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NUCLEOLAR PHOSPHOPROTEIN PHOSPHATASE FROM NOVIKOFF HEPATOMA AND RAT LIVER: CHARACTERIZATION AND PARTIAL PURIFICATION

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Summary

Phosphoprotein phosphatase which dephosphorylates ^{32}P-labeled nucleolar protein substrates was found in nucleoli of Novikoff hepatoma ascites cells and normal rat liver. The activity was extracted in high yield from nucleoli with 0.01 M Bis/Tris (pH 7.0). Low ionic strength was also required for activity: the activity was only 50% of maximum in 0.075 M NaCl. Activity was affected differently by various divalent cations: $MgCl_2$ had little effect; $CaCl_2$, $MnCl_2$ and $CoCl_2$ above 4 mM inhibited the activity 30—60%; $ZnCl_2$ above 2 mM completely destroyed the activity. EDTA had no effect, indicating that divalent cations are probably not required. The enzyme activity was enhanced 20% by 5—8 mM dithiothreitol and was inhibited 60% by 7—10 mM *N*-ethylmaleimide indicating a requirement for free sulfhydryl groups. The K_m of the extracted enzyme for ^{32}P-labeled nucleolar protein was 0.6 mg/ml. The phosphatase was capable of dephosphorylating the major phosphorylated nucleolar proteins C23-24 and B23-24 and also histone H_1. The enzyme was purified more than 200-fold on hydroxyapatite followed by DEAE-Sephadex, which resolved the activity into three major components. The activity of enzyme extracted from Novikoff hepatoma nucleoli was approximately 2.5 times greater than from normal liver nucleoli.

Introduction

The nucleolus is a unique subnuclear organelle which contains the cistrons for preribosomal RNA, the polymerase for their transcription and the system for assembly and processing of preribosomal particles [1,2]. It has been sug-

Abbreviation: EDTA, ethylenediaminetetraacetic acid.

gested that the numerous phosphorylated proteins present in nucleoli [3—5] are involved in the regulation of nucleolar processes. For example, the ^{32}P incorporation into nucleolar proteins in vitro roughly correlates with the rate of RNA synthesis of the nucleoli [6,7].

The kinases for the phosphorylation of most of the phosphoproteins of nucleoli are contained in nucleoli [8—11]. However, the steady state level of phosphorylation of the nucleolar proteins would be expected to be the result of the combined effects of the kinase and phosphatase systems. Since the turnover of phosphate groups in nuclear proteins is considered to be rapid [12,13], dephosphorylation is probably as important as the phosphorylation process.

The presence of phosphoprotein phosphatase in nucleoli was recently reported [14] and studies were undertaken to characterize further and fractionate this enzyme. This paper (a) describes the effects of various mono- and divalent cations and sulfhydryl reagents on the nucleolar phosphoprotein phosphatase activity, (b) examines the specificity of dephosphorylation of nucleolar phosphoprotein substrates, (c) presents evidence for multiple forms of phosphatase activity in nucleoli and (d) compares activities in normal liver and Novikoff hepatoma nucleoli.

Materials and Methods

Animals and tumor cells. Albino rats used in this study were obtained from Holtzman Co. (Madison, Wis.). The normal rats were killed by decapitation and the livers were perfused with an isotonic saline solution containing 0.13 M NaCl, 0.005 M KCl and 0.008 M $MgCl_2$. For Novikoff hepatoma preparations, the ascites cells were transplanted 6 days prior to the experiments. The tumor cells were filtered through cheesecloth and washed several times with the above isotonic saline solution.

Isolation of nucleoli. Nucleoli from normal liver or Novikoff hepatoma were isolated by the sucrose-calcium procedure [1] using sonic oscillation. Purified nucleoli were stored at —70°C prior to use.

^{32}P-labeled nucleolar protein substrates. For substrates labeled in whole cells, Novikoff hepatoma cells, washed as above, were suspended in the medium described by Mauritzen et al. [15] and incubated for 2 h at 37°C with [^{32}P]-orthophosphate (100 mCi/50 g cells). Nucleoli were isolated as above and the proteins were extracted with 0.2 M H_2SO_4, precipitated and washed with ethanol and dried in vacuo [4].

Nucleolar protein substrates were also labeled by incubation of isolated nucleoli with [γ-^{32}P]ATP by the method of Kang et al. [8] as modified by Mamrack et al. [16]. The protein was extracted as above with 0.2 M H_2SO_4 containing 1.3 mM ATP and 1.3 mM NaH_2PO_4.

In some experiments, in vitro ^{32}P-labeled proteins were subjected to preparative polyacrylamide gel electrophoresis [16]. Radioactive bands corresponding to spots C23-24, B23-24 and A17-19 [14] were electrophoretically eluted, dialyzed against 0.9 M acetic acid and lyophilized.

