

Protein phosphatase 2A is a specific protamine-kinase-inactivating phosphatase

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Purified preparations of a protamine protein kinase from bovine kidney cytosol [Damuni, Amick & Sneed (1989) *J. Biol. Chem.* **264**, 6412–6416] were inactivated after incubation with near-homogeneous preparations of protein phosphatase 2A₁ and protein phosphatase 2A₂. These protein phosphatase 2A-mediated inactivations of the protamine kinase were unaffected by highly purified preparations of inhibitor 2, but were prevented when the incubations were performed in the presence of 100 nM microcystin-LR, 100 nM okadaic acid or 0.2 mM-ATP. By contrast, highly purified preparations of protein phosphatase 2B, protein phosphatase 2C, the catalytic subunit of protein phosphatase 1, and two forms of a protein tyrosine phosphatase, designated PTPase 1B and T-cell PTPase, had little effect, if any, on protamine kinase activity. Purified preparations of the protamine kinase did not react with anti-phosphotyrosine antibodies, as determined by Western blotting and immunoprecipitation analysis. The results indicate that protein phosphatase 2A is a specific protamine-kinase-inactivating phosphatase.

INTRODUCTION

The major protamine kinase isolated from extracts of bovine kidney cytosol is a unique enzyme composed of a single polypeptide of apparent M_r approx. 45000 [1]. *In vitro*, this enzyme phosphorylates the mRNA cap-binding protein, eukaryotic protein-synthesis-initiation factor 4E [2], and the 40 S ribosomal protein S6 (S. A. G. Reddy, G. D. Amick & Z. Damuni, unpublished work). The protamine kinase is stimulated rapidly after incubation of isolated rat hepatocytes with insulin [3]. Thus this enzyme could contribute to the insulin-stimulated phosphorylation of ribosomal protein S6 [4–6] and initiation factor 4E [7,8]. Incubation with the catalytic subunit of protein phosphatase 2A (PP2A_c) inactivated purified preparations of the protamine kinase [9], indicating that this kinase itself was regulated directly by phosphorylation.

However, PP2A is only one of the four major protein serine/threonine phosphatases present in the cytoplasm of mammalian cells (reviewed in [10]). The other three are PP1, PP2B and PP2C. Furthermore, in cells, PP2A_c is complexed with other proteins. Thus, in addition to PP2A_c, which exhibits an apparent M_r of approx. 36000, the most commonly identified physiologically relevant forms of PP2A, i.e. PP2A₁ and PP2A₂, contain a subunit of apparent M_r approx. 60000, and PP2A₁ also contains a distinct subunit of apparent M_r approx. 55000. These subunit proteins can have a marked effect on the catalytic specificity of PP2A [10]. This effect depends on the substrate employed, and is in general inhibitory [10].

To characterize further the regulation of protamine kinase, the effects of highly purified preparations of native forms of PP2A, i.e. PP2A₁ and PP2A₂, as well as other protein phosphatases, including PP1, PP2B and PP2C, and two forms of a protein tyrosine phosphatase, designated PTPase 1B [11] and T-cell PTPase [12], on protamine kinase activity have been examined. In this paper, we show that, of all the protein phosphatase preparations tested, only PP2A₁ and PP2A₂ inactivated purified preparations of the protamine kinase. These results indicate

strongly that PP2A is a specific protamine-kinase-inactivating phosphatase.

EXPERIMENTAL

Materials

Microcystin-LR and okadaic acid were from Calbiochem. PTPase 1B [11] and T-cell PTPase [12] were provided by Dr. Nicholas Tonks (Cold Spring Harbor Laboratories). The catalytic subunit of protein kinase A and potato acid phosphatase were from Sigma. *Escherichia coli* and calf intestine alkaline phosphatases were from Boehringer Mannheim. Anti-phosphotyrosine antibody PY20 was from ICN Biomedicals. Goat anti-mouse IgG conjugated to alkaline phosphatase was from Bio-Rad. Sources of other materials are given elsewhere [1–3,9].

