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SPECIFICITY OF A PHOSPHATASE FOR PHOSPHOLIPID, Ca^{2+}-DEPENDENT PROTEIN KINASE-PHOSPHORYLATED HISTONE H1 RESIDES IN THE CATALYTIC SUBUNIT

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SUMMARY. A protein phosphatase from liver which acts preferentially on histone phosphorylated with phospholipid, Ca^{2+}-dependent protein kinase has been purified and the intrinsic specificity determined to reside in the catalytic subunit of the enzyme complex. Comparison with a preparation of pork heart protein phosphatase suggests that this specificity may be a general property of a class of protein phosphatases. Protein kinase C-phosphorylated histone H1 represents an improved substrate for phosphatase detection providing a five to tenfold greater sensitivity than other substrates including cAMP-dependent protein kinase phosphorylated H1.

The phospholipid, Ca^{+2}-dependent protein kinase (PKC) (1-3) has acquired considerable regulatory significance in a multitude of cell types because of its activation by diacylglycerol, a product of receptor-linked events (4,5), and by phorbol esters (6-8), a class of tumor promoting plant products. Previous studies with partially purified preparations of the kinase demonstrated the presence of a potent phosphatase activity for histone H1 phosphorylated by PKC with considerably less activity towards H2A, H2B, H3 or H4 phosphorylated by PKC (9). The same proteins phosphorylated by cAMP-dependent protein kinase (PKA) were poorer substrates for the phosphatase. Here we demonstrate that this specificity resides in the catalytic subunit of the enzyme and that it accounts for major phosphatase activities in liver and heart.

MATERIALS AND METHODS

Histone H1 (Type III-S) and H2B (Type VIII-S), phosphorylase b, cAMP-dependent protein kinase from bovine heart (P-5511) and phosphatidylserine from bovine brain were obtained from Sigma. [γ-^{32}P]ATP, 35 Ci/mmole was purchased from New England Nuclear. Chromatographic materials were acquired from the following sources: Ultrogel AcA34 (LKB), DE-52 (Whatman), CNBr-activated Sepharose 4B (Pharmacia) and the

Abbreviations: phospholipid, Ca^{+2}-dependent protein kinase - kinase C, PKC; cAMP-dependent protein kinase-kinase A, PKA; PKC phosphorylated histone H1 -H1 (PKC); PKA phosphorylated H1 - H1 (PKA); dithiothreitol, DTT

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MonoQ anion exchange HPLC column (Pharmacia). 1,6-diaminohexane (Aldrich) and histone H1 (10 mg/ml gel) were coupled to CNBr-activated Sepharose 4B by the method recommended by Pharmacia. Histones were phosphorylated by PKC in bulk in the following reaction mixture: 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.3 mM $CaCl_2$, 40 µg/ml phosphatidylserine, 250 µM histone, 250 µM ATP(0.25 µCi/nmole [γ-^{32}P]ATP). PKC isolated from rat brain by Mono Q anion exchange chromatography (9) was added to start the reaction at 30°C followed 15 min later by a second addition using sufficient PKC to attain maximal phosphorylation after 30 min. For PKA-phosphorylated histones the calcium and phosphatidylserine were replaced by 10 µM cAMP and 400 picomolar units (Sigma) of bovine heart cAMP-dependent protein kinase. Phosphorylated histones were recovered by precipitation with 20% (w/v) trichloroacetic acid and cycled through resolubilization-precipitation (10). Typically >95% of the recovered ^{32}P-histone was trichloroacetic acid-precipitable. Phosphorylase b was phosphorylated with phosphorylase kinase (11). Purity of the phosphorylated substrates and potential proteolytic degradation of substrates were monitored by SDS-gel electrophoresis (9). Phosphatase assays were performed with 25 µg of ^{32}P-H1 (12.5 nCi/µg), for 5 min at 30°C in 100 µl of 50 mM Tris-HCl, pH 7.6, 1 mM EDTA-1 mM dithiothreitol followed by precipitation with 1 ml of 20% trichloroacetic acid and filtration on 0.45 µm HAWP (Millipore) filters (9). Rat skeletal muscle phosphatase inhibitors were prepared by heat treatment (12). Heart protein phosphatase was purified according to (13) through the second DEAE step.

