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EVIDENCE THAT THE HEAT-STABLE PROTEIN ACTIVATOR OF PHOSPHORYLASE PHOSPHATASE IS HISTONE H1

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The heat-stable protein activator of phosphorylase phosphatase [FEBS Lett. 146, 331-334 (1982)] was located in the nuclear fraction. The activator was isolated from the nuclear fraction of various tissues. The largest amount was obtained from calf thymus and swine renal cortex. The activator protein from swine renal cortex was purified to apparent homogeneity. The activator had the same mobility as authentic histone H1 on sodium dodecyl sulfate or acetic acid/urea polyacrylamide gel electrophoresis. Phosphorylase phosphatase was activated by histone H1 or purified activator in an identical manner. Other histones were without effect or inhibited phosphorylase phosphatase activity. It is concluded that the heat-stable protein activator of phosphorylase phosphatase is histone H1.

Phosphorylase phosphatase activity is believed to be regulated by heat-stable protein modulators (1). Two heat-stable proteins (inhibitor-1 and inhibitor-2) have been purified from rabbit skeletal muscle and characterized (2,3). The activity of both proteins is modulated by phosphorylation (4,5). Recently, we reported the isolation of a heat-stable protein activator of phosphorylase phosphatase (6). The activator, which was purified from 2% trichloroacetic acid extracts of renal cortex, activated phosphatase activity by decreasing the Km of the enzyme for phosphorylase (6). There have been several other reports of heat-stable protein activators of phosphoprotein phosphatases (7-9). However, these activators did not stimulate the dephosphorylation of phosphorylase a (7,9).

In this communication, we report that the phosphorylase phosphatase activator is located in the nuclear fraction. The activator was extracted from crude nuclear preparations and purified to homogeneity. Evidence that the activator is histone H1 is presented. Activation of phosphorylase phos-

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phatase by authentic histone H1 was specific. Other histones did not stimulate the enzyme.

MATERIALS AND METHODS

Materials. Phosphorylase phosphatase activator was purified from swine kidney cortex through the phosphocellulose step as previously described (6). This is referred to as the standard activator preparation. The activator was further purified by chromatography on Phenyl-Sepharose (Pharmacia Fine Chemicals). Standard activator (1.6 mg) in 5 mM phosphate buffer (pH 7.0) containing 0.37 g ammonium sulfate/ml was applied to a 3 ml Phenyl-Sepharose column equilibrated in the same buffer. The column was washed with seven bed volumes of equilibration buffer and then eluted with a 31 ml descending linear gradient (0.37 to 0 g ammonium sulfate/ml in 5 mM phosphate buffer). The protein and activity peak which coeluted from the column between 0.18 and 0.06 g ammonium sulfate/ml was dialyzed against 50 mM imidazole and 5 mM EGTA (pH 7.4). The catalytic subunit of phosphorylase phosphatase was prepared from rabbit renal cortex (10). ^{32}P-Labeled rabbit muscle phosphorylase a was prepared from crystallized phoshorylase b (11). Catalytic subunit of cyclic AMP-dependent protein kinase, prepared as previously described (12), was a gift from Dr. Erwin Reimann. Histone H1 was purified from calf thymus (13) or was obtained from Boehringer Mannheim Biochemicals. Histone H2b and Histone H4 were purchased from Boehringer Mannheim Biochemicals. A mixture of core histones (H2a, H2b, H3 and H4) was prepared from mixed histones (Aldrich Chemical Co.). The mixed histones were dissolved in water and precipitated with 5% perchloric acid. The insoluble core histones were collected by centrifugation, dissolved in water, and the precipitation repeated 3 additional times. SDS-PAGE showed that histone H1 was completely removed from the core histones. High mobility group proteins (HMG 1 and HMG 2) were a gift from Dr. William Scovell. Molecular weight standards, low molecular weight range, were purchased from Bio-Rad Laboratories. Kidneys were obtained from a local slaughterhouse and transported to the laboratory on ice. The cortex was removed and used immediately or stored at -70°C.

Methods. Phosphorylase phosphatase was assayed by measuring the release of $^{32}P_i$ from [^{32}P] phosphorylase a (6). Phosphorylase phosphatase activator was assayed in a 100 µl incubation mixture containing 10 munits/ml phosphatase and 1.03 µM [^{32}P]phosphorylase a dimer. A unit of activator doubles the phosphatase activity under standard assay conditions. Proteins were determined by the Lowry method as modified by Hartree (14). SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed at the indicated acrylamide concentration according to the method of Laemmli (15). Acetic acid urea gel electrophoresis containing 6.25 M urea, 0.9 N acetic acid and 15% polyacrylamide was performed as described by Panyim and Chalkley (16). Proteins were stained overnight with Coomassie Brillant Blue R-250.

