Specificity of Protein Phosphotyrosine Phosphatases

COMPARISON WITH MAMMALIAN ALKALINE PHOSPHATASE USING POLYPEPTIDE SUBSTRATES*

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The specificity of cytosolic protein phosphotyrosine (PPT) phosphatases was investigated using different peptides and proteins that were phosphorylated on tyrosine residues by the EGF receptor kinase. The acidic phosphoproteins, serum albumin, casein, and myosin light chains, were dephosphorylated by the PPT phosphatases with apparent K m values of 1.2 to 12.5 μM and apparent velocities of 0.2 to 18 μmol/min/mg. In contrast, [Tyr(P)]histone and the phosphotyrosine peptides 

\[ \text{Val}^5 \text{angiotensin} \]

and RR-src, a peptide with sequence Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, were unreactive with the PPT phosphatases. However, each of these unreactive phosphopolypeptides was dephosphorylated under the same conditions by calf-intestine alkaline phosphatase. The data reveal how PPT phosphatase activity has been ascribed to different cellular enzymes. When acidic phosphotyrosine proteins were used as substrates in assays for PPT phosphatase activity the cytosolic enzymes were isolated, whereas when phosphotyrosine histones were used as substrates only the membrane-bound alkaline phosphatase was detected. Apparently the protein tyrosine kinase and the protein tyrosine phosphatases do not have the same specificity, so substrates such as histone, angiotensin, or RR-src are phosphorylated but not hydrolyzed. Therefore, these polypeptides would be ideal for the characterization of protein tyrosine kinases in cellular extracts.

Reversible phosphorylation of cellular proteins on tyrosine residues is believed to play a key role in the transformation of cells by a variety of oncogenes, in the action of growth factors, and in the action of insulin (for reviews see Refs. 1–3). The transforming proteins and the growth factor receptors display protein tyrosine kinase activity. These kinases act as their own substrates and thus can be labeled on tyrosine residues using [γ-32P]ATP. Analysis of the sequence of amino acids adjacent to the phosphorylated tyrosines revealed that two or three acidic residues typically preceded the tyrosine (4). A variety of synthetic peptides, including analogues of angiotensin, have been used to investigate the role primary structure plays in substrate recognition by the kinases (5–8).

Recently, this laboratory has purified two distinct forms of protein phosphotyrosine (PPT'); phosphatase from rabbit kidney (9). In this study we compared the reactivity of different Tyr(P) proteins and Tyr(P) peptides with the phosphatases to determine whether or not their specificity paralleled that of the kinases. We found that PPT phosphatases catalyzed the dephosphorylation of the acidic proteins serum albumin, casein, and myosin light chains from smooth muscle. In contrast, the basic protein histone and the peptides [Val P] angiotensin II and RR-src were not dephosphorylated by the PPT phosphatases but were dephosphorylated by mammalian alkaline phosphatase. Thus, our results show that experiments using Tyr(P) histone or Tyr(P) peptides as substrate would be expected to measure only the activity of alkaline phosphatase, not PPT phosphatases.

**EXPERIMENTAL PROCEDURES**

**Materials**

Supplies were purchased from the indicated companies as follows: ATP, 2-mercaptoethanol, epidermal growth factor, phenylmethylsulfonfonic acid, HEPES, MES, Tris, iodoacetamide, histone IIA, and bovine serum albumin fraction V (Sigma); acetic acid, trichloroacetic acid, MnCl 2, orthovanadate, acetonitrile, trifluoroacetic acid, and casein (Fishers); MgCl 2, ZnCl 2, and NH 4 HCO 3 (Mallinkrodt); Staphylococcus aureus V8 protease and 6 n HCl (Pierce); [Val P] angiotensin II (Vega Biochemical); 1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (Millipore); P 81 phosphocellulose paper (Whatman); [γ-32P]ATP, 2900 Ci/mmol (New England Nuclear). Scintillation fluid was prepared according to Brautigan et al. (20), except that pseudocumene (Fisher) was substituted for xylene. Myosin light chain and RR-src were generous gifts from the laboratory of Dr. Edwin G. Krebs, Howard Hughes Medical Institute, Seattle, WA.

