PHOSPHOPROTEIN PHOSPHATASE ACTIVITY OF NOVIKOFF HEPATOMA NUCLEOLI

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SUMMARY

Nucleoli from Novikoff hepatoma ascites cells contain phosphatase activity that acts upon ^{32}P-labeled nucleolar protein substrates. The activity is optimal near pH 7.0 and is inhibited by increasing concentrations of NaCl. The divalent cations $CaCl_2$, $MnCl_2$ and $CoCl_2$ at 6 mM inhibited phosphatase activity from 30-60%. $ZnCl_2$ completely inhibited the activity above 2 mM while EDTA and $MgCl_2$ had little effect. The activity was stimulated by dithiothreitol and inhibited by N-ethylmaleimide indicating a requirement for free sulfhydryl groups.

INTRODUCTION

Phosphorylation of nuclear proteins has been suggested to play a role in control of gene activity (1-5) or in the maturation of preribosomal particles (6). The rapid turnover of phosphate groups in these proteins (7,8) indicates that dephosphorylation is as important as phosphorylation. The multiple kinases of the forward reaction of phosphorylation have been studied extensively (9-12). However, little is known about nuclear phosphoprotein phosphatases.

Numerous nonhistone proteins in nucleoli of Novikoff hepatoma are phosphorylated (13,14) and the profile of ^{32}P labeling of proteins is dependent on the divalent metal ions added to the system (15). This study indicates the presence in nucleoli of phosphatase activity which dephosphorylates ^{32}P-labeled nucleolar protein substrates.

MATERIALS AND METHODS

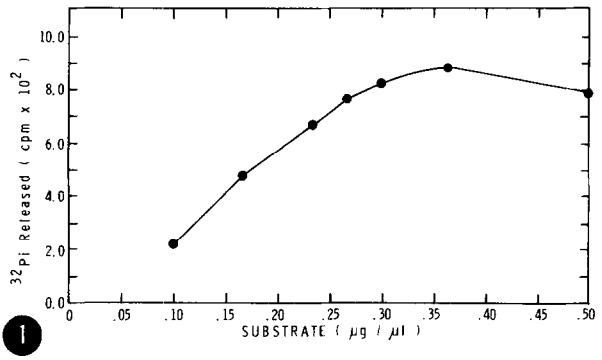
Nucleoli and Nucleolar Protein Substrates - Novikoff hepatoma ascites cells were transplanted into male albino rats (Holtzman Co., Madison, Wisc.) 6 days prior to the experiments. Nucleoli were isolated as previously described (16,17). For ^{32}P labeling of nucleolar proteins, Novikoff hepatoma cells were suspended in the medium described by Mauritzen et al (18) and incubated for 2 hours at 37° with ^{32}P-orthophosphate (100 mCi/50 g of cells). Nucleoli were isolated and the proteins were extracted with 0.4 N H_2SO_4, precipitated and washed with ethanol and dried in vacuo (13).

Phosphoprotein Phosphatase Assay - Release of ^{32}P as inorganic phosphate was measured by a system based on that of Ullman and Perlman (19). A typical substrate mixture contained (a) 0.1 ml 0.33 M Bistris, pH 7.2; (b) 0.1 ml of water or substances to be tested for stimulatory or inhibitory activity; and (c) 0.05 ml of ^{32}P-labeled 0.4 N H_2SO_4 extracted nucleolar proteins (20,000-180,000 cpm/mg protein) at a concentration of 1.0 mg/ml. The reaction was initiated by the addition of 0.05 ml nucleoli homogenized in water at a protein concentration of 1.6 mg/ml (determined by the A280 nm:A260 nm in 6 M guanidine hydrochloride). After various times of incubation at 37°, the reaction was stopped by addition of 25 µl of 0.1 M silicotungstic acid in 0.1 N H_2SO_4 and then cooled in an ice bath. To this was added 100 µl of 5% $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$ in 4 N H_2SO_4 followed by 100 µl of bovine serum albumin (10 mg/ml in water). The mixture was extracted with 0.5 ml of isobutanol:benzene, 1:1, and aliquots of the upper organic phase were counted in a Beckman LS 230 scintillation counter.

RESULTS

For Novikoff hepatoma nucleoli incubated with ^{32}P-labeled nucleolar protein substrates, the release of $^{32}P_i$ was linear out to about 30 minutes. Initial velocity time points for assays in this paper were taken at 20 minutes. Increasing amounts of substrate added to constant amounts of nucleoli (Fig. 1) resulted in saturation at a ratio of substrate protein to enzyme protein of 1.25:1 (w/w). This data plotted as the double reciprocal (20) was nonlinear, possibly indicating multiplicity of enzymes or substrates.

