Phosphotyrosyl-protein Phosphatase

SPECIFIC INHIBITION BY Zn^{2+}**

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David L. Brautigan*, Paul Bornstein, and Byron Gallis

From the Departments of Biochemistry and Medicine, University of Washington, School of Medicine, Seattle, Washington 98195


We show that the reverse reaction, catalyzed by a phosphotyrosyl-protein phosphatase associated with the membrane, is inhibited by Zn^{2+}. Dephosphorylation of phosphotyrosyl residues in the M, = 150,000 protein is completely inhibited by Zn^{2+} at concentrations as low as 10 µM, whereas other divalent cations have no substantial effect. Inhibition of the phosphatase was reversed by EDTA and the activity in membrane preparations was increased by EDTA or fluoride, agents commonly thought to be phosphatase inhibitors. Acid hydrolysis of the membrane proteins followed by analysis of phosphoamino acids by two-dimensional electrophoresis revealed that the phosphatase hydrolyzed phosphotyrosyl in preference to phosphoseryl residues. The specific inhibition of this phosphatase activity by low concentrations of Zn^{2+} may be indicative of the physiological importance of Zn^{2+} in the regulation of cellular phosphotyrosyl-protein levels.

Cohen and his colleagues (8-11) have shown that the mitogenic peptide epidermal growth factor stimulates specific phosphorylation of tyrosyl residues in a M, = 150,000 protein in membrane vesicles from human epidermoid carcinoma A-431 cells. The M, = 150,000 protein is rapidly dephosphorylated in vitro following labeling with [γ-32P]ATP by an enzyme present in the membrane vesicles (8). We examined the effects of various divalent cations on the phosphatase activity in these reaction mixtures because Mn^{2+} is known to specifically activate a skeletal muscle protein phosphatase (12). We found that Zn^{2+} concentrations as low as 10 µM were effective in completely inhibiting the activity of a phosphatase that preferentially hydrolyzed phosphotyrosyl residues and that the inhibition was reversed by addition of metal chelators. These properties distinguish this enzyme from other phosphoprotein phosphatases.

EXPERIMENTAL PROCEDURES

Human epidermoid carcinoma A-431 cells were a gift from Dr. Joseph De Larco and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum. EGF was purchased from Collaborative Research. Phosphothreonine and phosphoserine were purchased from Sigma Chemical Co. Phosphotyrosine was a gift from Dr. Linda Pike. [γ-32P]ATP (3000 Ci/mmol) was purchased from New England Nuclear.

Preparation of Membrane Vesicles—Membrane vesicles were prepared by a modification of the method of Carpenter et al. (8). Human A-431 cells were grown to confluency on 150-mm plates, washed twice with ice-cold PBS, scraped from the plate, and centrifuged for 5 min at 4000 × g in an HB-4 rotor. All subsequent procedures were carried out with samples maintained at 0 °C in an ice bath. Cells were resuspended in 2 volumes of 0.05 M sodium borate, pH 7.2, 0.15 M NaCl, 1 mM MgCl₂, 1 mM CaCl₂ and processed as previously described (8). The majority of nuclei were pelleted by centrifugation at 1000 × g for 45 s in the HB-4 rotor. The supernatants were removed and centrifuged for 30 min at 20,000 × g to pellet mitochondria and plasma membrane vesicles. The pellet was resuspended in PBS and layered on top of PBS containing 35% sucrose. The membranes were floated on the 35% sucrose by centrifugation at 40,000 × g for 45 min in an SW27 rotor, removed from the top of the sucrose layer, diluted 3-fold with PBS, and centrifuged for 30 min at 75,000 × g in a type 40 rotor. The pellet was resuspended at 3-4 mg of protein/ml in 20 mM Hepes, pH 7.4, quick-frozen, and stored in aliquots in liquid N₂. Kinase and phosphatase activities in the preparation were stable to repeated freezing and thawing.