**Methods**

Preparation of Tyr(P) Proteins—Bovine serum albumin was reduced and alkylated by the method of Schirmer and Brautigan (9). Casein was dissolved in dilute alkaline solution and precipitated by the addition of 100% trichloroacetic acid solution to a final concentration of 10%. The precipitated casein was collected by centrifugation at 10,000 x for 15 min, resuspended in 100 mM ammonium, and dialyzed exhaustively against the same solution before lyophilization. Smooth muscle myosin from chicken gizzards was purified by the method of Sobieszek (10). Myosin light chains were prepared using a modified version of the method of Holt and Lowey (11). Briefly, crude myosin was incubated overnight in a buffer containing 5 mM guanidinium, 2 mM EDTA, 0.3 M KCl, 2 mM diithiothreitol, and 50 mM Tris/Cl.HCl, pH 7.5. An equal volume of cold distilled water was added and the mixture was allowed to stand for 30 min. This step precipitated the myosin heavy chains which were pelleted by centrifugation at 10,000 x for 15 min. The soluble myosin light chains were precipitated by slowly adding cold ethanol to the supernatant to a final concentration of 60%. Mixed M r = 16,000 and 20,000 myosin light chains were resolved from the myosin light chain fraction by gel filtration on Sephadex G-100 in the presence of 5 mM guanidinium HCl.

Alkylated bovine serum albumin, acid-insoluble casein, myosin mixed light chains, and histone IIA were phosphorylated on tyrosine residues by calf-intestine alkaline phosphatase. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PPT, protein phosphotyrosine; EGF, epidermal growth factor; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; p-NPP, para-nitrophenylphosphate; Ser(P), phosphoserine; Tyr(P), phosphotyrosine; RR-src, peptide with sequence Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly; [Val P] angiotensin II, Asp-Arg-Val-Tyr-Val-His-Pro-Phe.
Dephosphorylation of Tyr(P) Polyptides

Phosphoprotein was determined by spotting 2-μl aliquots in triplicate onto squares of Whatman No. 50 paper and counting them in a Packard Model 300C Tri-Carb scintillation counter. The amount of 32P incorporated into proteins was calculated from the specific radioactivity of a 10-min solution of [32P]phosphate. Dephosphorylation of Peptide Substrates — The peptides [Val]angiotensin II and RR-arc were phosphorylated on tyrosine residues by the EGF receptor kinase in the same manner as for the protein substrates. The peptides were soluble in trichloroacetic acid, so they were isolated from the reaction mixture by ion-exchange chromatography on a 1 × 2-cm column of AG-1X2 matrix resin. After a 16-h incubation, the reaction mixture was acidified with an equal volume of 1 N acetic acid, applied to a 3-ml column and allowed to sit for a few minutes. Subsequently, the phosphopeptide was eluted with 1 N acetic acid whereas other reactants were retained on the resin. The Tyr(P) peptides were phosphorylated and dissolved in either 25 mM HEPES, pH 7.0, 1 mM EDTA for reaction with PPT phosphatase I, or in 50 mM MES, pH 5.0, 1 mM EDTA for reaction with PPT phosphatase II.

