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Isolation of Histone H1-Stimulated Phosphoprotein Phosphatase from Kidney and Skeletal Muscle (41906)

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Protein phosphorylation is an important mechanism for the regulation of numerous mammalian cellular processes. The extent of phosphorylation of any given protein is determined by the relative activity of protein kinases and protein phosphatases that catalyze the incorporation or removal of a protein-bound phosphate, respectively (1). Much is known about the regulation of a number of protein kinases (1) and it is becoming increasingly clear that protein phosphatases are also important sites for the regulation of cell function (2). Protein phosphatase activity is regulated by extrinsic (substrate-directed) and intrinsic (phosphatase-directed) mechanisms (1, 3). In the former, various metabolites bind to the phosphoprotein substrate, thus altering their susceptibility to dephosphorylation. In the latter, various ligands bind to the phosphatase and thus directly alter phosphatase activity.

Studies on protein phosphatases are complicated by the presence of multiple forms which dephosphorylate a variety of phosphoprotein substrates (1–3). Cohen and co-workers (2) have classified protein phosphatases as type 1 and type 2. Type 1 phosphatase is inhibited by heat-stable inhibitor proteins, is relatively insensitive to inhibition by ATP, and preferentially dephosphorylates the β subunit of phosphorylase kinase. Type 2 phosphatase is not inhibited by heat-stable inhibitors, is sensitive to inhibition by ATP, and preferentially dephosphorylates the α subunit of phosphorylase kinase. Ingebritsen and Cohen (2) have suggested that nearly all of the protein phosphatase activity of mammalian tissues can be attributed to four multifunctional phosphatases that they have designated protein phosphatases 1 (a type 1 phosphatase) and 2A, 2B, and 2C (type 2 phosphatases). The activity of phosphatase 2C is believed to be regulated by Mg^{2+}, and phosphatase 2B is dependent upon Ca^{2+} and calmodulin for activity. Phosphatase 1 appears to be the major form of protein phosphatase in skeletal muscle (4). Phosphatase 1 activity is regulated by heat-stable inhibitor proteins 1 and 2 (5, 6). Phosphatase 2A is the major protein phosphatase form in a number of tissues including heart, brain, and liver (4). It is not known how the activity of phosphatase 2A is regulated.

Isolation of a protein phosphatase activator. In an earlier study, we found heat-stable inhibitors with properties similar to those of skeletal muscle inhibitor 1 and inhibitor 2 present in extracts from rabbit kidney (7). In extracts prepared from medulla or cortex, most of the heat-stable inhibitor activity appeared to be complexed to high-molecular-weight native protein phosphatases (7). To study the interaction of renal heat-stable inhibitor proteins and renal protein phosphatases, we wanted to purify the inhibitors from renal cortex. In an attempt to purify inhibitor 1, we used the procedure developed by Foulkes and Cohen (8) for the purification of inhibitor 1 from rabbit skeletal muscle. Swine renal cortex was homogenized in 2% trichloroacetic acid and the extract was fractionated between 2 and 15% trichloroacetic acid (9). This fraction, which was expected to contain inhibitor 1 activity, actually stimulated the phosphorylase phosphatase activity of a relatively crude ethanol-treated protein phosphatase preparation ($M_r \simeq 35{,}000$) from rabbit renal cortex (Fig. 1). A preliminary study of the 2–15% trichloroacetic acid fraction revealed a heat-stable, nondialyzable, and trypsin-labile activator (9). The activator protein was purified to homogeneity (9, 10). On SDS–PAGE, the purified activator preparation had two protein bands with apparent molecular weights of 31,000 and 32,000 (10). The activator (both protein bands) was phosphorylated by cyclic AMP-dependent protein kinase to about 1 mole phosphate/mole protein. Activator activity was not altered by phosphorylation (Fig. 2). The purified activator protein lowered the K_m of the enzyme for phosphorylase *a* about 20-fold with little

