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IDENTIFICATION AND CHARACTERIZATION OF Mg^{2+}-DEPENDENT PHOSPHOTYROSYL PROTEIN PHOSPHATASE FROM RAT LIVER CYTOSOL

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Although highly purified preparations of Mg^{2+}-dependent phosphoseryl protein phosphatase (also designated phosphatase IA or phosphatase 2C) dephosphorylated phosphotyrosyl histone, the activity has been resolved from phosphatase IA by polyacrylamide gel electrophoresis at pH 9.5. This novel phosphotyrosyl-specific protein phosphatase absolutely requires Mg^{2+} or Mn^{2+} for activity, is inhibited by Zn^{2+}, vanadate and fluoride, and has an optimal pH of 9.0 and M_r = 50,000. Certain properties of this phosphatase so closely resemble those of phosphatase IA that the two enzymes tend to be copurified through various separation procedures.

Since the identification of $pp60^{src}$ as a tyrosyl protein kinase (1), attention has been focused on the intracellular phosphorylation/dephosphorylation system involving the tyrosyl residues of proteins. A considerable number of tyrosyl protein kinases (2) and PTPPs (3-13) from various tissues and cells have been reported. But the phosphoamino acid specificity of protein phosphatases is not so clarified as the amino acid specificity of protein kinases: the PTPPs thus far reported are not pure enough to give a convincing answer to this question, and no thorough investigation of the purified phosphoseryl (and phosphothreonyl) protein phosphatases has been performed in terms of their capacity for dephosphorylating the phosphotyrosine residues of proteins except for calcineurine, which was found to possess a potent PTPP activity even after purification to electrophoretic homogeneity (14). In this context, Li and coworkers (9,15,16) have reported that their Mg^{2+}-dependent phosphoseryl protein phosphatase, also called phosphatase IA (17,18) or phosphatase 2C (19), is capable of dephosphorylating the phosphotyrosine residues of proetins, although the purity of the phosphatase studied is not clear.

Phosphatase IA was first identified, purified and characterized by us from rat liver cytosol as a protein phosphatase acting on glycogen synthase (17,18).

Abbreviation: PTPP, phosphotyrosyl protein phosphatase.

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We therefore were interested in determining whether or not our highly purified phosphatase IA possesses PTPP activity. In this paper, we report that a novel Mg^{2+}-dependent PTPP was copurified with phosphatase IA but has been resolved from phosphatase IA by polyacrylamide gel electrophoresis. The results also establish that phosphatase IA itself does not act on the phosphotyrosine residues of proteins.

MATERIALS AND METHODS

Materials: Histone f2b and [γ-^{32}P]ATP were the products of Cooper Biochemical and New England Nuclear, respectively. The sources of other materials were described elsewhere (18,20,21).

Purification of phosphatase IA: Phosphatase IA was purified from rat liver by the previously described 7-step procedure (18).

Preparation of substrates: [^{32}P]Phosphoseryl histone was prepared as described previously (22). [^{32}P]Phosphotyrosyl histone was prepared as follows. Insulin receptor partially purified from rat epididymal fat pad as described previously (23 but up to the wheat germ agglutinin-Sepharose step) was kept at 4°C for 30 min in the presence of 10 mU/ml insulin, 0.01% (w/v) bovine serum albumin and 25 mM HEPES (pH 7.6); the whole mixture was then incubated at 25°C for 3 min in the presence of 80 μM [γ-^{32}P]ATP (2.8 Ci/nmol); after adding histone f2b to 10 mg/ml, the incubation was continued at 25°C for 4 h. The reaction was terminated by adding trichloroacetic acid to 30% (w/v) and the precipitated histone was collected by centrifugation. It was then washed as described by Meisler and Langan (24), dissolved in 10 mM Tris-HCl (pH 7.4), dialyzed against the same buffer and stored at -40°C until use. Under these conditions, more than 95% of the phosphate incorporated into histone f2b was on the tyrosine residues.

