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PURIFICATION AND PARTIAL CHARACTERIZATION OF A 19 KD/pI 4.5 NUCLEOLAR PHOSPHOPROTEIN

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Two-dimensional PAGE analysis of proteins associated with the slowly sedimenting "fibrillar" structures of HeLa nucleoli revealed a protein with a M_r of 19,000 and a pI of 4.5 which was highly labeled both with ^{32}P-orthophosphate and ^{35}S-methionine. The protein was isolated from Novikoff hepatoma nucleoli by extraction in 0.35 M NaCl and 5 mM DTT followed by chromatography in EDTA on DEAE-cellulose and Sephadex G-100. The protein was homogeneous with respect to two-dimensional PAGE, number of tryptic peptides and carboxyl terminal analysis. The protein contained an acidic/basic amino acid ratio of 2.1, 7 residues of methionine, 2 residues of cysteine, a blocked amino terminus and a carboxyl terminal lysylleucine.

The nucleolus is the site of both the synthesis of ribosomal precursor RNA (1-4) and the assembly and processing of preribosomal ribonucleoprotein particles (5-8) which are the precursors of mature cytoplasmic ribosomes. The phosphorylation of nonhistone proteins has been implicated in the regulation of nucleolar function (9,10) and several studies from this laboratory have described the isolation and characterization of two phosphoproteins, C23 (110 kD/pI 5.2) and B23 (37 kD/pI 5.2) (11-15). In addition, six RNA polymerase I subunits have been reported to be phosporylated *in vivo* (10).

The present studies describe the isolation and characterization of a nucleolar phosphoprotein, 19 kD/pI 4.5, found to be highly labeled with both ^{32}P and ^{35}S-methionine.

Abbreviations used: PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate

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Materials and Methods

HeLa cells (1g) were labeled by incubation for 20 hrs. either with 50 mCi of ^{32}P-orthophosphate in phosphate-free medium (Centichem, Houston, TX) or 5 mCi of ^{35}S-methionine (Amersham, Arlington Heights, IL) (16). Novikoff hepatoma ascites cells were transplanted into male albino rats (Holtzman Co., Madison, WI). The cells were harvested and washed three times with NKM (0.13 M NaCl/0.05 M KCl/0.008 M $MgCl_2$).

Isolation of Nucleoli. Isolation of nucleoli was essentially the same for HeLa and Novikoff hepatoma cells. Cells were swollen at room temperature in 10 volumes RSB (10 mM Tris-HCl(pH 8.0)/10 mM NaCl/8 mM $MgCl_2$) for 10 min, centrifuged at 1200 x g for 10 min and resuspended in 10 volumes RSB/0.5% Nonidet P-40 (Sigma, St. Louis, MO). The cells were homogenized in a Dounce homogenizer and nuclei pelleted by centrifugation at 1200 x g for 10 min. Nuclei were washed in 0.88 M sucrose/5 mM $MgCl_2$ and then sonicated in 5 volumes of 0.34 M sucrose/0.5 mM $MgCl_2$. The sonicate was layered over 0.88 M sucrose and centrifuged at 4000 x g for 20 min. The isolated nucleoli were washed in 0.88 M sucrose/5 mM $MgCl_2$. Nucleoli from Novikoff cells not used immediately were stored at -80°C.

Extraction of RNP particles from nucleoli. Extraction of RNP particles from HeLa nucleoli, as described by Prestayko et al. (17), was in 10 mM Tris-HCl (pH 7.4)/10 mM KCl/5 mM $MgCl_2$/20 mM DTT/0.2% sodium deoxycholate, followed by incubation for 10 min at room temperature and for 10 min at 4°C. The suspension was centrifuged at 25,000 x g and the supernatant loaded on 5-45% sucrose density gradients in 10 mM Tris-HCl(pH 7.4)/10 mM KCl/5mM $MgCl_2$/0.5 mM DTT/1 mM PMSF/5% glycerol followed by centrifugation for 18 hr at 80,000 x g.

Polyacrylamide gel electrophoresis. One-dimensional SDS-PAGE was according to Laemmli (18). Two-dimensional PAGE was done by isoelectric focusing (19) and SDS-PAGE as above. Gels were stained either with 0.3% Coomassie Brilliant Blue R (Sigma) or with silver (20). For detection of ^{32}P or ^{35}S, the gels were dried and autoradiographed using Kodak XRP-1 film and Dupont Chronex intensifier screens at -70°C.