To study the subcellular distribution of the activator, swine renal cortex was homogenized in the presence of 10 volumes of 30 mM histidine, 1 mM EDTA and 250 mM sucrose, pH 7.2. The homogenate was fractionated by differential centrifugation at 1,000 x g for 10 min, 6,000 x g for 15 min and 48,000 x g for 30 min. The pellets were washed by resuspending in homogenizing buffer and repeating the centrifugation step. The recentrifuged pellets were suspended in 4 mM EDTA and trichloroacetic acid was added to each suspension and the 48,000 x g supernatant to a concentration of 1.7%. Insoluble material was removed by centrifugation and the supernatants were fractionated between 1.7 and 15% trichloroacetic acid. The precipitate was dissolved in 500 mM K_2HPO_4, 1 mM EDTA and if necessary adjusted to pH 7 with 6 N NH_4OH. The samples were then extensively dialyzed against 10 mM phosphate, 1 mM EDTA, pH 6.9. Crude nuclear fractions were prepared from several tissues as described above for the 1,000 x g pellet and purified rat liver

nuclei were prepared by sucrose density centrifugation as described by Blobel and Potter (17). Activator was prepared from the 1.7 to 15% trichloroacetic acid fractions as described above.

RESULTS

The standard activator preparation, purified from 1.7% trichloroacetic acid extracts of swine renal cortex, had two major bands on SDS-PAGE with apparent molecular weights of 32,000 and 31,000 (FIgure 1A). There was also a minor band at 28,000. The latter contaminating protein could be removed from the activator preparation by hydrophobic chromatography on Phenyl-Sepharose (Figure 1B). Activator increased the activity of phosphorylase phosphatase by as much as 300% and had a specific activity of about 4,000 activator units/mg protein (Figure 2).

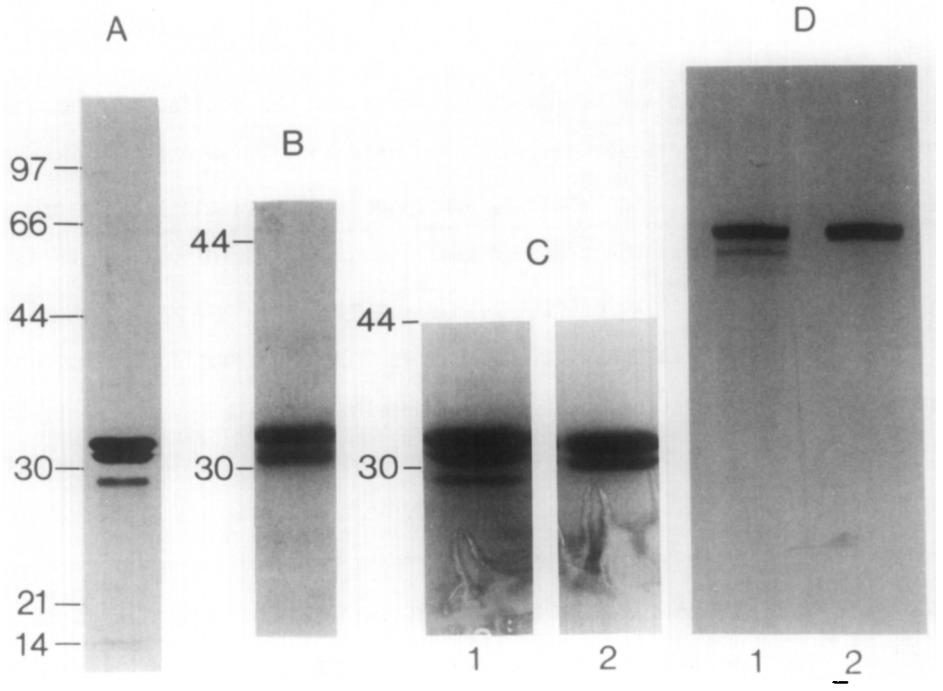

Figure 1. Polyacrylamide gel electrophoresis of activator purified from swine kidney cortex and histone H1. Electrophoresis was performed as described in Materials and Methods. Positions of the standard protein markers are indicated by arrows (molecular weight $x10^{-3}$). A. Standard activator (5 µg) was subjected to electrophoresis on 0.1% SDS and 10% polyacrylamide gel. B. Activator (2 µg) purified through the Phenyl-Sepharose step was subjected to electrophoresis on 0.1% SDS and 10% polyacrylamide gel. C. In Lane 1 standard activator (5 µg) and in Lane 2 histone H1 (5 µg) were subjected to electrophoresis on 0.1% SDS and 15% polyacrylamide gel. D. In Lane 1 standard activator (7 µg) and in Lane 2 histone (7 µg) were subjected to electrophoresis on the acetic acid/urea polyacrylamide gel system.