Assay of protein phosphatase activity: Phosphatase IA was assayed by measuring the release of ^{32}P from [^{32}P]Phosphoseryl histone in the presence of 5 mM $MgCl_2$. The detailed procedure was already described (22). One unit of the enzyme was defined as the amount which catalyzed the release of 1 nmol of phosphate in 1 min. The standard assay mixture for PTPP activity contained 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 μM [^{32}P]phosphotyrosyl histone and enzyme (60 μl). The release of [^{32}P]phosphate was measured as described previously (22). One unit of the enzyme was defined as the amount which was required for the release of 1 pmol of phosphate in 1 min.

RESULTS AND DISCUSSION

Li and coworkers (9,15,16) reported that their Mg^{2+}-dependent protein phosphatase corresponding to our phosphatase IA (17,18) was capable of dephosphorylating the phosphotyrosine residues of proteins. In our hands also, phosphatase IA purified from rat liver by the previously described 7-step procedure (18) was found to dephosphorylate [^{32}P]phosphotyrosyl histone in the presence of 5 mM Mg^{2+}. When subjected to polyacrylamide gel electrophoresis at pH 9.5, the preparation gave one major and one minor protein band with Rf values of 0.60 and 0.55, respectively (Fig. 1, top), confirming the previous observation (18). The previous paper (18) has also shown that the major Rf 0.60 band represents the intact and active form of phosphatase IA. A duplicate, unstained gel was sliced into thin segments, which were then extracted and assayed. Fig. 1 (lower part) shows that PTPP activity as revealed with phospho-

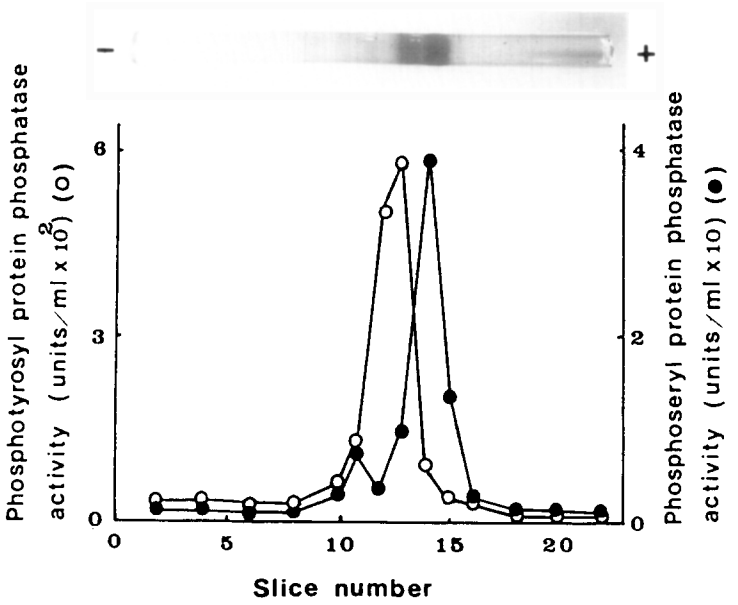

Fig. 1. Polyacrylamide gel electrophoresis of purified phosphatase IA. Concentrated step-7 enzyme (10 μg) was electrophoresed in duplicate on 7% polyacrylamide gel (8.8 cm in length) at pH 9.5 using the buffer system of Davis (25). A current of 2 mA/tube was applied for 2.5 h. One gel was stained with 0.125% Coomassie blue (top). The second gel was sliced into 4-mm segments, each of which was extracted with 0.25 ml of buffer A (10 mM Tris-HCl, pH 7.4, 5 mM mercaptoethanol, 2%, v/v, glycerol, 0.1 mM EDTA and 0.05 M NaCl) containing 5 mM $MgCl_2$ for 30 min at 30°C and assayed.

tyrosyl histone as substrate forms a sharp single peak and is distinct from a peak active to phosphoseryl histone, which corresponds to the major Rf 0.60 band and is phosphatase IA. Although the PTPP appears to comigrate with the Rf 0.55 protein, the latter protein has been identified as a modified and inactivated form of phosphatase IA (18).