Extraction of 19 kD/pI 4.5 from Novikoff hepatoma nucleoli. Nucleoli (3g wet weight) were washed twice with 25 ml of 10 mM Tris-HCl(pH 8.0)/5 mM $MgCl_2$/0.1 mM PMSF and extracted twice with 25 ml of 0.15 M NaCl followed by 0.35 M NaCl/5 mM DTT in wash buffer. The 0.35 M NaCl extract was centrifuged at 144,000 x g for 3 hr and the supernatant dialyzed against 10 mM Tris-HCl(pH 8.0)/0.1 M NaCl/1 mM EDTA/ 1 mM DTT/0.1 mM PMSF. It was then clarified by centrifugation at 25,000 x g for 10 min.

Ion-exchange chromatography. The clarified supernatant was applied to a DE-52 (Whatman) column (1 x 18 cm). After washing with dialysis buffer until no protein was detected, a 120 ml 0.10-0.35 M NaCl linear gradient in 10 mM Tris-HCl(pH 8.0)/1 mM EDTA/1 mM DTT/0.1 mM PMSF was employed and 2 ml fractions collected. The fractions with 19 kD/pI 4.5 protein were pooled and concentrated in dialysis tubing against Sephadex G-200.

Molecular sieve chromatography. The concentrate was applied to a Sephadex G-100 column (1.5 x 90 cm) equilibrated with 10 mM Tris-HCl(pH 8.0)/1 mM EDTA/1 mM DTT/0.1 mM PMSF and eluted in 2 ml fractions.

Protein assay. Protein concentrations were determined by the method of Bradford (21) using a Bio Rad kit.

Amino acid analysis. For amino acid compositions, 10-20 µg of protein was hydrolyzed with 5.7 N HCl at 110°C. for 22 hr and analyzed. Half-cystine and methionine were determined by

hydrolysis after performic acid oxidation (22). Tryptophan was determined by hydrolysis with mercaptoethane sulfonic acid (23).

Hydrolysis with carboxypeptidases. Carboxyl-terminal amino acids were determined by digestion with carboxypeptidases A, B and Y (24-26). A 1 nmole sample of 19 kD/pI 4.5 was incubated with 1 µg of the respective enzyme at 37°C for varying times in 0.2 M N-ethylmorpholine acetate (pH 8.5). Digestions were stopped by heating at 110°C for 2 min. Subsequent digestions were performed in the same manner. After lyophilization, the digests were analyzed with a Beckman Model 121-M amino acid analyzer.

Results

Sucrose density gradient centrifugation. Figure 1 shows the analysis of phosphoproteins by one-dimensional SDS-PAGE when nucleolar RNP particles extracted from HeLa cells labeled with ^{32}P-orthophosphate were fractionated on sucrose density gradients. A highly labeled phosphoprotein of M_r about 19,000 was found mainly associated with the upper part or fibrillar region of the gradient (Fig. 1, fractions 6-20). When these fractions were pooled and subjected to two-dimensional PAGE and autoradiography (Figure 2A), the 19 kD phosphoprotein had an

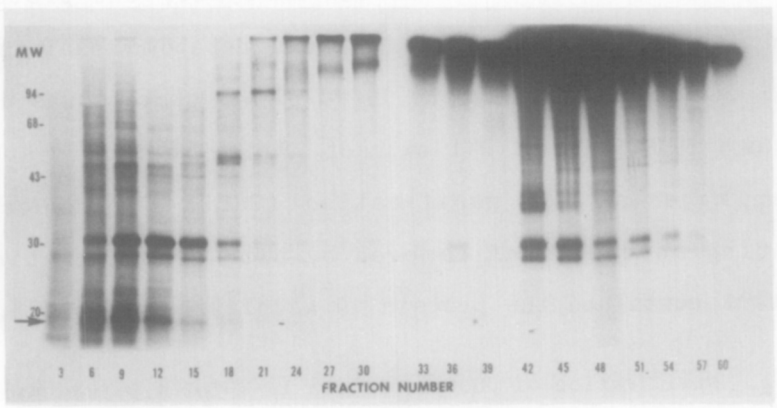

Figure 1. Autoradiograph of ^{32}P-labeled HeLa proteins after fractionation on a sucrose density gradient and one-dimensional SDS-PAGE (12.5%). HeLa cells (1g) were labeled 20 hrs with 50 mCi of ^{32}P-orthophosphate and nucleoli were isolated and extracted with 2 ml of 10 mM Tris-HCl (pH 7.4)/10 mM KCl/5 mM $MgCl_2$/20 mM DTT/0.2% sodium deoxycholate for 10 min at room temperature. The supernatant was subjected to sucrose density gradient (5-45%, 35 ml) centrifugation for 18 hrs at 80,000 x g in an SW-27 rotor (4°C) and collected in 0.6 ml fractions. Increasing fraction number corresponds to increasing sucrose density. The arrow points to the position of the 19 kD phosphoprotein.