Rat liver cytosol was obtained (9) in the presence of 50 µg/ml phenylmethylsulfonyl fluoride, 100 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 µg/ml antipain and 1 mM EDTA to forstall proteolytic degradation in a buffer containing 50 mM Tris-HCl-0.25 M sucrose, pH 7.4. All manipulations were carried out at 4°C or below.

RESULTS AND DISCUSSION

Standard methods were adapted for the purification of rat liver H1 (PKC) phosphatase. The activity was precipitated between 30 and 55% saturated ammonium sulfate. The pellet was resuspended in 1/20 volume of 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM DTT (Buffer A) and dialyzed for 6 hours against Buffer A. After removing insoluble material by centrifugation, the ammonium sulfate fraction was applied to a 280 ml Ultrogel AcA 34 column equilibrated with Buffer A and 10 ml fractions collected. The peak of phosphatase activity, eluted as previously described (9), was loaded onto a DE-52 column and eluted as indicated in the legend to Fig. 1A. Considerable further purification was afforded by aminohexyl-Sepharose 4B chromatography of the dialyzed DE-52 enzyme (Table 1, Fig. 1B). Subsequent chromatography of the enzyme on histone H1-Sepharose 4B and elution with a salt gradient did not yield greater purification. Either isoelectric focusing or preparative native gel electrophoresis in a Laemmli buffer system without SDS resulted in poor recovery.

By contrast, gel permeation chromatography of the enzyme in 6 M urea effectively separated enzyme activity from the bulk of the residual contaminating protein (Fig. 1C). This treatment dissociated the catalytic unit from the M_r 150,000 holoenzyme providing a homogeneous protein of M_r 34,000 (Fig. 1D) on SDS-gel electrophoresis. Similar purifica-

Table 1. Purification of Rat Liver H1 (PKC) Phosphatase

Step	Specific Activity (nmoles min^{-1} mg^{-1})	Total Activity (% recovery)	Fold-purification
1. 30-55% ammonium sulfate pellet	1.4	8148(100)	1
2. AcA34 gel filtration	10	3177(39)	7.1
3. DEAE-cellulose	40	2600(33)	28.6
4. Aminohexyl-Sepharose	400	1652(20)	286
5. AcA34-urea	3215	165(2)	2300

tion but with poorer reproducibility could be achieved by treatment with ethanol (16) followed by gel filtration.

In order to determine the relationship of H1 (PKC) phosphatase to other protein phosphatases, the relative substrate specificity and regulatory properties of the enzyme