Phosphoprotein phosphatase assay. Release of ^{32}P as inorganic phosphate was measured as previously described [14] by a system similar to that of Ullman and Perlman [17]. The reaction mixture typically contained 500 μg/ml

^{32}P-labeled 0.2 M H_2SO_4-extracted nucleolar protein substrates (20000—180000 cpm/mg protein) and isolated nucleoli (10—666 μg protein/ml) or enzyme extracted as described below. The reaction was run in 0.3 ml 6 mM dithiothreitol and 0.005 M Bis/Tris (adjusted to pH 7.2 with acetic acid). After various times of incubation at 37°C, the reaction was stopped by addition of 25 μl 0.1 M silicotungstic acid in 0.05 M H_2SO_4. The mixture was cooled in ice and 100 μl bovine serum albumin (10 mg/ml water) was added. The mixture was agitated vigorously, allowed to stand in ice for 10 min, and extracted with isobutanol/benzene (1 : 1, v/v; 0.5 ml). Aliquots of the upper organic phase were counted in a Beckman LS 230 scintillation counter after addition of 5 ml Handifluor (Malinckrodt, St. Louis, Mo.).

In some experiments, nucleolar proteins labeled by the in vitro method (with [γ-^{32}P]ATP) were subjected to dephosphorylation and analyzed by paper electrophoresis. The reaction, run at pH 7.2 in 0.03 M *N*-ethylmorpholine (Pierce, Rockford, Ill.), was stopped by acidification to pH 3 with acetic acid. After centrifugation, the supernatant was spotted on Whatman 3 MM paper and analyzed by electrophoresis at pH 1.8 (2% formic acid/8% glacial acetic acid, v/v, in water) followed by autoradiography on Kodak RP Royal X-omat film.

Preparation of crude phosphoprotein phosphatase extract. To determine optimal conditions for extraction, nucleoli were suspended in 0.01 M Bis/Tris (pH 7.2) at 1.6 mg protein/ml. The mixtures (0.15 ml) were incubated at 37°C for 30 min and centrifuged at 7500 × *g* for 3 min. The pellets were extracted twice as above and all supernatants were combined and assayed for phosphatase activity. For large scale preparations the procedure was scaled up accordingly.

Specificity of dephosphorylation of nucleolar proteins. The specificity of dephosphorylation was tested on the whole mixture of ^{32}P-labeled proteins in the substrate and on individual isolated nucleolar protein. To minimize the interference of RNA present in the enzyme extract with electrophoretic analysis, the phosphatase extract was predigested with ribonuclease A. Aliquots of phosphatase extract containing 10 μg protein were preincubated with 0.5 μg of ribonuclease A (Worthington, Freehold, N.J.) at 37°C for 30 min in 0.1 ml 0.01 Bis/Tris (pH 7.2). Subsequently, aliquots of 80 μg in vitro ^{32}P-labeled nucleolar proteins (125 cpm/μg) in the same buffer containing 0.2 mM phenylmethanesulfonyl fluoride were added. After incubation the reaction mixtures were made 0.9 M acetic acid, 10 M urea and 1% β-mercaptoethanol. The samples were allowed to stand overnight at room temperature and were then applied to the first dimension of the polyacrylamide gel electrophoresis system previously described [5,18]. The gels were stained with Buffalo Black and destained as previously described [8]. The gels were then sliced and the radioactivity counted in a scintillation counter [5].

Fractionation of phosphoprotein phosphatase on hydroxyapatite. The crude extract of phosphoprotein phosphatase was applied to columns (1.7 × 9 cm) of hydroxyapatite (BDH Biochemicals, Gallard-Schlesinger, Carle Place, N.Y.) and eluted by a method similar to that of Patel [19]. Briefly, 2.5-ml portions of the extract were dialyzed against 2 M KCl, 1 M urea and 1 mM potassium phosphate buffer (pH 8.0) and applied to the column equilibrated with the same buffer. The column was eluted at 20 ml/h with 10 ml portions of the following

buffers: 1 mM potassium phosphate (pH 8.0), 0.05 M potassium phosphate (pH 8.0), 0.25 M potassium phosphate (pH 8.0), 0.5 M potassium phosphate (pH 6.8), each containing 2 M KCl and 1 M urea. Aliquots of the fractions were dialyzed against 0.01 M Bis/Tris (pH 7.2) and assayed for phosphatase activity and protein and nucleic acid content by absorbance at 260 and 280 nm.

DEAE-Sephadex. The fractions eluting from hydroxyapatite at 0.05 M phosphate were pooled and dialyzed against 0.01 M Bis/Tris (pH 7.2) and applied to a column (0.9 × 29 cm) of DEAE-Sephadex equilibrated with the same buffer. The column was eluted at 20 ml/h with 10-ml portions of NaCl at 0.1, 0.3, 0.5, 0.7 and 1.0 M, all containing 0.01 M Bis/Tris (pH 7.2). The 1-ml fractions were dialyzed against 0.01 M Bis/Tris (pH 7.2) and assayed for phosphatase activity.