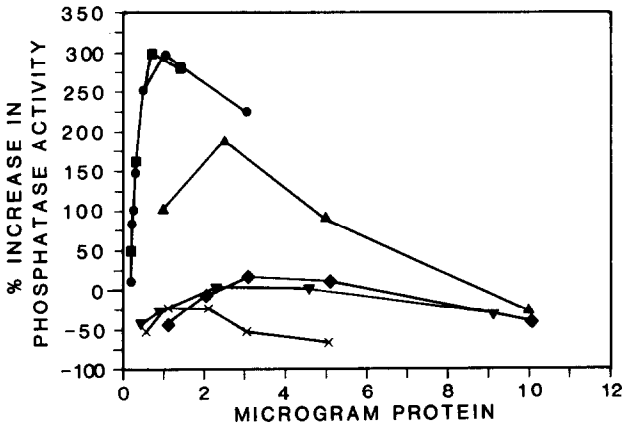

Figure 2. Effect of activator and various histones on the activity of phosphorylase phosphatase. The assay was performed as described in Materials and Methods in the presence of various concentrations of standard activator [■], histone H1 [●], mixed histones [▲], core histones [▼], histone H2B [◆] or histone H4 [×].

The subcellular distribution of the activator was studied by fractionation of the renal cortical homogenate by differential centrifugation. Nearly all of the activator activity was associated with the 1,000 x g insoluble crude nuclear fraction. A small amount of activity was associated with the 6,000 x g insoluble fraction. All other fractions were without activator activity. Activator was also prepared from crude nuclear fractions of rat liver, bovine heart, rabbit skeletal muscle, and calf thymus. Activator activity was obtained from each with the greatest amount isolated from calf thymus followed by swine kidney cortex, bovine heart, rat liver and rabbit skeletal muscle. The specific activity of each of the preparations, ranged from 2,000 to 2,500 activator units/mg protein. On SDS-PAGE, the 1.7 to 15% activator fractions prepared from each of the tissues showed two prominent protein bands and a number of minor protein bands. The lower mobility prominent band obtained from all tissues had the same mobility as the lower mobility band in the standard kidney preparation. There were slight variations in the mobility of the other prominent band. Activator could also be isolated from purified liver nuclei. On SDS-PAGE, the latter had the same two prominent bands as activator isolated from the crude liver nuclear fraction.

Based on the above evidence, the activator appeared to be a major protein which could be acid extracted from nuclei. Two classes of proteins are known to be acid extracted from nuclei: HMG proteins and histone H1 (13). Histone H1 proteins were purified from calf thymus and their mobility on SDS-PAGE compared with the standard activator preparation. Histone H1 was separated into two subfractions, with the same mobility as the activator protein bands (Figure 1C). As previously reported (18), histone H1 subfractions with molecular weights of approx. 21,000 gave anomalously high apparent molecular weights on SDS-PAGE. HMG 1 and HMG 2 (data not shown) and HMG 14 and HMG 17 (19) have a higher mobility than the H1 subfractions and are clearly different than the proteins in the purified activator preparation. Acetic acid/urea polyacrylamide gel electrophoresis separates histone H1 from other classes of histones (16). The activator had the same mobility as histone H1 in the acetic acid/urea system (Figure 1D). Thus it appeared that the activator was histone H1.

Activation of phosphorylase phosphatase by histone H1 purified from calf thymus is shown in Figure 2. Increasing concentrations of histone H1 activated phosphorylase phosphatase in a manner virtually identical to that obtained with activator purified from kidney cortex. Mixed histones, which contain histone H1, caused some activation. Other histones were without effect or inhibited phosphorylase phosphatase activity.

DISCUSSION

Our present findings indicate that the heat-stable protein activator of phosphorylase phosphatase which we have purified from swine renal cortex is histone H1. The physiological significance of this nuclear protein in the regulation of cytoplasmic glycogen metabolism is unknown. It is of interest that of all the histones, histone H1 specifically activates the phosphorylase phosphatase at a low concentration (0.1 μM histone H1 doubles the activity in the standard assay). Phosphorylase phosphatase activity is increased primarily by decreasing the Km of the phosphatase for phosphorylase (6). Specifically decreasing the Km for one substrate of a multifunctional

phosphoprotein phosphatase would be a mechanism for increasing the substrate specificity of the phosphatase. Even if histone H1 is not the physiologically important ligand, it has revealed a possible mechanism whereby an unknown ligand could specifically modulate the dephosphorylation of phosphorylase a.

It will be of interest to investigate the effect of histone H1 on nuclear phosphoprotein phosphatases. Recently, Dopere and Stalmans (20) reported that about one-half of the phosphorylase phosphatase activity of rat liver extracts was associated with the nuclear fraction. In a preliminary study, we have isolated from a liver nuclear fraction a phosphorylase phosphatase which was stimulated by histone H1 (unpublished data). It will be important to determine the effect of histone H1 on the dephosphorylation of nuclear phosphoproteins.

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