Phosphorylation Reaction—Phosphorylation reactions were carried out as described by Carpenter et al. (8), modified to use 0.1 µM ATP and detergent-containing buffer. The reaction (100 µl) contained A-431 membrane vesicles (10 µg of protein), 100 ng of EGF, 0.2% NP-40, 0.1 mg/ml of bovine serum albumin, 2 mM manganese acetate, and 0.1 µM [γ-32P]ATP in 20 mM Hepes, pH 7.4. Reactions were carried out for 5 min in an ice bath to maintain the temperature at 0 °C. Reactions were monitored by removing 10-µl aliquots that were spotted on 1.5-cm squares of Whatman No. 50 paper. The filter paper was dropped into ice-cold 10% trichloroacetic acid containing 0.01 M sodium pyrophosphate and washed as described (8). For polyacrylamide gel analysis, reactions were terminated by boiling for 3 min in an equal volume of SDS-sample buffer (13) containing 5 mM dithiothreitol.

SDS-Polyacrylamide Gel Electrophoresis—Ten per cent SDS-polyacrylamide slab gels were run according to the method of Laemmli (13). Gels were stained, destained, and dried as previously described (14).

Hydrolysis and Two-dimensional Electrophoresis of 32P-labeled Membrane Vesicle Proteins—Reactions containing phosphorylated membrane proteins were digested for 10 min at 30 °C by addition of 5 µl of 175 µM trypsin. The reactions were taken to dryness under a stream of N₂ in test tubes (6 × 50
mm) (Corning), sealed under vacuum into a large ampule with 1 ml of 6 N HCl and heated in a 110 °C oven for 2 h. Cellulose thin layer plates (Brinkmann, 20 × 20 cm) were spotted with 1-5 μl of reaction mixture dissolved in pH 1.9 electrophoresis buffer and 0.5 μl each of neutralized phosphoserine, phosphothreonine, and phosphotyrosine at 20 mg/ml. Electrophoresis in two dimensions was performed for 75 min at pH 1.9 and then at pH 3.5 for 60 min as described by Hunter and Soskin (1). Following electrophoresis, the thin layer plates were dried and stained with buffered ninhydrin (15) containing tungstic acid (Pierce Chemical Co.) to locate the position of the phosphoamino acids. Phosphotyrosine gave a distinct gray color when stained with an associated protein phosphatase, even in the presence of 10 μM Zn²⁺ (Fig. 2) because the rapid removal of phosphate is prevented while the kinase is still active. Zn²⁺ does not activate a kinase, because in the absence of Mn²⁺, there is no incorporation of labeled phosphate into protein (data not shown).

Following incubation with EGF and MnATP to phosphorylate the membranes, we found that a 10-fold or greater dilution of samples in detergent-containing buffers was also effective in preventing the dephosphorylation reaction. This probably indicates that the M₅₀, 150,000 protein substrate is no longer accessible to the phosphatase following dilution.

**Effects of Divalent Cations, EDTA, and Fluoride Ions on Phosphotyrosyl-protein Phosphatase Activity**—Zinc ions specifically inhibit the phosphotyrosyl-protein phosphatase activity in a reversible manner. Membrane vesicles from A-431 cells were phosphorylated for 5 min at 0 °C in the presence of EGF (Fig. 1, lane B). Individual reactions were then made 0.1 mM with Zn²⁺, Mn²⁺, Mg²⁺, Ca²⁺, or Co²⁺ by addition of stock solutions so that the total volume was increased by 1% or less.

Following incubation for 60 min at 30 °C to allow dephosphorylation by the membrane-associated phosphatase, duplicate samples of each reaction were spotted on filter paper, washed in trichloroacetic acid, and counted. As shown in Fig. 2, decay of ³²P from A-431 membrane vesicles in the presence and absence of Zn²⁺. Reactions containing ³²P-labeled M₅₀, 150,000 protein, as shown in Fig. 1, Lane B, were incubated for 30 min at 30 °C in the absence (○) or in the presence (■) of 10 μM Zn²⁺. Duplicate 10-μl aliquots were removed and precipitated on filter paper at the times indicated.