The phosphatase activity of Novikoff hepatoma nucleoli was maximal near pH 7.0 (Fig. 2) although the activity was relatively high over the broad range of pH 5.5-8. The enzyme activity was inhibited by increasing concentrations of NaCl with 50% inhi-



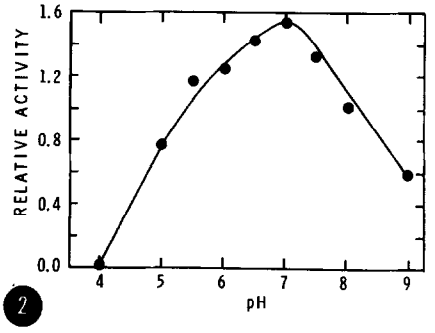


Figure 1 Phosphoprotein phosphatase activity vs substrate concentration for Novikoff hepatoma nucleoli. Assay conditions were as described in the Materials and Methods section. Nucleoli containing identical amounts of protein (50 µg) were incubated with increasing amounts of ^{32}P-labeled nucleolar protein substrate for 20 minutes at 37° in 0.011 M Bistris buffer, pH 7.2, containing 6 mM dithiothreitol in a total volume of 0.3 ml.

Figure 2 The pH dependence of phosphatase activity of isolated Novikoff hepatoma nucleoli against ^{32}P-labeled acid extracted nucleolar proteins. Each point represents a 20 minute assay at 37° using 0.011 M Bistris buffer adjusted to the desired pH with acetic acid. Each tube contained 80 µg of ^{32}P-labeled nucleolar protein substrate and nucleoli containing 50 µg of protein in a total volume of 0.3 ml. The pH 8.0 point (150 cpm released/20 min) was arbitrarily chosen as 1.0 on the relative activity scale.

bition at approximately 0.075 M. Subsequent assays were done near neutrality (pH 7.2) and at low or no salt to maintain optimal activity.

Divalent cations were generally inhibitory to phosphoprotein phosphatase activity of nucleoli. At 6 mM, $MgCl_2$ had little if any effect whereas $MnCl_2$, $CaCl_2$ and $CoCl_2$ inhibited activity from 30-60%. At concentrations greater than 1 mM, $ZnCl_2$ destroyed essentially all of the activity. EDTA had no effect at any concentration up to 20 mM suggesting that divalent cations are not required for phosphatase activity.

The effects of a reducing agent, dithiothreitol, and a sulf-

hydryl blocking agent, N-ethylmaleimide, were also tested. Dithiothreitol stimulated activity at all concentrations tested but was maximal in its stimulation (20%) at approximately 6 mM. N-ethylmaleimide was inhibitory at all concentrations but maximal inhibition (60%) was attained near 8 mM.

DISCUSSION

The steady state level of phosphorylation of nucleolar phosphoproteins is dependent on the rate of attachment of phosphoryl groups combined with the rate of dephosphorylation. Previously, nucleoli were shown to contain enzymes capable of phosphorylating nucleolar proteins (15,21) or exogenous substrates (22,23). The present studies indicate that nucleoli also contain phosphatase activity which acts on homologous nucleolar protein substrates. Unlike the kinase activity which requires divalent cations (15), the phosphatase activity is not metal ion dependent and is generally inhibited by their presence. Although the precise role or content of metal ions in nucleoli is not known, localized concentrations of divalent cations may participate in regulatory processes.

Inhibition by the sulfhydryl blocker N-ethylmaleimide indicates that free sulfhydryl groups may be required for activity. The inhibition by the heavier divalent metals, zinc and cobalt, may also be due to interference with free sulfhydryl groups.

In contrast to the inhibition of this activity by zinc. *E. coli* (24) or chicken intestine (25) alkaline phosphatases are zinc metalloenzymes. A phosphoprotein phosphatase activity with a similar response to divalent metals but an opposite response to monovalent cations was recently found in tobacco hornworm (26). This nucleolar phosphatase also differs from rat liver histone phosphatase (27) in that the latter requires monovalent

ions at ionic strengths of 0.1-0.2. Thus, this phosphatase activity appears to be unique in several characteristics. Studies on the specificity and function of this phosphatase activity will require its purification and further characterization.

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