Enzyme preparations

Protamine kinase was purified to apparent homogeneity as described previously [1]. PP2A₁ and PP2A₂ were resolved by chromatography of bovine kidney extracts on a column (2.5 cm × 20 cm) of DEAE-cellulose, and then purified to apparent homogeneity as described previously [13], except that chromatography of each enzyme on aminohexyl-Sepharose and poly(L-lysine)-agarose was repeated twice.

PP1_c [14] and PP2B [15] were purified to apparent homogeneity from extracts of bovine brain as described in the references. PP2C was purified from extracts of bovine kidney up to step 5 of a procedure described by McGowen & Cohen [16]. Inhibitor 2 was partially purified from extracts of bovine kidney up to step 4 of a procedure described by Tonks & Cohen [17]. Pyruvate dehydrogenase complex [18] and pyruvate dehydrogenase phosphatase [19] were purified to apparent homogeneity from extracts of bovine kidney mitochondria [18] as described in the references.

Assay of protein phosphatases

³²P-labelled phosphorylase [9], ³²P-labelled phosphorylase kin-

Abbreviations used: PP2A, protein phosphatase 2A; PP2A₁, protein phosphatase 2A₁; PP2A₂, protein phosphatase 2A₂; PP2A_c, catalytic subunit of protein phosphatase 2A; PP1_c, catalytic subunit of protein phosphatase 1; PP2B, protein phosphatase 2B; PP2C, protein phosphatase 2C; PTPase, protein tyrosine phosphatase.

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ase [9] and ^{32}P -labelled pyruvate dehydrogenase complex [19] were prepared as described in the references. The phosphatase assay mixtures contained 50 mM-imidazole/HCl, pH 7.3, 10% (v/v) glycerol, 1 mM-benzamidine, 0.1 mM-phenylmethanesulphonyl fluoride, 14 mM- β -mercaptoethanol, 0.1 mg of BSA, phosphatase sample and ^{32}P -labelled substrate in a final volume of 0.05 ml. ^{32}P -labelled substrates were added after equilibration of the mixtures for 5 min at 30 °C in a plastic micro-centrifuge tube. After a 5 min reaction period, 0.1 ml of 12% (w/v) trichloroacetic acid was added. The mixture was centrifuged at 12000 g for 2 min in a micro-centrifuge. A sample (0.12 ml) of the supernatant was then added to 1 ml of scintillant (Ready-Solv, Beckman) and the radioactivity determined. Phosphatases were omitted from controls. Assay of PP1 and PP2A was performed in the presence of 1 mM-EDTA with ^{32}P -labelled phosphorylase (5 mg/ml) as substrate. Assay of PP2B activity was performed in the presence of 0.2 mM- Ca^{2+} and 0.1 μM -calmodulin with ^{32}P -labelled phosphorylase kinase (2 mg/ml) as substrate. PP2C activity was determined in the presence of 10 mM- Mg^{2+} and 1 mM-EGTA with ^{32}P -labelled pyruvate dehydrogenase complex (5 mg/ml) as substrate. Pyruvate dehydrogenase phosphatase activity was determined with ^{32}P -labelled pyruvate dehydrogenase complex (5 mg/ml) in the presence of 10 mM- Mg^{2+} and 0.1 mM- Ca^{2+} . One unit of phosphatase activity was defined as the amount of each phosphatase that catalysed the release of 1 nmol of [^{32}P]P_i/min from ^{32}P -labelled substrate. To ensure linearity, the extent of [^{32}P]P_i release was limited to < 10%.

Assay of inhibitor-2 activity was based on the measurement of PP1 activity as described above in the absence and presence of the inhibitor preparations. The reaction mixture contained 0.02 units of PP1/ml in 35 μl of 50 mM-imidazole/HCl, pH 7.3, 1 mM-EDTA, 10% glycerol, 1 mM-benzamidine, 0.1 mM-phenylmethanesulphonyl fluoride, 0.1 mg of BSA and inhibitor sample (5 μl). After incubation of this solution for 10 min at 30 °C in a plastic micro-centrifuge tube, the reaction was initiated with 10 μl of ^{32}P -labelled phosphorylase and the assay was continued as described above. One control tube was performed in the absence of the inhibitor protein and another in the absence of both inhibitor protein and PP1. Inhibition of PP1 was linear up to 50%. One unit of inhibitor 2 activity was defined as the amount of protein that inhibited 1 unit of PP1 by 50% in the standard assay.