Protein, RNA, DNA and phosphate determination. Protein was determined by the Bio-Rad protein assay according to the procedure outlined in Bio-Rad technical bulletin number 1051 (Bio-Rad Laboratories, Richmond, California 94804). In some cases, protein was quantitated on a Beckman 121MB amino acid analyzer after hydrolysis in 5.7 M HCl for 22 h at 110°C in vacuo. RNA was determined by the orcinol method described by Ashwell [20]. DNA content was determined by the modified diphenylamine reaction according to the procedure of Richards [21]. Total phosphate was determined with the inorganic phosphorous kit number 670 of Sigma (St. Louis, Mo.) after ashing [22].

Results

Phosphoprotein phosphatase assay. The phosphoprotein phosphatase activity in Novikoff hepatoma nucleoli was assayed at pH 7.2 with the system previously described [14]. For the initial studies in vivo labeled nucleolar protein was used as the substrate. However, proteins with much higher specific activities could be obtained by in vitro labeling [8]. Therefore, the latter substrate was used for most studies described in this paper.

One unit of enzyme activity was defined as the amount of enzyme that liberates 1 nmol phosphate from the nucleolar protein substrate in one minute at pH 7.2 at 37°C. The phosphate liberated was calculated from the proportion of the total phosphate as ^{32}P-labeled phosphate. Since the phosphate may not be removed uniformly from all sites, the unit is approximate and applies only to the above substrate.

To insure that the phosphatase removed only phosphate from protein, the release of radioactivity from the ^{32}P-labeled substrate was analyzed by paper electrophoresis at pH 1.8 followed by autoradiography. All released radioactivity was in the form of [^{32}P]orthophosphate at all time points up to 90 min of incubation.

Effects of salt, divalent cations and sulfhydryl reagents. The effects of salt and metal ions on the enzyme (as whole nucleolar suspensions) were tested using in vivo labeled nucleolar protein substrate. The enzyme activity was inhibited approx. 50% by 0.075 M NaCl. At a concentration above 0.5 M, inhibition was nearly complete. Therefore, all assays were done at low ionic strength (0.01 M Bis/Tris, pH 7.2).

Divalent cations were generally inhibitory to phosphoprotein phosphatase

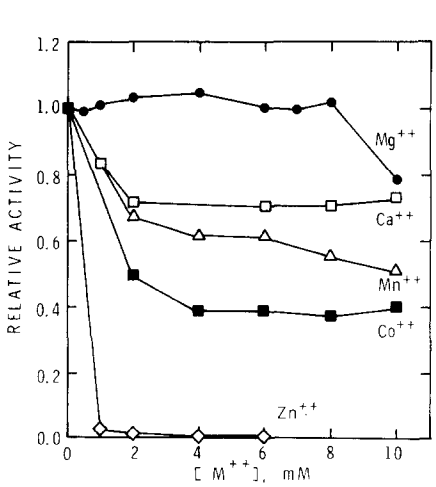
activity (Fig. 1). Above 2 mM, $MnCl_2$, $CaCl_2$ and $CoCl_2$ were moderately inhibitory. At concentrations greater than 1 mM, $ZnCl_2$ completely destroyed the phosphoprotein phosphatase activity. However, $MgCl_2$ had no effect below 8 mM.

To test the effect of removal of metal ions, EDTA was added to the system (Fig. 2). No effect occurred at concentrations to 20 mM. Thus, it is unlikely that any divalent cations are required for the phosphoprotein phosphatase activity.

The effect of KF, an inhibitor of phosphoprotein phosphatase [23,24] was tested. A maximal inhibition of approx. 86% was achieved with 4 mM KF.

To investigate the possible requirement of free sulfhydryl groups for phosphatase activity, the enzyme was assayed in the presence of dithiothreitol or *N*-ethylmaleimide. Dithiothreitol stimulated enzyme activity slightly at all concentrations but was maximal in its stimulation at 5—8 mM (Fig. 2). *N*-ethylmaleimide, after a 30-min preincubation period, was inhibitory at all concentrations (Fig. 2). However, under these conditions, a plateau of about 66% inhibition was attained above 7 mM. Thus, free sulfhydryl groups appear to be required for the bulk of phosphoprotein phosphatase activity.

Extraction of phosphoprotein phosphatase. Phosphoprotein phosphatase activity was readily extracted from nucleoli at low ionic strength (0.01 M Bis/Tris, pH 7.2). Incubation at 37°C rather than at lower temperatures was required for maximal release of activity (Table I). The release did not appear to be dependent on endogenous proteases since phenylmethanesulfonyl fluoride, a protease inhibitor, had no effect on the extraction.