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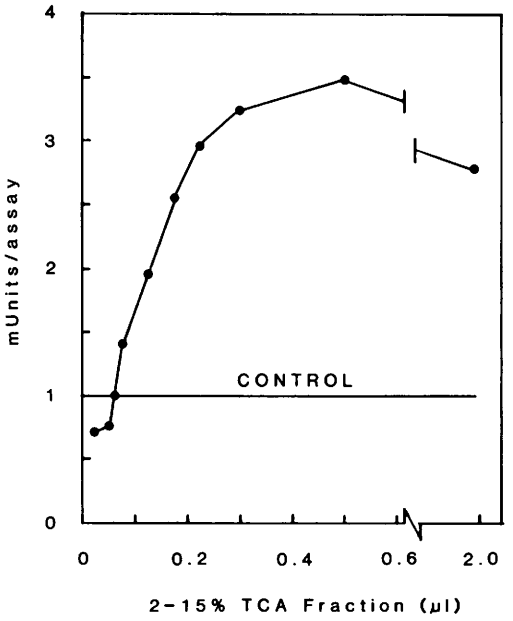

FIG. 1. Effect of a 2–15% trichloroacetic acid fraction on the activity of an ethanol-treated phosphatase prepared from rabbit renal cortex. A 2–15% trichloroacetic acid fraction was prepared from rabbit renal cortex and assayed as described in (9). Control activity (no additions) is shown as a solid horizontal line.

if any effect on the V_{max} (9). Thus, the degree of activation was dependent upon the concentration of phosphorylase *a* in the assay.

Identification of the activator as histone H1. The subcellular distribution of the activator was investigated by differential centrifugation of renal cortical homogenates. Nearly all of the activator activity was found in the 1000*g* insoluble crude nuclear fraction (Fig. 3). Activator was also prepared from the crude nuclear fraction of a number of other tissues (Table I). Activator activity was found in each of the nuclear fractions tested with the greatest amount isolated from calf thymus. The activator was also isolated from highly purified liver nuclei (10). When the 2–15% trichloroacetic acid fraction from the renal cortical nuclear pellet was subjected to SDS–PAGE, there were a number of proteins deteced (Fig. 3). Even after this modest purification, the two major proteins detected (Fig. 3) were the same as in the homogeneous activator preparation (10).

Based on the above evidence, the activator appeared to be a major phosphorylatable protein which could be extracted from nuclei with 2% trichloroacetic acid. Histone H1 is the major protein which is extracted from nuclei by acids (11). Histone H1 proteins were purified from calf thymus (11) and their mobilities on SDS–PAGE and acetic acid/urea PAGE were compared with purified activator. In both systems histone H1 and activator had identical mobilities (10). As previously noted (12) histone H1 subfractions (M_r = 21,000) gave anomalously high apparent molecular weights on SDS–PAGE [M_r = 31,000 and 32,000] (Fig. 3 and Ref. (10)).

Activation of phosphorylase phosphatase activity by authentic histone H1 purified from calf thymus was compared with activation by the activator prepared from swine renal cortex. Histone H1 activated phosphorylase phosphatase activity in a manner

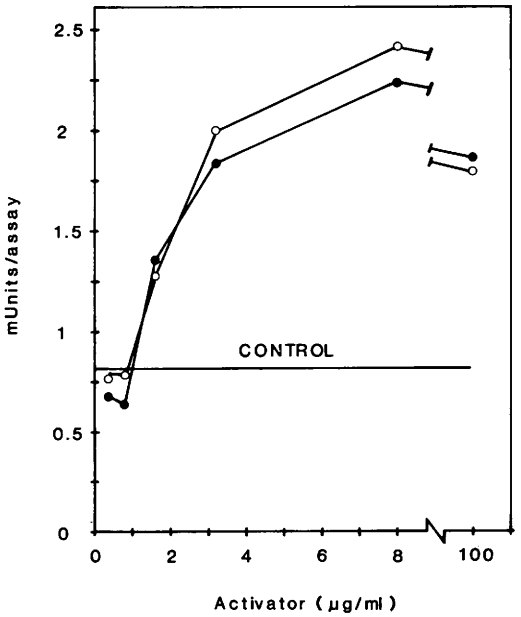

FIG. 2. Effect of phosphorylation on the activity of purified activator. Activator was purified from swine renal cortex (9) and phosphorylated by incubation with the catalytic subunit of cyclic AMP-dependent protein kinase and [γ-^{32}P]ATP (15). The degree of phosphorylation plateaued at approx 1 mole ^{32}P/mole activator protein. Autoradiography after SDS–PAGE showed that both bands of activator protein were phosphorylated. Phosphatase activity in the presence of nonphosphorylated (○) or phosphorylated activator (●) was determined as in (9).