Protamine kinase assay

Protamine kinase activity was determined as described previously [1], except that reactions contained 100 nM microcystin-LR and were terminated after 3 min of incubation. One unit of protamine kinase activity was defined as the amount of the enzyme that incorporated 1 nmol of ^{32}P into protamine/min.

Protein was determined as described in [20]. Homogeneity of enzyme preparations was determined by SDS/PAGE [21]. Protein bands were detected by staining with Coomassie Blue or silver [22]. To identify radioactive bands, after SDS/PAGE of ^{32}P -labelled substrates, Kodak X-Omat AR-5 film was employed. Immunoprecipitation and Western blotting with anti-phosphotyrosine antibody were performed as recommended by the manufacturer.

RESULTS AND DISCUSSION

Native forms of PP2A inactivate protamine kinase

PP2A₁ and PP2A₂ inactivated the protamine kinase in a time- (Fig. 1) and concentration- (Fig. 2) dependent manner. These

PP2A₁- and PP2A₂-mediated inactivations of the protamine kinase occurred at rates similar to those obtained with purified preparations of PP2A_c when the activities were normalized with phosphorylase *a* as substrate (Figs. 1 and 2). In this regard, it is noteworthy that the standard procedure employed in this study to dissociate PP2A_c from the native forms of PP2A results in about a 4–6-fold increase in activity with phosphorylase *a* as substrate [10,13]. Therefore, since the activities of PP2A₁, PP2A₂ and PP2A_c were normalized with phosphorylase *a* as substrate (Figs. 1 and 2), the protamine kinase-inactivating activity of PP2A also appears to be suppressed by the regulatory subunits of PP2A₁ and PP2A₂.

Inactivation of the protamine kinase by PP2A₁, PP2A₂ or PP2A_c was unaffected by highly purified preparations of inhibitor 2 at concentrations that inhibited PP1 completely with ^{32}P -labelled phosphorylase *a* as substrate. By contrast, in the presence of 100 nM okadaic acid or 100 nM microcystin-LR, inactivation of the protamine kinase by PP2A₁, PP2A₂ and PP2A_c was prevented. Okadaic acid and microcystin-LR are potent inhibitors of PP1 and PP2A [23–25]. In the presence of 0.2 mM-ATP, a specific inhibitor of PP2A [26,27], inactivation of the protamine kinase by the PP2A preparations was prevented. Inactivation of the protamine kinase preparations by the PP2A preparations was also prevented by the general phosphatase inhibitors NaF (20 mM), P_i (10 mM) and PP_i (10 mM).

The rate of inactivation of the protamine kinase by PP2A₁, PP2A₂ and PP2A_c was unaffected by Mg^{2+} (up to 10 mM), Ca^{2+} (up to 0.5 mM), EDTA (up to 1 mM) or EGTA (up to 1 mM). The rates of the PP2A₁- and PP2A₂-mediated inactivations of the protamine kinase were stimulated 3-fold in the presence of 2 mM- Mn^{2+} . In the presence of this concentration of Mn^{2+} , the phosphorylase phosphatase activities of the PP2A₁ and PP2A₂ preparations were stimulated by about 1.3–1.7-fold.