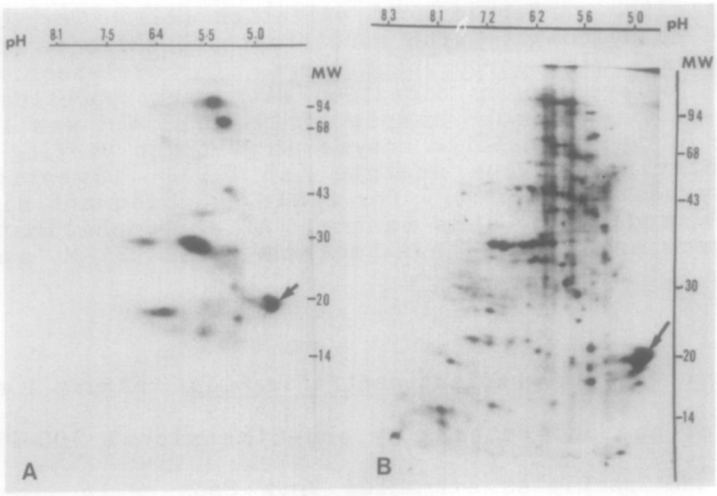

Figure 2. Autoradiographs of two-dimensional isoelectric focusing/SDS-PAGE (12.5%) of (A) ^{32}P and (B) ^{35}S-methionine labeled proteins from pooled sucrose density gradient fractions 6-20 of the HeLa nucleolar extract (figure 1). The arrows show the position of phosphoprotein 19 kD/pI 4.5.

apparent pI of 4.5. A corresponding analysis of the labeled proteins from this fraction showed strikingly high incorporation of ^{35}S-methionine into the 19 kD/pI 4.5 protein (Figure 2B).

Purification of phosphoprotein 19 kD/pI 4.5. To obtain sufficient quantities for chemical analysis, the protein was purified from the nucleoli of Novikoff hepatoma ascites cells. Inasmuch as essentially all the 19 kD/pI 4.5 protein was detected in the 0.35 M NaCl/5 mM DTT extract, this fraction was used as starting material for purification (Table I). When heavy sedimenting material was removed by ultracentrifugation, the supernatant contained the protein 19 kD/pI 4.5 enriched 1.5 fold.

TABLE 1: Purification of phosphoprotein 19 kD/pI 4.5 from Novikoff ascites cell nucleoli

	Protein in Fractions Containing 19 kD/pI 4.5	Protein Recovered in All Fractions	Fold Purification
0.35 M NaCl extract	98 mg	98 mg	1.0
144,000 x g supernatant	65 mg	92 mg	1.5
DEAE-cellulose	3.1 mg	74 mg	32
Sephadex G-100	0.5 mg	2.8 mg	202