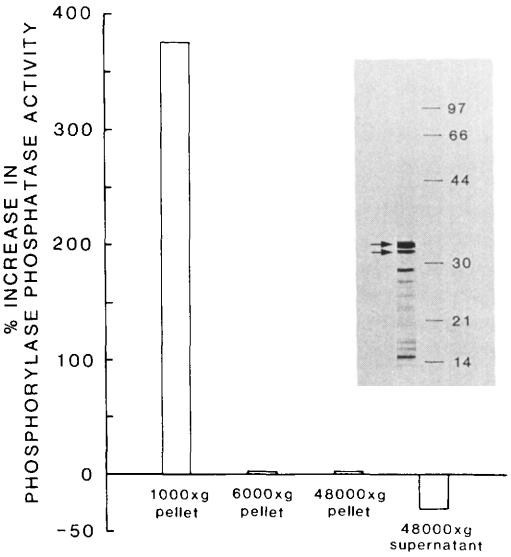

FIG. 3. Subcellular distribution of activator activity in swine renal cortex. Cortical homogenate was prepared in buffer containing 250 mM sucrose as previously described (10). The homogenate was fractionated by differential centrifugation at 1000g for 10 min, 6000g for 15 min, and 48,000g for 30 min. Each of the pellets was washed and 2–15% trichloroacetic acid fractions were prepared from the pellets as well as the 48,000g supernatant and assayed as in (10). The 1000g 2–15% trichloroacetic acid fraction was subjected to SDS-PAGE (16) and stained with Coomassie brilliant blue R-250 (insert). Positions of standard protein markers are indicated (molecular weight $\times 10^{-3}$).

virtually identical to that obtained with swine renal cortex activator (10). The concentration required for half-maximal activation was about 0.1 μM. Core histones (H2a, H2b, H3, and H4) were without effect or were inhibitory. It was concluded that the heat-stable protein activator was histone H1.

TABLE I. ACTIVATOR ACTIVITY ISOLATED FROM CRUDE NUCLEI OF VARIOUS TISSUES

Tissue	Units/g tissue
Bovine calf thymus	6487
Swine kidney cortex	1855
Bovine heart	210
Rat liver	117
Rabbit skeletal muscle	61

Note. The trichloroacetic acid fraction (2–15%) of the 1000g pellet from each tissue was prepared and assayed as described in Fig. 3 and Ref. (10).

Renal histone H1-stimulated phosphoprotein phosphatase. All of the above phosphatase activator studies were done with a poorly characterized ethanol-treated phosphatase ($M_r \simeq 35{,}000$) from rabbit renal cortex which was a mixture of type 1 and type 2 phosphatases. About 75% of the activity measured in the absence of histone H1 was inhibited by heat-stable inhibitor 1 (Fig. 4). However, when the effect of inhibitor 1 was determined in the presence of histone H1, the histone-stimulated activity was not inhibited. Thus it appeared that histone H1 was stimulating an inhibitor 1-insensitive, i.e. type 2, protein phosphatase.

To determine the effect of histone H1 and inhibitor 1 on the phosphorylase phosphatase activity of presumably native renal protein phosphatase(s), cortical extracts were prepared and assayed for phosphorylase phosphatase activity (Table II). The results were very similar to those obtained with the ethanol-treated preparation. Addition of histone H1 stimulated the activity > twofold. About 70% of the activity measured in the absence of histone H1 was inhibited by inhibitor 1, whereas most of the activity determined in the presence of histone H1 was not inhibited by inhibitor 1. Chromatography of a renal cortical extract on DEAE Sephacel revealed two major regions of histone H1-stimulated phosphatase activity (Fig. 5). One eluted at about 160 mM chloride (peak I) and the

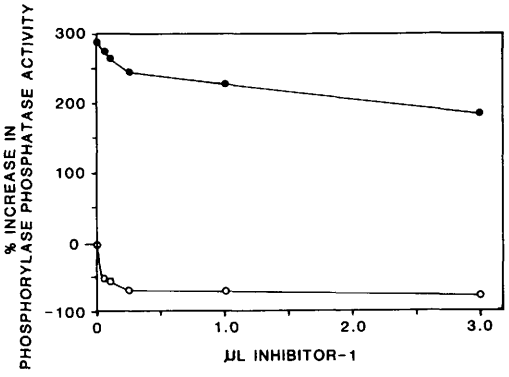

FIG. 4. Effect of inhibitor 1 on the activity of ethanol-treated phosphatase from rabbit renal cortex assayed in the absence (○) or presence (●) of histone H1. Inhibitor 1 was purified as in (8). Activator activity was determined as described in (10).