Effect of other protein phosphatases

By contrast with PP2A₁, PP2A₂ and PP2A_c, purified preparations of PP1_c (up to 50 units/ml), PP2B (up to 10 units/ml) and PP2C (up to 10 units/ml) had little effect, if any, on the activities of purified preparations of the protamine kinase (Table 1). Similarly, purified preparations of pyruvate dehydrogenase phosphatase (up to 10 units/ml), *E. coli* or calf intestine alkaline phosphatase (up to 10 units/ml) and potato acid phosphatase (up to 10 units/ml) exhibited little or no effect on protamine kinase activity. Purified preparations of two forms of a protein tyrosine phosphatase, designated PTPase 1B (up to 5 units/ml) and T-cell PTPase (up to 5 units/ml), also displayed little or no effect on the activities of purified preparations of the protamine kinase (Table 1). Together, these results indicate that PP2A is a specific protamine kinase-inactivating phosphatase. The possibility that protamine kinase-inactivating phosphatases other than PP2A exist cannot yet be entirely ruled out, however. Experiments performed with extracts of bovine kidney were inconclusive, because of proteolysis of the protamine kinase during the incubations (results not shown).

Other than the protamine kinase, four other distinct insulin-stimulated protein kinases have been shown to be inactivated by incubation with PP2A. These enzymes include two different insulin-activated ribosomal protein S6 kinases [28,29], a distinct mitogen-activated protein kinase [29] and an insulin-stimulated Kemptide kinase [30]. PP1 was far less effective in inactivating these kinases [28–30]. However, the effects of PP2B and PP2C were not determined. Thus, although the information is limited, together with the results presented herein, the observations are consistent with the idea that PP2A may play an important role in the control of insulin-stimulated protein phosphorylation.

Three lines of evidence indicate that PP2A₁, PP2A₂ and PP2A_c

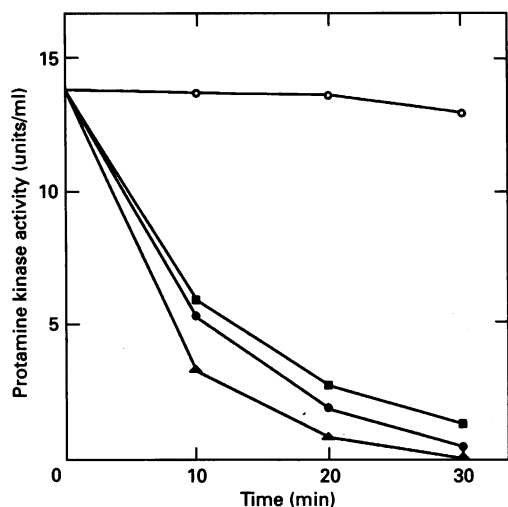


Fig. 1. Inactivation of protamine kinase by PP2A

Highly purified protamine kinase (155 units/ml) was incubated at 30 °C in the absence (○) or the presence of PP2A₁ (●), PP2A₂ (▲) and PP2A_c (■) (5 units/ml) in 50 mM-imidazole/HCl, pH 7.3, containing 10% glycerol, 0.1 mM-phenylmethanesulphonyl fluoride, 1 mM-benzamidine and 14 mM-β-mercaptoethanol in a final volume of 0.05 ml. At the indicated times, a 0.005 ml sample of the incubations was used to determine protamine kinase activity as described in the Experimental section.

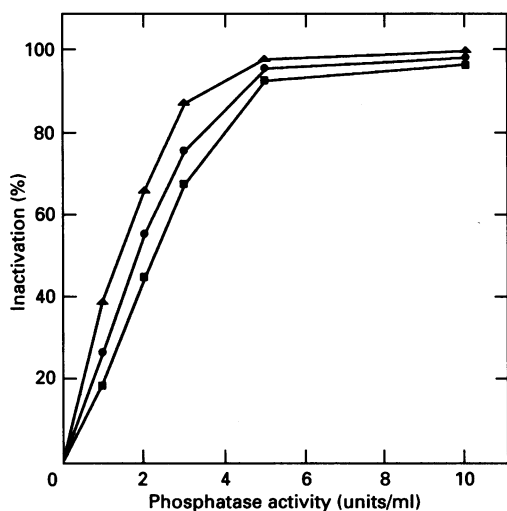


Fig. 2. Dependence of inactivation on the concentration of PP2A

Protamine kinase was incubated in the presence of the indicated concentrations of PP2A₁ (●), PP2A₂ (▲) and PP2A_c (■) as described in the legend to Fig. 1. After a 30 min incubation at 30 °C, protamine kinase activity was determined as described in the Experimental section.

