phosphatases are primarily responsible for the hydrolysis of P-Tyr proteins in vivo as suggested by Swarup et al. (1981).

Preparation of a suitable substrate for dephosphorylation of protein-bound P-Tyr posed an initial obstacle to the investigation of this enzyme. Several requirements for the substrate needed to be fulfilled including the following: (1) availability in relatively large amounts; (2) solubility at neutral pH and at high concentration; (3) accessible Tyr residues for reaction with EGF receptor kinase; (4) insolubility in trichloroacetic acid. Skeletal muscle phosphorylase, modified by reduction, carboxymethylation, and succinylation, was found superior to casein and to other proteins for use as a phosphatase substrate. However, this protein is phosphorylated at multiple residues, each of which is modified to a very small extent, and the protein is denatured. This must be taken into account when extrapolating from these results to the properties of the enzyme in vivo.

Even though largely a single molecular size species of phosphatase was detected in cellular extracts, there may well be multiple phosphotyrosyl-protein phosphatases present in these cells. More than half of the phosphatase was not retained by DEAE-Sephadex A-50, and the enzyme that was bound was occasionally resolved into two peaks of activity. Further evidence for different forms of the phosphatase comes from the separation of the enzyme into two fractions by stepwise elution of Zn^{2+}–agarose with 20 and 60 mM histidine. Whether these results are due to the presence of different phosphatases or result from variable modification of a single protein remains to be determined.

From previous work (Brautigan et al., 1981; Gallis et al., 1981), we know that phosphotyrosyl-protein phosphatase dephosphorylates the EGF receptor kinase, as well as phosphotyrosine-containing proteins of M_r 36 000, 50 000, and 68 000 in RSV-transformed rat cells and a protein of M_r 36 000 in normal rat cell membrane vesicles. The M_r = 50 000 protein may be the same as a phosphotyrosine-containing protein of identical molecular weight found complexed with pp60^{src} in immunoprecipitates and lysates of RSV-transformed cells (Hunter & Sefton, 1980; Brugge et al., 1981; Oppermann et al., 1981). Another phosphotyrosine-containing protein (M_r 36 000) found in both uninfected and RSV-transformed cells can undergo rapid changes in its state of phosphorylation as a function of the temperature in cells infected with RSV coding for a temperature-sensitive transforming protein (Radke & Martin, 1979; Radke et al., 1980; Erikson & Erikson, 1980). A homologous protein (M_r 36 000) is phosphorylated in response to EGF in A431 cells (Hunter & Cooper, 1981; Cooper & Hunter, 1981). Thus, phosphotyrosine-containing proteins similar in molecular weight to those which serve as substrates for the Zn^{2+}-inhibited phosphotyrosyl-protein phosphatase are more highly phosphorylated in RSV-transformed cells or when EGF is added to cells. This suggests that both tyrosyl protein kinase and phosphatase activities play roles in determining the level of tyrosine phosphorylation in these proteins.

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