TABLE II. PHOSPHORYLASE PHOSPHATASE ACTIVITY OF RENAL CORTICAL AND SKELETAL MUSCLE EXTRACTS

Addition		Phosphatase activity (units/g)	
Inhibitor 1	Histone H1	Cortex	Muscle
−	−	37.2	37.4
+	−	11.9	8.7
−	+	85.0	38.8
+	+	76.9	23.9

Note. Extracts were prepared from fresh rabbit renal cortex or fresh rabbit skeletal muscle as described in Fig. 5. Phosphorylase phosphatase activity was assayed as described in (10).

other eluted at about 300 m*M* chloride (peak II). Peak II was clearly separated from inhibitor 1-sensitive phosphatase activity and thus did not appear to be a type 1 phosphatase. Peak I eluted from the column at about the same position as inhibitor-sensitive phosphatase activity. Inhibitor 1-sensitive phosphatase and histone H1-stimulated phosphatase of peak I could be separated by chromatography on Bio-Gel A-1.5. Thus, renal cortical extract contains a large amount of histone H1-stimulated phosphatase which does not appear to be a type 1 phosphatase.

Skeletal muscle histone H1-stimulated phosphoprotein phosphatase. Since the protein phosphatases of skeletal muscle have been extensively studied and characterized (1–3), we decided to look for histone H1-stimulated phosphatase in rabbit skeletal muscle. When extracts were assayed for phosphorylase phosphatase activity, there was no histone H1 stimulation of phosphatase activity (Table II). About 75% of the activity of the extract was sensitive to inhibitor 1. The most striking result was that in the presence of inhibitor 1 (where type 1 phosphatase activity was inhibited), one could observe a marked stimulation of phosphatase activity by histone H1 (Table II). Chromatography of the extract on DEAE cellulose revealed several peaks of phosphatase activity (13). A peak of activity eluting at about 160 m*M* NaCl was almost fully inhibited by histone H1. Another peak of activity which eluted at 300 m*M* chloride, was activated twofold by histone H1. Subsequent studies showed that highly purified catalytic subunit of rabbit muscle phosphatase 1 was inhibited by histone H1 (13). Thus, the lack of stimulation of phosphatase activity in skeletal muscle extracts assayed in the absence of inhibitor 1 (Table II), probably was due to a dual effect of histone H1, i.e., an inhibition of type 1 phosphatase and a stimulation of another phosphatase(s). When the extracts were assayed in the presence of inhibitor 1, only the latter effect of histone H1 occurred and histone H1 stimulation could be observed (Table 2).

Rabbit skeletal muscle histone H1-stimulated phosphoprotein phosphatase was partially purified by successive chromatography on DEAE cellulose, Bio-Gel A-1.5, and