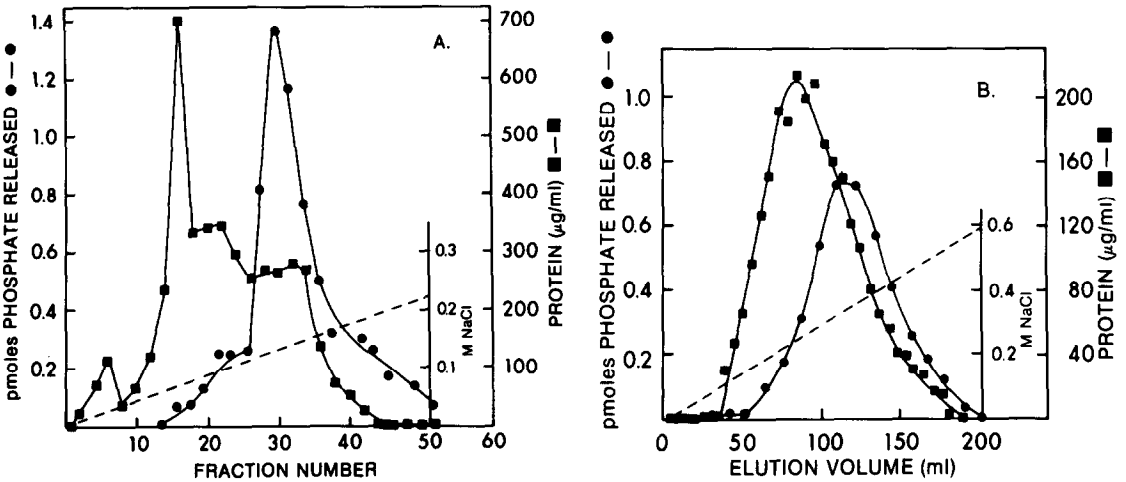

Figure 1: A and B

A. Elution Profile of H1 (PKC) Phosphatase from DEAE-Cellulose. One hundred milliliters of AcA34 phosphatase activity peak (Step 2) was loaded at 2.5 ml/min onto a 50 ml DE-52 column equilibrated with 20 mM Tris-HCl-1 mM EDTA-0.5 mM DTT-2% (v/v) glycerol pH 7.4. After washing with 4-column volumes of buffer, a linear gradient from 0-0.35 M NaCl was applied collecting 10 ml fractions. Five µl aliquots were assayed for phosphatase activity.

B. Elution Profile of H1 (PKC) Phosphatase From Aminohexyl-Sepharose-4B. One hundred and forty milliliters of the DEAE-cellulose phosphatase activity peak (Step 3) was concentrated to 20 ml with 60% ammonium sulfate, dialyzed overnight against 2 ℓ of 20 mM Tris-HCl-1 mM EDTA-0.5 mM DTT - 2% (v/v) glycerol, pH 8.0, and applied to a 3 ml aminohexyl-Sepharose-4B column equilibrated with the same buffer at 4°C. After washing with 60 ml of the column buffer, a 200 ml gradient of 0-0.6 M NaCl was run at 1 ml/minute collecting 6.7 ml fractions. Five µl aliquots were assayed for phosphatase activity.

were investigated. The activity profiles of the following three protein phosphatase preparations were compared: the purified catalytic subunit of the H1 (PKC) phosphatase, its corresponding holoenzyme (purified through Step 4), and a partially purified porcine heart phosphatase. In these experiments, activity was standardized such that the concentration of each of the enzymes tested showed equal digestion of H1 (PKC). It is clear from Table 2 that the relative specificity of the liver enzyme H1 (PKC) over H1 (PKA) resides in its catalytic subunit and that a similar specificity exists in the heart enzyme in a Mn^{+2}-sensitive form. Table 3 shows that H1 (PKC) phosphatase was not sensitive to divalent cation chelators, $MnCl_2$, $MgCl_2$ + ATP, Ca^{+2} + calmodulin, or the skeletal muscle inhibitor.

Results presented in Tables 2 and 3 are consistent with the classification of H1 (PKC) phosphatase as Type 2A according to the criteria summarized by Cohen (17). The