**Table I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>[³²P]Phosphate in membrane protein</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1,800</td>
</tr>
<tr>
<td>+0.1 mM Zn²⁺</td>
<td>20,550</td>
</tr>
<tr>
<td>+0.1 mM Mn²⁺</td>
<td>3,500</td>
</tr>
<tr>
<td>+0.1 mM Mg²⁺</td>
<td>2,900</td>
</tr>
<tr>
<td>+0.1 mM Ca²⁺</td>
<td>3,500</td>
</tr>
<tr>
<td>+0.1 mM Co²⁺</td>
<td>5,300</td>
</tr>
<tr>
<td>+10 mM F⁻</td>
<td>1,550</td>
</tr>
<tr>
<td>+5 mM EDTA</td>
<td>600</td>
</tr>
<tr>
<td>+0.1 mM Zn²⁺, 1 h; add EDTA, 1 h</td>
<td>3,500</td>
</tr>
<tr>
<td>+0.1 mM Zn²⁺, 2 h</td>
<td>14,500</td>
</tr>
</tbody>
</table>

Human A-431 membrane vesicles were phosphorylated in the presence of EGF for 5 min at 0 °C as described under “Experimental Procedures.” Reactions were pooled and redistributed so that each initial sample contained 19,150 cpm of protein-bound [³²P]phosphate/10 μl. To measure the phosphatase activity, samples with the indicated additions were incubated for 60 min at 30 °C. Protein-bound radioactivity was determined by precipitation of duplicate 10-μl aliquots onto filter paper. Following a 60-min incubation in the presence of Zn²⁺, EDTA was added to one sample while Zn²⁺ remained in another during a second 60-min incubation at 30 °C.

**Fig. 1.** Autoradiograms of an SDS-polyacrylamide gel of human A-431 membrane vesicles. Vesicles were incubated for 5 min on ice with [γ-³²P]ATP in the absence (Lane A) or in the presence (Lane B) of EGF. EGF-stimulated reactions in Lanes C and D were incubated for 30 min at 30 °C in the presence and absence of Zn²⁺, respectively. Equal aliquots of each reaction were removed, boiled in Laemmli buffer containing SDS and reducing agent, and then subjected to electrophoresis on a 10% polyacrylamide slab gel. The gel was stained with Coomassie blue R-250, destained, and dried, and autoradiography was done for about 1 h.
Table I, Zn\(^{2+}\) entirely inhibited the release of phosphate from the \(M_0 = 150,000\) protein. The inhibition by Zn\(^{2+}\) is specific; other divalent cations had little effect. The addition of Co\(^{2+}\) mimicked the effect only to a limited extent, even at a 10-fold higher relative concentration (compare Fig. 2 and Table I). Phospholipids of the membrane vesicles and serum albumin in the buffer are both expected to bind Zn\(^{2+}\), hence lowering the effective Zn\(^{2+}\) concentration. For this reason, and because accurate determination of the unbound ion would prove difficult, the values we cite represent only the upper limits of the actual free Zn\(^{2+}\) concentration. Characterization of the isolated phosphatase will include a more complete concentration dependence of Zn\(^{2+}\) inhibition.

The phosphatase is activated by addition of chelators such as EDTA (Table I). Fluoride, commonly thought to be a true free Zn\(^{2+}\) concentration. Characterization of the isolated enzyme will include a more complete concentration dependence of Zn\(^{2+}\) inhibition.