Determination of PPT Phosphatase Activity — Dephosphorylation of [Tyr(P)]serum albumin, [Tyr(P)]casein, or [Tyr(P)]myosin light chains was catalyzed by the enzyme from PPT phosphatase I, from the preparation used. Purified PPT phosphatase I was reconstituted with trichloroacetic acid as described previously (9). Dephosphorylation of [Tyr(P)]histone, [Tyr(P)][Val]angiotensin II, or [Tyr(P)]RR-arc was measured by loss of 32P. Aliquots of the reaction mixture were pipetted onto 1-cm squares of Whatman 81 phosphorcellulose paper which bound the polypeptides. The papers were immediately spotted with equal volumes of 25 mM HEPES, pH 7.0, 1 mM EDTA, 50 mM 2-mercaptoethanol, 1 mg/ml of serum albumin, and 5 μl of H2O. Activities were calculated relative to samples containing no enzyme but where 20 μl of the buffer was added to bring the final volume of the reaction mixture up to 50 μl. Reactions were incubated for 10 min at 30°C and were terminated by the addition of 0.1 ml of 25 mg/ml of serum albumin immediately followed by the addition of 0.85 ml of 1 M Na2CO3. The reaction mixtures were placed on ice for 15 min and centrifuged in a microcentrifuge. The amount of radioactivity released was determined by dissolving 0.5 ml of the supernatants in 20 ml of 15% trichloroacetic acid for 10 min and finally were rinsed with 100 ml of 1 M Na2CO3. The concentration of p-nitrophenol was calculated from the absorbance of the solution at 410 nm, using a millimolar extinction coefficient of 17.5. The amount of enzyme that produced 1 nmol of p-nitrophenol/min at 30°C at the optimum pH was defined as 1 unit.

RESULTS

Characterization of Tyr(P) Polypeptide Substrates — To compare the activities of PPT phosphatases and alkaline phosphatase, several different substrates containing Tyr(P) were prepared. Each of the proteins and peptides used were phosphorylated on tyrosine residues by the EGF receptor kinase in membranes from A431 cells. As is well known, in most cases the extent of phosphorylation of added proteins is less approximately 0.01 to 0.1 mol of 32P/mol of protein. Amino acid analyses of the labeled albumin and histone by thin layer electrophoresis revealed that more than 80% of the radioactivity was present as Tyr(P) (data not shown). The substrates were phosphorylated to different extents as follows: alkylated serum albumin, 1 nmol of 32P/mg; histone, 1 nmol of 32P/mg; casein, 0.08 nmol/mg; myosin mixed light chains, 1.5 to 2.9 nmol/mg; angiotensin, 7.3 nmol of 32P/mg, and RR-arc, 0.53 nmol of 32P/mg.

Phosphorylation of myosin light chains was dependent on the preparation used. Purified Mr = 20,000 light chain was phosphorylated to the highest extent (13), 0.85 mol of 32P/mol of protein, whereas a mixture of myosin light chains of Mr = 16,000 and 20,000 was phosphorylated to 0.42 mol of 32P/mol of protein. The myosin mixed light chain fraction used as phosphatase substrate was labeled with only 0.03 to 0.06 mol of 32P/mol of protein. In every case, most of the label was located in myosin light chain of Mr = 20,000 as revealed by autoradiography, following polyacrylamide gel electrophoresis in the presence of dodecyl sulfate.

Dephosphorylation of Acidic Proteins by PPT Phosphatase, Type I and II — Rabbit kidney cytosolic PPT phosphatases types I and II, have parallel substrate specificity, PPT phosphatase, type I, displays optimal activity at pH 7.0 and requires mercaptans for activity. In contrast, PPT phosphatase, type II, exhibits optimal activity at pH 5.0 and does not require mercaptans for activity. As shown in Table I, both PPT phosphatases dephosphorylated [Tyr(P)]-alkylated serum albumin and [Tyr(P)]casein with apparent Km values ranging from 1.2 to 12.5 μM. In addition, PPT phosphatase, type I, dephosphorylated [Tyr(P)]myosin light chains with an apparent Km of 1.4 μM. Reactions with each of these substrates displayed saturation kinetics and gave linear double reciprocal plots. Thus, these acidic phosphoproteins served as high-affinity substrates for PPT phosphatases. The reactivity of [Tyr(P)]myosin light chains with PPT phosphatase, type II, was not measured because of the low solubility of the substrate at pH 5.0.