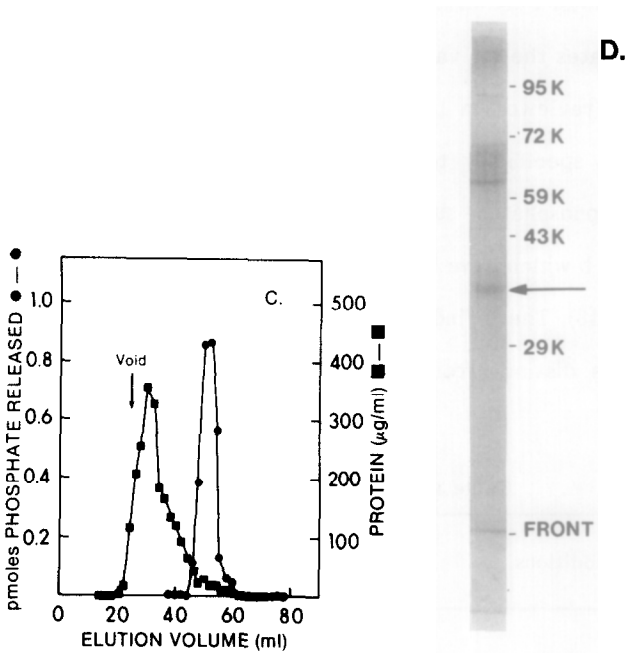

Figure 1: C and D

C. Purification of Catalytic Subunit of H1 (PKC) Phosphatase on Ultrogel AcA 34 in Urea. Twenty-four milliliters of H1 (PKC) phosphatase purified through the amino-hexyl-Sepharose-4B (Step 4) was concentrated to 4 ml with an Amicon YM-10 membrane and the buffer changed to 50 mM Tris-HCl, 1 mM EDTA - 1 mM DTT, pH 8.0. This enzyme was made 6.5 M in urea by the addition of solid, incubated on ice for 30 minutes, then applied to an 80 ml column of Ultrogel AcA 34 equilibrated in the same buffer containing 6 M urea at 4°C. Two-milliliter fractions were collected at 0.12 ml/min and 20 µl assayed for activity in a 200 µl volume.

D. Purified Catalytic Subunit of Liver H1 (PKC) Phosphatase. Silver stained (14) 10% SDS-gel electrophoresis in the system of Laemmli (15) of pool of fractions from AcA 34 column in 6 M urea (Fig. 1C). Molecular weight markers electrophoresed as indicated. Arrow indicates catalytic subunit, * indicates staining artifact.

Table 2. Substrate Specificity of Phosphatase Preparations

Enzyme	Substrate			
	H1 (PKC)	H1 (PKA)	H2B (PKA)	Phosphorylase a
Liver	1	0.17	0.08	0.09
Liver, catalytic	1	0.15	0.10	0.31
Heart	1	0.20	0.15	0.14

Amounts of phosphatase used in each case was determined to catalyze equivalent dephosphorylation of H1 (PKC). 2 mM (free) $MnCl_2$ was included in all assays because of the $MnCl_2$ requirement of the heart enzyme. No effect of $MnCl_2$ was observed on either of the liver enzyme preparations for any substrate. Concentrations of substrates used: H1 (PKC), H1 (PKA), and H2B (PKA) - 12.5 μM; phosphorylase a - 6.4 μM. A 5-min assay at 30°C was used.

catalytic specificity shared with the $MnCl_2$-sensitive heart phosphatase may reside in similar catalytic subunits. Furthermore, the M_r value of the active subunit of H1 (PKC) phosphatase approximates the M_r values reported for the active subunits of certain other protein phosphatases (reviewed in 17). These considerations suggest that phosphoprotein phosphatase catalytic specificity towards H1 (PKC) may represent a more primitive specificity than other phosphatase substrate specificities such as those for phosphorylase a and glycogen synthase b which have been shown to be conferred by the regulatory subunits of the heart enzyme (18). These findings indicate that the phosphorylation site on histone H1 for PKC which is distinct from that selected by PKA (4) is a preferred site of

Table 3. Phosphatase Regulators

Additions	Activity (nmoles phosphate released)
None	1.00
1 mM EDTA or EGTA	1.09
2 mM $MnCl_2$	1.02
10 mM $MgCl_2$ + 2 mM ATP	0.91
200 μM $CaCl_2$ + calmodulin (10 μg/assay)	1.06
Skeletal muscle inhibitor (12 μg protein/assay	1.16

Five μl of aminohexyl-Sepharose 4B-purified phosphatase (Step 4) were assayed by the standard procedure using H1 (PKC) as a substrate with the noted additions.

dephosphorylation for one or more similar protein phosphatases. The relevance of these findings to the phosphorylation-dephosphorylation of H1 in vivo may be associated with growth-related events involving H1 (9,19-21) and may also obtain for other PKC substrates. Finally, H1 (PKC) represents a new tool for use in the detection, purification, and characterization of phosphatases since activities observed with this substrate are over fivefold higher than those observed with H1 (PKA). H1 (PKC) dephosphorylation may prove to be a suitable novel criterion for the classification of such enzymes.

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