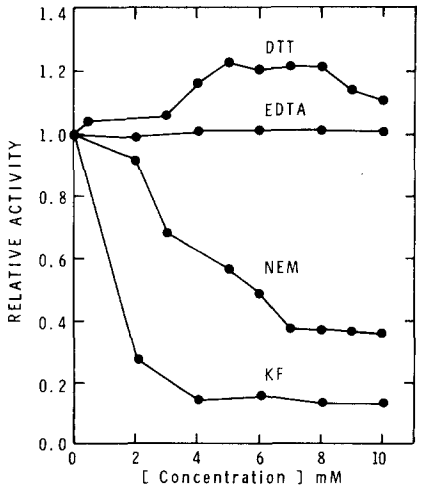


Fig. 1. The effect of divalent metals on phosphatase activity. Assays were performed with increasing concentrations of divalent cations. (●) $MgCl_2$, (□) $CaCl_2$; (△) $MnCl_2$; (■) $CoCl_2$; (◇) $ZnCl_2$. (1.0 = 150 cpm released).

Fig. 2. Effect of dithiothreitol, EDTA, *N*-ethylmaleimide and KF on phosphoprotein phosphatase activity. Assays were performed with increasing concentrations of added reagents. In the case of *N*-ethylmaleimide, the nucleoli were preincubated with the reagent for 30 min at 25°C prior to addition of substrate. Curves are indicated from top to bottom: DTT, dithiothreitol, upper curve; EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; KF, potassium fluoride, lower curve. (1.0 = 190 cpm released).

TABLE I

RECOVERY OF EXTRACTED PHOSPHOPROTEIN PHOSPHATASE ACTIVITY

Nucleoli were suspended in deionized water at a concentration of 1.6 mg protein/ml. The mixtures (0.15 ml) were incubated at 37°C for 30 min and centrifuged at 7500 X *g* for 3 min. The pellet was then re-extracted twice more as above. Assays were performed for 30 min on identical aliquots of control nucleoli, on the 3 extracts and on the pellet after the three extractions. PMSF, phenylmethanesulfonyl fluoride.

	cpm/30 min	% of control	Protein (μg)
Unextracted control nucleoli	31 747	100	364
Extraction at 4°C	10 461	33.0	N.D.
1st extraction (37°C)	13 948	43.9	160
1st extraction (37°C, 0.1 mM PMSF)	13 956	44.0	N.D.
2nd extraction (37°C)	8 368	26.4	17
3rd extraction (37°C)	949	3.0	60
Pellet	6 686	21.1	167
Total extracted *	23 265	73.3	237
Total recovered **	29 951	94.3	404

* Includes total activity and protein solubilized in the 3 sequential extractions.
** Includes total extracted plus pellet.

The extraction was also pH-dependent. In a 30-min extraction at 37°C, a pH of 7.0 or greater was required for maximal release of activity. Therefore, subsequent extractions were done at pH 7.2 in 0.01 M Bis/Tris buffer.

To determine the extent of extraction of phosphatase activity by this method, nucleoli were extracted with 0.01 M Bis/Tris (pH 7.2) at 37°C with three 30-min sequential extractions. Table I indicates that extractions 1, 2 and 3 released approx. 44%, 26% and 3% of the activity, respectively. Thus, nearly three-quarters of the phosphoprotein phosphatase activity in the nucleoli was removed by this extraction method.

K_m of phosphoprotein phosphatase. The crude phosphatase extract was diluted 50-fold and incubated for 20 min at 37°C with various concentrations of in vitro ^{32}P-labeled nucleolar protein substrate. When the data were plotted as the double reciprocal [25] the K_m was found to be 0.6 mg/ml (Fig. 3).

Dephosphorylation of nucleolar proteins. The specificity of dephosphorylation was tested on the whole mixture of extracted nucleolar proteins and on isolated nucleolar proteins. When the mixture of in vitro ^{32}P-labeled nucleolar proteins was incubated with extracted enzyme for a period of 1.5 h at 37°C the ^{32}P was released from both of the major phosphorylated peaks (Fig. 4) previously identified as protein bands C23-24 and B23-24 [4].

Analysis of the stained gels (Fig. 5) of the nucleolar proteins indicated that the major radioactive bands, C23-24 and B23-24 did not decrease in staining intensity after a 2-h incubation with extracted phosphatase. Furthermore, there was no evidence of appearance of new bands at lower molecular weight. Thus, it is not likely that the decrease in radioactivity of the protein peaks was due to degradation of protein during incubation.