In order to elucidate the relationship of the Mg^{2+}-dependent PTPP activity to phosphatase IA further, the elution profiles of the two activities were compared at each chromatographic step in the course of the 7-step purification of phosphatase IA (18). Although one unique feature of phosphatase IA was an acceleration of its elution from DEAE-cellulose by Mg^{2+} (18), the bulk of the PTPP activity coeluting with phosphatase IA at step 4 (Fig.2A) also underwent this accelaration (Fig. 2B). At the histone-Sepharose-4B step (step 6) too, PTPP activity largely coeluted with phosphatase IA (Fig. 2C). When the step-6 enzyme was further chromatographed on Sephadex G-100 (step 7), however, phosphatase IA eluted with a M_r value of 40,000 while PTPP activity emerged slightly earlier (M_r = 50,000) (Fig. 3, upper part). The active fractions were pooled into three portions, A, B and C as shown in Fig. 3. Each portion was then concentrated and separately subjected to polyacrylamide gel electrophoresis at pH 9.5. Fig. 3 shows that the Rf 0.55 band is more intense in portion C than

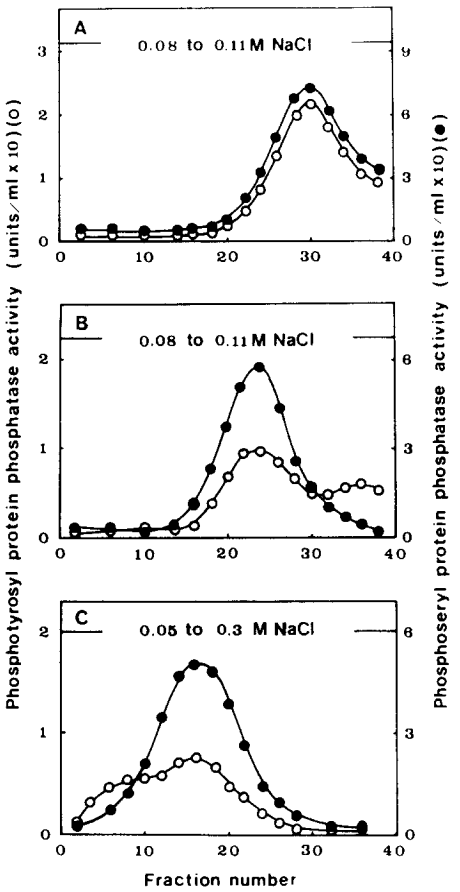

Fig. 2. PTPP activity in the course of phosphatase IA purification. A: Step-3 enzyme (from 370 g rat liver) (18) was applied to a DEAE-cellulose column (2.5 X 10 cm) previously equilibrated with buffer A and eluted with a linear 0.08-0.11 M NaCl gradient in 800 ml of buffer A; fractions were collected in 20 ml and assayed. B: Fractions 25-35 from A were pooled and adjusted to 5 mM $MgCl_2$; chromatography and assay were performed as described for A except that buffer A was substituted by buffer A containing 5 mM $MgCl_2$. C: Fractions 20-28 from B were pooled, applied to a histone-Sepharose-4B column (1.5 X 4 cm) and eluted with a linear 200 ml 0.05-0.3 M NaCl gradient using buffer A containing 5 mM $MgCl_2$; fractions were collected in 5 ml and assayed.

in A while PTPP activity is much higher in portion A than in C. These results argue against the possibility that the modified and inactivated form of phosphatase IA exhibits PTPP activity.

The PTPP freed from phosphatase IA by gel electrophoresis was found to have an absolute requirements for Mg^{2+} or Mn^{2+} for activity, the maximal activity being attained at 2.0 and 0.2 mM, respectively (Fig. 4); it was inhibited 100, 40 and 100% by 0.1 mM Zn^{2+}, 0.1 mM vanadate and 40 mM fluoride, respectively; and it exhibited an optimal pH of 9.0. The enzyme was inactive not only toward phosphoseryl histone but also toward glycogen synthase D and phosphorylase a thereby considered to be highly specific for the phosphotyrosine residues of proteins.