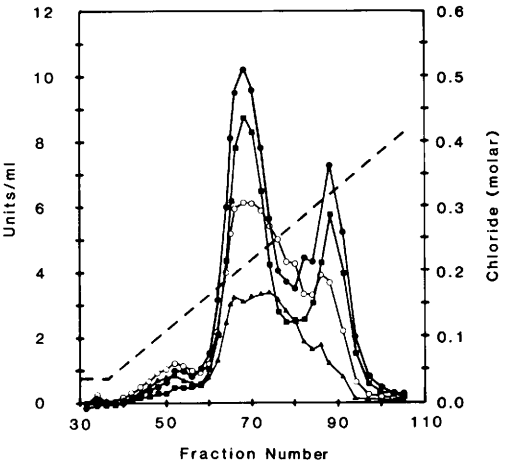

FIG. 5. DEAE Sephacel chromatography of an extract from rabbit renal cortex. Seven grams of fresh rabbit renal cortex was homogenized in 18 ml of buffer A (50 m*M* imidazole–HCl, 5 m*M* EGTA, 1 m*M* dithiothreitol, 0.25 m*M* phenylmethylsulfonyl fluoride, 10 m*M* benzamidine, and and 1μg pepstatin A/ml, pH 7.4 at 4°C) containing in addition 250 m*M* sucrose (all operations at 4°C). The homogenate was centrifuged for 20 min at 20,000*g*. A 16-ml sample of the resulting supernatant was applied to a 1.6 × 10-cm column of DEAE Sephacel equilibrated with buffer A and washed with 3 bed volumes of buffer A. The column was then eluted with a linear gradient of 0–600 m*M* NaCl in 240 ml of buffer A. Two-milliliter fractions were collected and assayed for phosphorylase phosphatase activity (10) without additions (○), with histone H1 (●), or with histone H1 and inhibitor 1 (■). Inhibitor-sensitive activity (▲) was estimated by subtracting the activity determined in the presence of inhibitor 1 from activity determined without additions.

phenyl-Sepharose (13). The histone H1-stimulated phosphatase activity of the enzyme purified through phenyl-Sepharose chromatographed as a 5.5-nm form on Bio-Gel A-1.5. A number of properties of the enzyme were studied (13). The phosphorylase phosphatase activity was stimulated 5- to 10-fold by histone H1 using standard assay conditions. Histone H1 dramatically lowered the K_m of the enzyme for phosphorylase *a* but it did not significantly change the V_{max} (Table III). Core histones did not activate the enzyme. Protamine or poly-L-lysine ($M_r \simeq 22{,}000$) activated phosphorylase phosphatase activity. Various low-molecular-weight polyamines were either without effect or inhibited activity. Phosphatase activity was not inhibited by inhibitor 1 whether tested in the presence or absence of histone H1. Activity was inhibited by 0.1 m*M* ATP. Freezing and thawing the enzyme in the presence of 0.2 *M* β-mercaptoethanol converted the phosphatase to a low-molecular-weight form (Fig. 6). The low-molecular-weight enzyme was activated by histone H1 (Fig. 6), it was inhibited by ATP, and it was not inhibited by inhibitor 1. When the histone H1-stimulated phosphatase was assayed with phosphorylase kinase as the substrate, dephosphorylation was markedly stimulated by histone H1 (14). Thus, histone H1 stimulation was not restricted to the dephosphorylation of phosphorylase *a*. Only the α subunits were dephosphorylated in the absence or presence of histone H1.

Discussion. There have been several other reports of heat-stable protein activators of phosphoprotein phosphatase (18–20). However, these activators have chemical and physical properties quite different than histone H1 (18–20), and they were without effect on phophorylase phosphatase activity (18, 19). There is also a low-molecular-weight ($M_r \simeq 9000$) heat-stable deinhibitor protein which has been isolated by Merlevede and co-workers (21, 22). The deinhibitor protein neutralizes the inhibitory effect of either inhibitor 1 or inhibitor 2. Thus in the presence of these inhibitors, the deinhibitor protein would appear to be a phosphatase activator. Histone H1 does not reverse the inhibition of phosphatase 1 by inhibitor 1 (13). Indeed, histone H1 is itself an inhibitor of the phos-

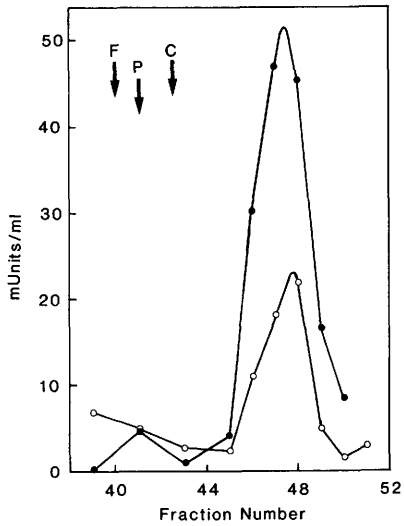

FIG. 6. Bio-Gel A-1.5m chromatography of skeletal muscle H1-stimulated phosphoprotein phosphatase after freeze-thawing in the presence of β-mercaptoethanol. The H1-stimulated phosphatase (13) was incubated in the presence of 0.2 *M* β-mercaptoethanol for 1 hr at −20°C, thawed, and centrifuged to remove insoluble protein. The sample in a volume of ca. 0.5 ml was applied to a 1.0 × 45-cm column of Bio-Gel A-1.5 m equilibrated with 50 m*M* imidizole-HCl, 0.2 m*M* EGTA, 2 m*M* dithiothreitol, 0.1 *M* NaCl, pH 7.4. Fractions of 0.64 ml were collected and assayed for phosphorylase phosphatase activity in the absence (○) or presence (●) of histone H1. The marker proteins ferritin (arrow F, 5.85-nm Stokes radius) and catalase (arrow C, 5.15-nm Stokes radius) were chromatographed separately. Histone H1-stimulated phosphatase without freeze-thawing in the presence of β-mercaptoethanol eluted from the column at arrow P.