[Tyr(P)]Histone Is Not a Substrate for PPT Phosphatases — Surprisingly, PPT phosphatases I or II did not dephosphorylate [Tyr(P)]histone, a basic protein. During the course of a 90-min incubation with PPT phosphatase I, less than 1% of the 32P was released from labeled histone. In a parallel experiment, the same sample of enzyme hydrolyzed 20% of the 32P from [Tyr(P)]-alkylated serum albumin in only 20 min. Similarly, when PPT phosphatase, type II, was incubated with [Tyr(P)]histone for 90 min at pH 5.0, less than

| [Tyr(P)]protein | Type I | Type II |
|-----------------|--------|--------|
| protein         | Km     | Vmax   | Vmax/Km | Km     | Vmax   | Vmax/Km |
| μM              | μmol/min/μg | μmol/min/μg | μmol/min/μg | μmol/min/μg | μmol/min/μg |

Alkylated serum albumin

| Protein | Km (μM) | Vmax (μmol/min/μg) | Vmax/Km |
|---------|---------|-------------------|--------|
| Casein  | 1.4     | 5.6               | 0.3    | ND     | ND     |
| Myosin light chains | 3.6 | 2.0 | 0.6 | 6.6 | 0.54 | 0.08 |
| Histone | NR | NR | NR | NR | NR |
| RR-arc | NR | NR | NR | NR | NR |
| Angiotensin | NR | NR | NR | NR | NR |
12% of the $^{32}$P was released from the protein (Fig. 1). The lack of reactivity with histone presumably reflects the specificity of the PPT phosphatases.

In contrast, when calf intestine alkaline phosphatase was incubated with (Tyr(2$^{32}$P))histone at pH 8.5 the reaction appeared complete within 30 min and 70% of the $^{32}$P was released from the protein, as shown in Fig. 1. Furthermore, in a similar experiment at pH 7.0, alkaline phosphatase released greater than 60% of the $^{32}$P from labeled histone. The relative amount of activity added for PPT phosphatase I-PPT-phosphatase II-alkaline phosphatase was 0.27:1.0:1.2. The ratio was determined using $p$-nitrophenylphosphate as substrate under the conditions described. It appeared that (Tyr(2$^{32}$P))histone served as a substrate for calf intestine alkaline phosphatase but not for PPT phosphatases.

**Reactivity of Tyr(P) Peptides with PPT Phosphatases and Alkaline Phosphatase**—PPT phosphatases were unreactive with Tyr(P) phosphopeptides. When [Tyr(2$^{32}$P)][Val]angiotensin or with PPT phosphatase, type I, (at pH 7) or with PPT phosphatase, type II, (at pH 5) only 1% of the $^{32}$P was released during the course of 2 h. Both phosphatases were also unreactive with the peptide (Tyr(2$^{32}$P))RR-src. Neither phosphatase produced a detectable level of dephosphorylation when incubated for up to 2 h with 110 $\mu$M phosphopeptide. With RR-src the range of variation between samples processed at various times of incubation was $\pm$10% of the initial 200,000 cpm. Phosphatase activity in the enzyme samples added to the peptides was confirmed by the hydrolysis of $p$-nitrophenylphosphate.

In contrast to the PPT phosphatases, calf intestine alkaline phosphatase dephosphorylated both angiotensin and RR-src. More than 70% of the $^{32}$P was released from [Tyr(2$^{32}$P)]RR-src when incubated with alkaline phosphatase at 30 °C, pH 7.0. Similarly, when [Tyr(2$^{32}$P)][Val]-angiotensin was incubated with alkaline phosphatase under the same conditions as those used for RR-src, over 65% of the $^{32}$P was released. These results show that the peptides were enzymatically dephosphorylated by alkaline phosphatase, but not by PPT phosphatases.