The specificity of dephosphorylation was also tested by incubating individual ^{32}P-labeled proteins isolated by preparative gel electrophoresis with the extracted phosphatase. Fig. 6 indicates that all of the isolated bands were susceptible to dephosphorylation but that none were completely dephosphorylated. Approximately the same percentage of the total radioactivity (13—16%)

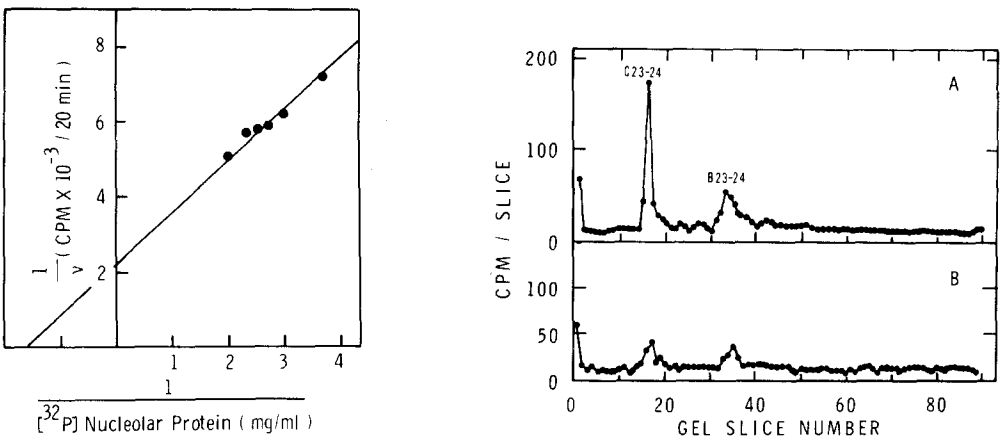


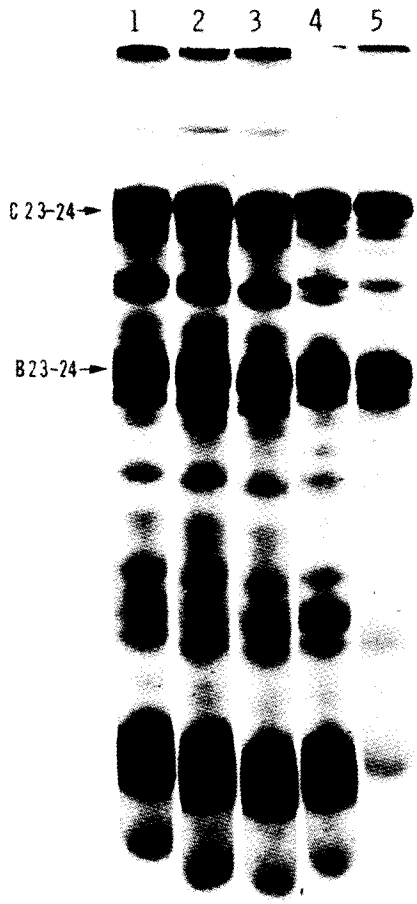
Fig. 3. Kinetics of action of nucleolar phosphoprotein phosphatase on nucleolar protein substrates. Extracted nucleolar phosphoprotein phosphatase was diluted 1/50 and identical aliquots were incubated for 20 min at 37°C with various concentrations of ^{32}P-labeled nucleolar protein substrate. The released $^{32}P_i$ and substrate concentration were plotted as reciprocals.

Fig. 4. Analysis of dephosphorylation of nucleolar proteins by polyacrylamide gel electrophoresis. Phosphoprotein phosphatase extract was prepared containing 10 μg of protein and incubated for 90 min with 80 μg in vitro ^{32}P-labeled nucleolar proteins in phenylmethanesulfonyl fluoride, and 6 mM dithiothreitol in a total volume of 0.2 ml. The reaction was stopped by making the solution to 0.9 M acetic acid, 10 M urea and 1% 2-mercaptoethanol. After standing overnight at room temperature 200-μl aliquots of the samples were run in an acid-urea 6% polyacrylamide gel electrophoresis system. (A) control, unincubated substrate. (B) substrate digested 90 min with phosphoprotein phosphatase extract.

was released from bands C23-24 and B23-24 after 90 min of incubation. No additional radioactivity was released when an equal amount of fresh enzyme was added after 60 min incubation indicating that the plateau was not due to inactivation of the enzyme. Approx. 45% of the radioactivity was removed from band A17-19, which corresponds to the H_1 histone [4].

To test whether the lack of complete dephosphorylation of isolated nucleolar proteins is due to "buried" phosphorylated sites, the phosphatase assays were also run in the presence of 1 M urea. Essentially complete activity was maintaned at this concentration. The percentage of dephosphorylation was similar in the absence and in the presence of urea for proteins A17-19 and C23-24. However, urea increased the amount of dephosphorylation of protein B23-24 from approx. 15% to 35%, suggesting that more sites are exposed by the presence of urea.