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**Effects of Zn\(^{2+}\) on the Phosphoamino Acid Content of Human A-431 Membrane Vesicles**—We found that the Zn\(^{2+}\)-inhibited phosphatase preferentially hydrolyzed phosphotyrosyl residues. Membrane vesicles were phosphorylated and the reaction was split into equal portions; half was incubated for 60 min at 30 °C with 10 μM Zn\(^{2+}\) and the other half was incubated without Zn\(^{2+}\). After digestion with trypsin and partial acid hydrolysis, the phosphoamino acids were resolved by two-dimensional electrophoresis. Autoradiograms of the thin layer plates reveal the distribution of [\(^{32}\)P]phosphoamino acids (Fig. 3). About 90% of the label present in amino acids was present as phosphotyrosine with the remainder in phosphoserine (see below), proportions that agree with the results of Ushiro and Cohen (11). Thus, Zn\(^{2+}\)-inhibition of phosphate release from the \(M_0 = 150,000\) protein of membrane vesicles involved a phosphotyrosyl-protein phosphatase.

To determine the relative specificity of the Zn\(^{2+}\)-inhibited phosphatase, the ninhydrin-positive areas, demarcated by dotted lines in Fig. 3, were scraped from the plates and counted in scintillation fluid. The phosphotyrosine content was 6.0-fold lower in control samples than in those containing Zn\(^{2+}\) (1229 versus 6809 cpm) whereas phosphoserine content decreased by only 1.5-fold (442 versus 714 cpm). This evidence indicates that the phosphatase preferentially hydrolyzed phosphotyrosyl residues, even though, like other protein phosphatases, it may use other substrates.

**DISCUSSION**

We have corroborated the results of Cohen and co-workers (8–11), showing that EGF stimulates phosphorylation of tyrosyl residues in a \(M_0 = 150,000\) protein in membrane vesicles from human A-431 epidermoid carcinoma cells. Rapid dephosphorylation occurs by the action of a membrane-associated phosphotyrosyl-protein phosphatase that is specifically inhibited by Zn\(^{2+}\), activated by EDTA, and not inhibited by fluoride. These characteristics distinguish this enzyme from other phosphatases. Alkaline phosphatase from Escherichia coli requires Zn\(^{2+}\) as an essential cofactor (16). Phosphoprotein phosphatases are in several cases activated by divalent cations, especially Mn\(^{2+}\) or Co\(^{2+}\) (12, 17–20). Weak inhibition of protein phosphatases has been observed with Zn\(^{2+}\) (19, 21–23). However, these effects were not always specific for Zn\(^{2+}\) and required a 10- to 500-fold higher concentration of Zn\(^{2+}\) than that which fully inhibits the phosphotyrosyl-protein phosphatase. Metal chelators such as EDTA, either alone or in combination with fluoride or pyrophosphoryl ions, cause inactivation of E. coli alkaline phosphatase and some protein phosphatases (15, 19–23), in contrast to the activation we observe. Fluoride, perhaps the best known and most commonly used phosphatase inhibitor, had a negligible effect on the dephosphorylation of human A-431 membrane vesicle proteins and at higher concentrations caused a slight increase in phosphatase activity.

Inhibition by Zn\(^{2+}\) may result from interaction either with the substrate protein, rendering it unreactive, or with the phosphatase itself. Addition of Zn\(^{2+}\) preferentially blocked dephosphorylation of phosphotyrosyl residues in the \(M_0 = 150,000\) membrane protein. However, the effect is not specific for this substrate because the dephosphorylation of phosphotyrosyl-proteins of \(M_0 = 67,000, 50,000,\) and 37,000 in rat fibroblasts is also inhibited by micromolar Zn\(^{2+}\). Although it is possible that in each case the presence of the phosphorylated amino acid provides a specific, high affinity site for the binding of Zn\(^{2+}\), it seems more likely that Zn\(^{2+}\) interacts with the phosphatase. Since 10 μM Zn\(^{2+}\) completely blocks dephosphorylation of phosphotyrosyl-proteins in membrane vesicles from human carcinoma and Rous sarcoma virus transformed rat cells, the phosphatases in these cells appear to be closely related. Thus, Zn\(^{2+}\) may be a physiologically important inhibitor of the phosphotyrosyl-protein phosphatases and thereby play a role in the elevation of cellular phosphotyrosine levels during stimulation by EGF or transformation by RNA tumor viruses.

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**REFERENCES**


Zinc Inhibition of Phosphotyrosine Phosphatase