TABLE III. KINETICS OF THE ACTIVATION OF HISTONE H1-STIMULATED PHOSPHOPROTEIN PHOSPHATASE

	K_m (μ*M* phos. *a* dimer)	V_{max} (munits/ml)
(−) Histone H1	19.4 ± 0.4	102 ± 32
(+) Histone H1	0.4 ± 0.08	73 ± 4

Note. The kinetics of histone H1-stimulated phosphoprotein phosphatase for phosphorylase *a* were determined in the absence and presence of 4 μg histone H1/ml. The enzyme was isolated from rabbit skeletal muscle as described in (13) and assayed as described in (9). Kinetic constants were calculated by fitting the data to the Michaelis–Menten equation by nonlinear regression analysis (17).

phorylase phosphatase activity of phosphatase 1 (13).

The isolation of histone H1-stimulated phosphoprotein phosphatase from renal cortex and skeletal muscle in our laboratories, and from vascular smooth muscle by DiSalvo and co-workers (23) raises several interesting questions concerning the nature of the stimulatable phosphatase, the mechanism of activation, and the possible physiological significance. The insensitivity of histone H1-stimulated phosphoprotein phosphatase to inhibitor 1, the inhibition by low concentrations of ATP, and the specificity for the α subunits of phosphorylase kinase (13, 14) (Table III, Figs. 4, 5) suggest that the enzyme is a type 2 phosphatase according to the classification of Ingebritsen and Cohen (2). DiSalvo *et al.* (23) reached a similar conclusion studying histone H1-stimulated phosphatase isolated from vascular smooth muscle. Of the well-characterized skeletal muscle phosphatases (2), phosphatase 2A most closely resembles the histone H1-stimulated phosphatase we have prepared from skeletal muscle (13). However, it is possible that histone H1-stimulated phosphoprotein phosphatase is a totally new type 2 phosphatase.

Using partially purified histone H1-stimulated phosphoprotein phosphatase, we have been able to obtain some information on the mechanism of histone H1 activation when phosphorylase *a* is the substrate. Histone H1 increases the activity by decreasing the K_m of the phosphatase for phosphorylase *a* (9) (Table III). A decrease in the K_m could be the result of either an interaction of histone H1 with phosphorylase *a* (substrate-directed) or an interaction with the phosphatase (phosphatase-directed). Several lines of evidence suggest that histone H1 activates by a phosphatase-directed mechanism. Maximum activation of phosphorylase phosphatase activity could be achieved in the presence of a 10-fold molar excess of phosphorylase *a* relative to histone H1 (9). Also, histone activation is observed with at least two protein substrates (phosphorylase *a* and phosphorylase kinase) [14]. Recently, we found that *p*-nitrophenylphosphatase activity of a skeletal muscle histone H1-stimulated phosphatase preparation was stimulated by histone H1 (unpublished result). A more complete understanding of histone H1 activation will require more detailed studies on highly purified histone H1-stimulated phosphoprotein phosphatase.

The physiological significance of a histone H1-stimulated phosphoprotein phosphatase in the cytoplasm which acts on cytoplasmic phosphoprotein substrates remains to be clarified. Although histone H1 occurs predominately in the nucleus, it is synthesized in the cytoplasm and there is some evidence that under certain conditions it can accumulate in the cytoplasm (24). Thus it is possible that histone H1 itself or a histone H1-like activator could play a previously undescribed role in regulating phosphoprotein dephosphorylation. Even if histone H1 is not a physiologically important activator, it has revealed a possible mechanism whereby the dephosphorylation of phosphoprotein substrates could be modulated. One of the major problems in understanding a multifunctional phosphoprotein phosphatase is how the activity of a phosphatase which acts on several protein substrates is selectively regulated (1, 3). An activator which can decrease the K_m for certain substrates and has no effect or even increases the K_m for other substrates provides a mechanism by which relative substrate specificity can be conferred on a multisubstrate enzyme.

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