Fractionation of phosphoprotein phosphatase. The extracted enzyme contained much associated nucleic acid (approx. 45%, Table II) which interfered with chromatographic separation. The bulk of the nucleic acid was effectively removed by chromatography of the extract on hydroxyapatite columns run in the presence of 2 M KCl and 1 M urea. The major peak of phosphatase activity was eluted with 0.05 M phosphate (Fig. 7). This peak was clearly separated from the following peak which eluted at 0.25 M phosphate and contained most of the nucleic acid. However, some RNA remained in the major peak of activity (Table II) and appeared to be closely associated with the enzyme. The hydroxyapatite step resulted in a 14-fold purification of the phosphoprotein phosphatase and appeared to activate the enzyme, possibly by removal of



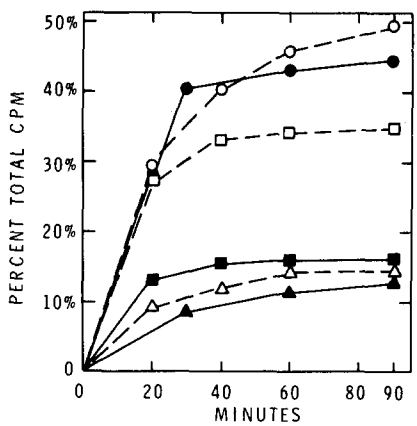


Fig. 5. Polyacrylamide gel electrophoresis of nucleolar protein substrates before and after nucleolar phosphoprotein phosphatase treatment. Nucleolar protein substrates before or after treatment with phosphatase were dialyzed overnight against 0.9 M acetic acid, 10 M urea, and 1% 2-mercaptoethanol. The samples were applied to the system of polyacrylamide gel electrophoresis as above and run for 5 h at 120 V constant voltage. The gels were stained with Buffalo Black. (1) 80 μg nucleolar acid extract, +46 μg extracted phosphoprotein phosphatase, unincubated; (2) 80 μg nucleolar acid extract +46 μg phosphatase incubated 2 h at 37°C; (3) same as (2) but containing 0.2 mM PMSF; (4) 80 μg nucleolar acid extract; (5) 46 μg phosphoprotein phosphatase extract.

Fig. 6. Dephosphorylation of isolated ^{32}P-labeled nucleolar proteins. Isolated in vitro ^{32}P-labeled nucleolar proteins were incubated for various times with 138 μg of crude phosphoprotein phosphatase extract in 0.011 M Bis/Tris (pH 7.2) and 6 mM dithiothreitol in a total volume of 0.43 ml. Released ^{32}P was measured. Results are expressed as percent of total CPM in each time point. (▲) 48 μg protein C23-24 containing 3000 cpm; (■) 46 μg protein B23-24 containing 2095 cpm; (●) 46 μg of protein A17-19 (histone H_1) containing 602 cpm: open circles, squares and triangles indicate parallel reactions which were run in reaction mixtures containing 1 M urea.

competing unlabeled substrates.

The major peak of activity was further chromatographed on DEAE-Sephadex and eluted stepwise with increasing concentrations of NaCl (Fig. 8). 3 major peaks of activity were consistently observed, suggesting multiple components of phosphoprotein phosphatase activity. The DEAE-Sephadex chromatography resulted in a purification of more than 200-fold for each of the

TABLE II

PURIFICATION OF PHOSPHOPROTEIN PHOSPHATASE

Aliquots from the three stages of purification were dialyzed against 0.01 M Bis/Tris (pH 7.2) and assayed for phosphoprotein phosphatase activity, or protein, RNA and DNA content. All assays were performed with the same batch of in vitro ^{32}P-labeled nucleolar protein substrate. All activities were derived from 15-min assays and were standardized to the total activity in nucleoli containing 2.5 mg protein.

Fraction	Total activity (nmol/min)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	% yield	Total RNA (mg)	Total DNA (mg)
Nucleoli	3.21	2.5	1.28	—	100	3.4	0.58
pH 7.2 extract	3.26	1.3	2.51	2.0	100	0.9	0.17
Hydroxyapatite, 0.05 M phosphate	5.69	0.3	18.9	14.8	174	0.14	0
DEAE-Sephadex peak 1	1.51	0.0041	368	287	46	N.D.	0
DEAE-Sephadex peak 2	1.12	0.0035	320	250	34	N.D.	0
DEAE-Sephadex peak 3	1.21	0.0039	310	242	37	N.D.	0

peaks of activity (Table II).

Phosphatase activities of Novikoff hepatoma and normal liver nucleoli. The levels of nucleolar phosphoprotein phosphatase activity were measured in two tissues with different rates of ribosome synthesis; normal liver and Novikoff hepatoma. Released $^{32}P_i$ was measured using whole nucleoli (Fig. 9a) or nucleo-