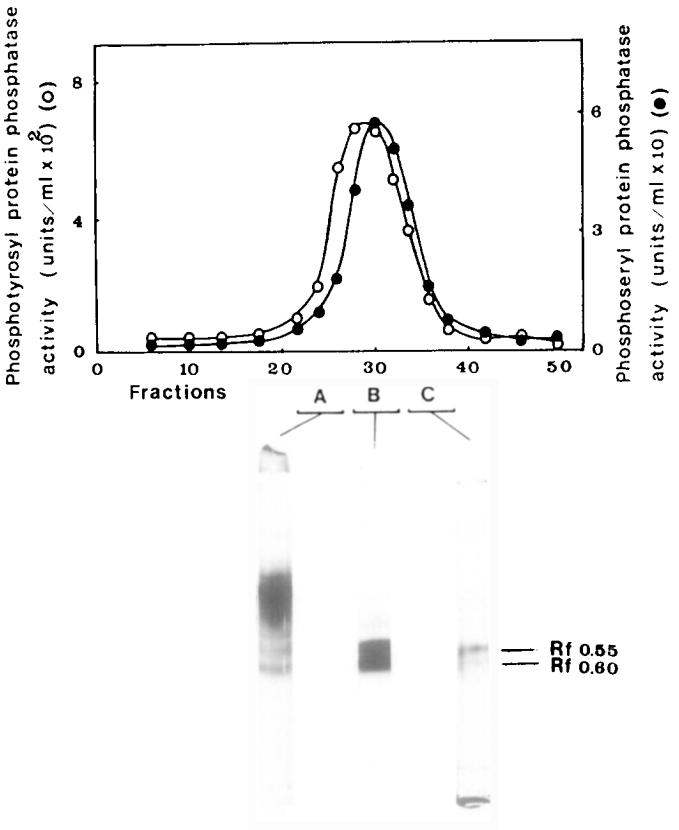

Fig. 3. Chromatography on Sephadex G-100 followed by polyacrylamide gel electrophoresis of phosphatase IA. Fractions 11-22 from Fig. 2C were pooled, concentrated to 3 ml using Amicon YM-10 membrane, applied to a Sephadex G-100 column (2.5 X 60 cm) previously equilibrated with 10 mM Tris-HCl (pH 7.4) containing 5 mM mercaptoethanol, 2% glycerol, 0.1 mM EDTA, 0.1 M NaCl and 5 mM $MgCl_2$ and eluted with the same buffer. The eluate was collected in 6 ml and assayed (upper part). The column had been calibrated with molecular weight markers (18). The active fractions were then combined to give A, B and C. Each portion was concentrated to 0.1 ml and its 30 µl was electrophoresed and stained as described for Fig. 1 except that the gel length was 5 cm (lower part).

It should be noted that although phosphatase IA had been freed from the major alkaline phosphatase activity at early steps of its purification (22), the PTPP described here exhibited a weak *p*-nitrophenylphosphatase activity with an optimal pH of 9.0.

These properties are closely similar to those described by Li *et al.* (15) for the PTPP activity of phosphatase IA. The present work, however, clearly demonstrates that the PTPP activity of highly purified phosphatase IA is due to a Mg^{2+}-dependent PTPP, which has been copurified with phosphatase IA through various separation procedures because of their similarities in certain properties. This PTPP appears to be a novel PTPP since it differs from any of the PTPPs so far reported (3-13).

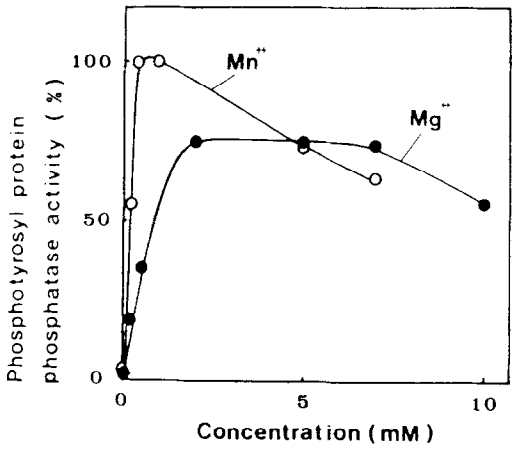

Fig. 4. Effects of Mg^{2+} and Mn^{2+} concentrations on the activity of PTPP freed from phosphatase IA by gel electrophoresis. Gel segment 12 in Fig. 1 was used.

In the present studies, insulin-receptor kinase was employed for the first time to prepare PTPP-substrate. Using this substrate, we have been able to identify, in rat liver homogenate, several forms of PTPP besides the one described in the present paper (data not shown). The ease of preparation of insulin receptor compared to that of other tyrosine-specific protein kinases would offer an advantage to the investigation of PTPPs.

ACKNOWLEDGEMENTS

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