inactivate the protamine kinase by dephosphorylating serine and/or threonine residues rather than tyrosine residues. First, preincubation of PP2A with ATP prevented the inactivation of the protamine kinase with this phosphatase. Incubation with ATP inactivates the protein serine/threonine phosphatase activity of PP2A and converts this phosphatase into a protein tyrosine phosphatase [31–33]. Second, purified preparations of PTPase 1B and T-cell PTPase had little or no effect on protamine

Table 1. Effect of protein phosphatases on protamine kinase

Purified protamine kinase (120 units/ml) was incubated at 30 °C in the presence of PP2A₁ (5 units/ml), PP2A₂ (5 units/ml), PP2A_c (5 units/ml), PP1_c (50 units/ml), PP2C (10 units/ml), PP2B (10 units/ml), pyruvate dehydrogenase phosphatase (PDHP; 10 units/ml), PTPase 1B (5 units/ml) or T-cell PTPase (5 units/ml). Incubations containing PP2C and PDHP contained 10 mM-Mg²⁺. Incubations with PP2B included Ca²⁺ (0.5 mM) and calmodulin (0.5 μM). After 30 min of incubation, protamine kinase activity was determined as described in the Experimental section. The values shown were obtained relative to control incubations in which the kinase was incubated for 30 min at 30 °C in the absence of phosphatases. The percentage inactivation after incubation of the kinase with PP2B was obtained relative to controls in which the kinase was incubated in the presence of calmodulin (0.5 μM) and Ca²⁺ (0.5 mM). The values for PP2C and PDHP were obtained relative to control incubations in which the kinase was incubated in the presence of 10 mM-Mg²⁺. Incubation in the presence of Ca²⁺ (0.5 mM) and calmodulin (0.5 μM) or Mg²⁺ (10 mM) had little effect, if any, on the activity of the protamine kinase (results not shown).

Protein phosphatase	Inactivation (%)
PP2A ₁	98
PP2A ₂	95
PP2A _c	97
PP1 _c	10
PP2B	8
PP2C	4
PDHP	6
PTPase 1B	-5
T-cell PTPase	-6

kinase activity (Table 1). Third, all attempts to immunoprecipitate the protamine kinase or to detect it by Western blotting with anti-phosphotyrosine antibody failed (results not shown). Nevertheless, it is possible that the protamine kinase could be phosphorylated on tyrosine residues and that this phosphorylation is lost during the purification of the enzyme. However, it is noteworthy in this regard that several attempts to phosphorylate the protamine kinase with purified preparations of the insulin receptor failed (results not shown).

We suggested previously that the mechanism underlying the insulin-stimulated increase in protamine kinase activity may occur via increased phosphorylation of the kinase [3]. This possibility was raised by three observations. First, the effect of insulin on protamine kinase activity was stable to chromatography, and the enzyme from control and insulin-stimulated cells exhibited the same apparent *M_r* [3]. Second, the effect of insulin was unaffected by preincubation of the cells with the protein-synthesis inhibitor cycloheximide [3]. Third, the enzyme from control and insulin-treated cells was inactivated completely after incubation with PP2A_c [3]. Increased phosphorylation and activation of the protamine kinase could occur via stimulation of the protamine kinase-activating kinase(s) and/or inactivation of the inactivating phosphatase(s). Therefore, one possibility raised by the results in this paper is that insulin may cause inactivation of PP2A in the intact cells. Inactivation of PP2A may be of the allosteric type [10,34] and/or may occur via covalent modification, e.g. phosphorylation [34]. Regulation of a protein phosphatase by phosphorylation is well established for PP1, where phosphorylation of regulatory subunits modulates the activity of two forms of this enzyme [35,36]. Studies to evaluate the possibility that PP2A is regulated by phosphorylation are needed.

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