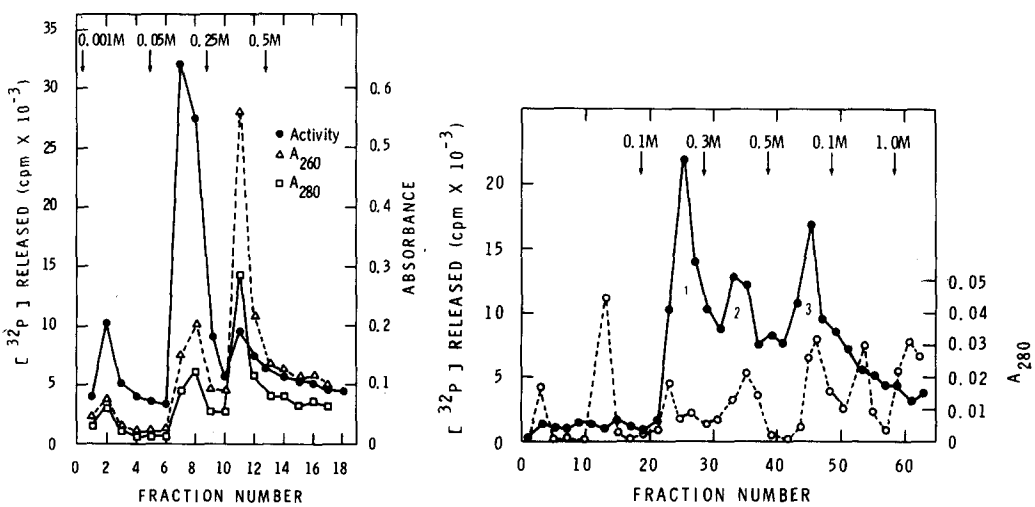


Fig. 7. Chromatography of nucleolar phosphoprotein phosphatase on hydroxyapatite. Crude extracts (2.5 ml) of nucleoli prepared as in text were dialyzed against 2 M KCl/1 M urea/1 mM K phosphate (pH 8.0) and applied to a column (1.7 X 9 cm) of hydroxyapatite equilibrated with same buffer. The column was stepwise eluted with increasing concentrations of phosphate in the KCl/urea buffer as indicated by the arrows on the top of the figure. The flow rate was 20 ml/h and 1 ml fractions were collected. 150-μl aliquots were dialyzed against 0.01 M Bis/Tris (pH 7.2) and assayed for activity. ●———●, phosphoprotein phosphatase activity; △———△, A_{260}; □———□, A_{280}.

Fig. 8. Chromatography of nucleolar phosphoprotein phosphatase on DEAE-Sephadex. The fractions eluted from hydroxyapatite at 0.05 M phosphate were pooled and dialyzed against 0.01 M Bis/Tris (pH 7.2) and applied to a column (0.9 X 29 cm) on DEAE-Sephadex equilibrated with the same buffer. The column was eluted at 20 ml/h with increasing concentrations of NaCl in the 0.01 M Bis/Tris buffer. 1-ml fractions were dialyzed against 0.01 M Bis/Tris and assayed for phosphatase activity. ●———●, phosphoprotein phosphatase activity; ○———○, A_{280}.

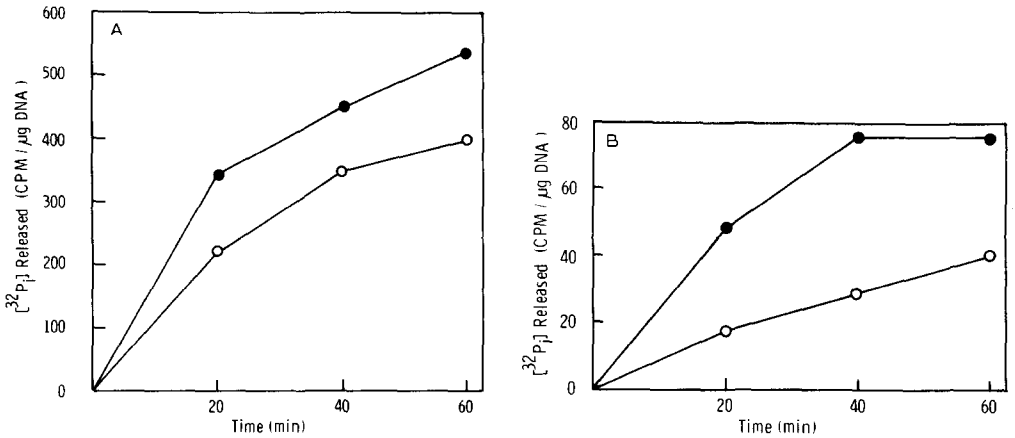


Fig. 9. Time course of phosphoprotein phosphatase activity of Novikoff hepatoma and normal liver nucleoli. (a) Nucleolar suspensions containing identical amounts of DNA (77 µg) were diluted 1/25 and incubated with identical amounts of in vitro ^{32}P-labeled nucleolar protein substrate (150 µg) for various times at 37°C in 0.011 M Bis/Tris (pH 7.2)/6 mM dithiothreitol in a total volume of 0.3 ml. (b) Extracted phosphoprotein phosphatase from nucleoli containing identical amounts of DNA (77 µg) were diluted and incubated with substrate as above. ●———●, Novikoff hepatoma nucleoli; ○———○, normal liver nucleoli.

lar extracts (Fig. 9b) as enzyme sources. The comparison was based on the DNA content of the nucleoli (Fig. 9a) or of the nucleolar suspension used for the extraction of enzyme (Fig. 9b). For whole nucleoli (Fig. 9b) the rate of ^{32}P released/µg DNA at 20 min was approx. 50% greater in the Novikoff nucleoli than in normal liver nucleoli. The differences in activity were greater when the extracted enzyme was used (Fig. 9b); Novikoff hepatoma nucleolar phosphatase activity was about 2.5 times that of normal liver. Similar results were obtained in three separate comparisons from different preparations of nucleoli.