**DISCUSSION**

Our results show that PPT phosphatases only dephosphorylate certain acidic Tyr(P) protein substrates which is indicative of their relatively high degree of specificity. The PPT phosphatases do not parallel the specificity of the EGF receptor kinase, which was used to phosphorylate all the polypeptides tested as substrates. Regardless of the net charge of the polypeptides, protein tyrosine kinases phosphorylated tyrosine residues that were preceded by several acidic side chains (5–8, 13). Thus, it appears that the primary structure surrounding the phosphorylated tyrosine residue, and not the overall charge of the substrate, plays a principal role in determining kinase specificity. In contrast to the kinase, PPT phosphatases I and II reacted solely with the acidic phosphoproteins. Even though they are distinct enzymes, the two cytosolic phosphatases appear to have parallel specificity. Of the proteins tested, Tyr(2$^{32}$P)-alkylated serum albumin was the best substrate for both PPT phosphatases, types I and II. The data indicate that (Tyr(2$^{32}$P))histone was not a substrate for either of the PPT phosphatases. The lack of reactivity with histone is intriguing because it helps to clarify conflicting reports in the literature. Swarp et al. (15) used Tyr(P) histone, phosphorylated by the EGF-receptor kinase, to monitor protein Tyr(P) phosphatase activity in homogenates of TCRC-2 cells. They found about 10-fold higher specific activity in the particulate versus the soluble fraction. A membrane-bound glycoprotein was recovered and characterized as an alkaline-type phosphatase. The authors concluded “that alkaline phosphatases may be involved in the dephosphorylation of membrane-bound phosphotyrosine-containing proteins.” Our results confirm that Tyr(P) histone is indeed a substrate for mammalian alkaline phosphatase. However, this phosphoprotein is not dephosphorylated by cytosolic PPT phosphatases. Using acidic Tyr(P) proteins rather than Tyr(P) histone to measure PPT phosphatase activity, Horlein et al. (16), Foulkes et al. (17), and Shrirer and Brautigan (9) have characterized cytosolic enzymes that are distinct from alkaline phosphatase. Based on these results, we believe that if one uses Tyr(P) histone to measure PPT phosphatase activity, only the membrane-bound alkaline phosphatase will be detected.

In addition to phosphomonoesters such as $p$-nitrophenylphosphate alkaline phosphatases react with Ser(P) and Tyr(P) residues in proteins (14, 18) and exhibit some specificity for Tyr(P) relative to Ser(P) (14). Nonetheless, PPT phosphatases appear to be far more specific for Tyr(P) proteins than alkaline phosphatases. Relative to intestine alkaline phosphatase, Foulkes et al. (17) have shown that cytosolic PPT phosphatase was nearly 3000 times more active with Tyr(P) casein than with $p$-nitrophenylphosphate as substrate. We have been unable to detect hydrolysis of Ser(P) in proteins with PPT phosphatases. Furthermore, PPT phosphatases,
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... types I or II, were unreactive with Tyr(P) peptides, RR-src, or [Val]angiotensin, though alkaline phosphatase dephosphorylated each of these phosphopeptides under nearly the same conditions. We conclude that the cytosolic PPT phosphatases prefer substrates with a net negative charge with a more extensive tertiary structure than afforded by a tridecapeptide such as RR-src.

Although the in vivo substrates of protein tyrosine kinases and PPT phosphatases continue to elude identification, in vitro studies employing artificial substrates and synthetic peptides have proven useful in the characterization of these enzymes. An important conclusion of this report, with direct practical applications, is that neither Tyr(P) histone, nor Tyr(P) peptides are dephosphorylated by cytosolic PPT phosphatases. Thus, histone IIA would be a preferred substrate for measuring protein tyrosine kinase activity in cell extracts. Removal of the particulate fraction containing membrane-bound alkaline phosphatase should eliminate nearly all of the Tyr(P) histone phosphatase activity. Moreover, use of histone as substrate in a reaction mixture containing orthovanadate, a potent and effective PPT phosphatase inhibitor (19), would be ideal for investigating the kinetics and specificity of protein tyrosine kinases.

Finally, differences in substrate specificity between cytosolic PPT phosphatase and membrane-bound alkaline phosphatase may yet prove to be of physiological significance. It will be interesting to discover whether there are both acidic and basic amino acid sequences surrounding Tyr(P) in the proteins phosphorylated in vivo.

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