Discussion

This paper and a previous report [14] demonstrate that nucleoli of both normal rat liver and Novikoff hepatoma contain phosphatase activity which acts upon nucleolar phosphoprotein substrates. Since nucleoli also contain kinase activity for phosphorylation of most nucleolar phosphoproteins [8] the nucleolus contains the system for attachment and removal of the phosphoryl groups of its own proteins.

As rate of phosphate moieties transferred per unit DNA the protein kinase activity is estimated to be several times greater than phosphoprotein phosphatase activity (Olson, M., unpublished data). Thus, the equilibrium seems to favor the phosphorylated form of proteins in the Novikoff hepatoma nucleolus.

The effects of various divalent cations were tested because previous studies indicated that the profile of proteins labeled as well as the total ^{32}P uptake from [γ-^{32}P]ATP was dependent on the specific divalent metal ions added to the isolated whole nucleolar system [8]. For example, the presence of 5 mM

$ZnCl_2$ produced a 3-fold greater ^{32}P uptake than $MgCl_2$ and resulted in a unique pattern of protein labeling. The present study shows that $ZnCl_2$ is a potent inhibitor of phosphoprotein phosphatase. Therefore, the net effect of $ZnCl_2$ in isolated nucleoli may be the result of phosphatase inhibition rather than activation of specific kinases. A similar phenomenon, the enhanced phosphorylation of H1 histone has been observed when $ZnCl_2$ is added to hepatoma cells in culture [26].

The nucleolar phosphoprotein phosphatase does not appear to have a requirement for divalent metal ions (Fig. 4). However, free sulfhydryl groups are necessary for full activity. It is likely that the marked inhibition by $ZnCl_2$ is related to the free sulfhydryl requirement. The divalent cation of zinc is known to have a particularly high affinity for SH groups [27]. Zinc has been shown to be concentrated in nucleoli of human prostatic cancer cells [28] and to move in and out of the nucleolus during the cell cycle of starfish oocytes [29]. Although the exact role of zinc in the nucleolus is unknown, localized concentrations of the divalent metals may exist as control factors for the dephosphorylation of nucleolar proteins.

The K_m for the crude phosphatase against nucleolar phosphoproteins was found to be 0.6 mg/ml. This value is within the range of K_m values obtained for phosphoprotein phosphatases from rabbit liver using a variety of substrates [30].

The crude extract of nucleolar phosphoprotein phosphatase exhibited little specificity in that it removed phosphate from all substrates tested. The major phosphorylated nucleolar proteins (C23-24 and B23-24) were nearly completely dephosphorylated when incubated as the total substrate mixture with the enzyme. However, isolated proteins were dephosphorylated no more than 16%. This was increased to about 35% for protein B23-24 in the presence of urea. In the absence of other nucleolar proteins to interact with, the major phosphoproteins may assume a conformation in which many of the phosphorylated sites are buried.

The lysine-rich H1 histone (spot A17-19) was also dephosphorylated by this enzyne. The nucleolar phosphatase differs from the histone phosphatase described in Meisler and Langan [31] in that it requires very low ionic strengths for maximal activity. Nucleolar phosphoprotein phosphatase also differs from nuclear histone phosphatase described by Tan et al. [32] in metal ion requirements and ionic strength optima.

Chromatography on DEAE-Sephadex resulted in three peaks of activity each purified more than 200-fold. However, the activities were very unstable at this stage, and it was not possible to determine whether differences in specificity existed in these peaks. The role of multiple species of these enzymes must await methods to stabilize their activities.

Earlier studies indicated that uptake of ^{32}P into nucleolar proteins from [γ-^{32}P]ATP is much greater in nucleoli of tissues with a high rate of ribosome synthesis (e.g. regenerating liver and Novikoff hepatoma) than in tissues with a lower rate such as normal liver [6,7]. Although these differences in uptake could be due to differences in kinase activities, the reverse reaction must also be taken into account. The present studies indicate that phosphoprotein phosphatase is found in higher concentrations in Novikoff hepatoma nucleoli than

in normal liver nucleoli. Therefore, differences in ^{32}P uptake in vitro are possibly due to differences in both phosphatase and kinase levels.

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