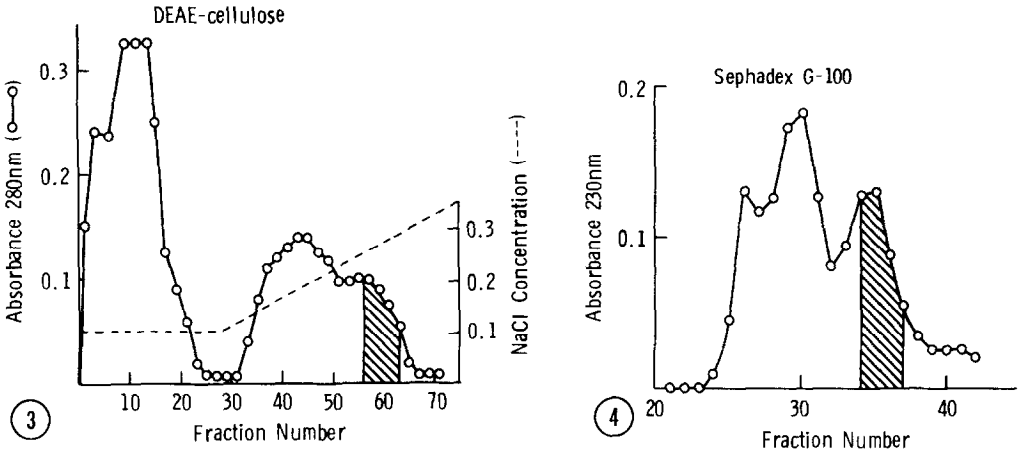

Figure 3. Elution profile of Novikoff nucleolar proteins on DEAE-cellulose. After dialysis against 10 mM Tris-HCl (pH 8.0)/1 mM EDTA/1 mM DTT/0.1 mM PMSF/0.1 M NaCl and clarification by centrifugation, the sample was applied to a DEAE-cellulose column (1 x 18 cm). The column was washed with dialysis buffer and collected in 5 ml fractions. A linear NaCl gradient (0.1-0.35 M, 120 ml) in the above buffer was applied and collected in 2 ml fractions. The shaded area indicates fractions with detectable protein 19 kD/pI 4.5 and these fractions were pooled and concentrated.

Figure 4. Elution profile of 19 kD/pI 4.5 fractions pooled and concentrated from DEAE-cellulose on a Sephadex G-100 column (1.5 x 90 cm). The column was run with 10 mM Tris-HCl (pH 8.0)/1 mM EDTA/1 mM DTT/0.1 mM PMSF and collected in 2 ml fractions. The shaded area indicates the pooled fractions containing phosphoprotein 19 kD/pI 4.5.

The supernatant was dialyzed, clarified by centrifugation and subjected to DEAE-cellulose chromatography, eluted with a linear NaCl gradient (Figure 3). After analysis by one-dimensional SDS-PAGE, the fractions (#56-63, Figure 3) which contained protein 19 kD/pI 4.5 enriched 32-fold were pooled and concentrated.

Further purification of protein 19 kD/pI 4.5 was accomplished on Sephadex G-100 (Figure 4). Fractions 34-37 contained the purified protein which moved as a single component with its characteristic M_r of 19 kD and pI of 4.5 on two-dimensional PAGE (Figure 5). Under the conditions of the Sephadex G-100 column, the 19 kD/pI 4.5 protein elutes as a single polypeptide.

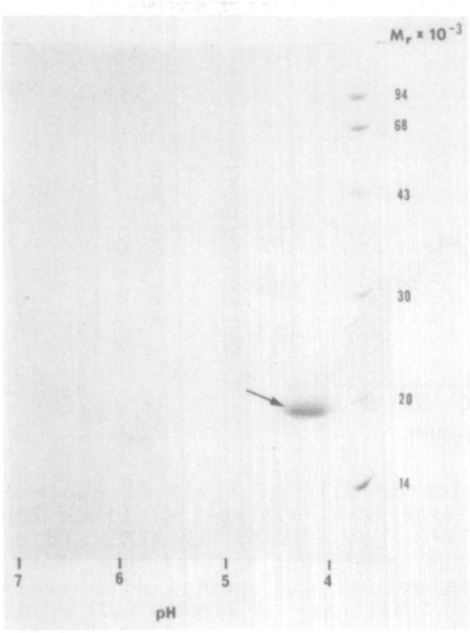

Figure 5. Two-dimensional isoelectric focusing/SDS-PAGE (12.5%) of purified 19 kD/pI 4.5 (40 µg) from pooled Sephadex G-100 fractions. The arrow shows the position of phosphoprotein 19 kD/pI 4.5.

Chemical Characterization. As shown in Table II, protein 19 kD/pI 4.5 had a high content of aspartic acid plus asparagine and glutamic acid plus glutamine. The ratio of acidic to basic amino acids is 2.1. Seven methionine residues were present. Two cysteine residues were detected. No tryptophan was detected after hydrolysis with mercaptoethane sulfonic acid.

Reaction of protein 19 kD/pI 4.5 with Dansyl chloride (29) and phenylisothiocyanate (30-32) as well as digestion with leucine aminopeptidase (33) were negative indicating the amino-terminus was blocked. Hydrazinolysis (34) released only leucine in 24% molar yield. Carboxypeptidase A digestion released only leucine (molar yield), after which, carboxypeptidase B digestion released lysine in molar yield. Subsequent digestions with carboxypeptidases A, B and Y released no further amino acids. Thus, the carboxyl-terminal sequence is lysylleucine.

TABLE II: Amino Acid Composition of 19 kD/pI 4.5

Amino Acid	Residues
Asx	22
Thr	6
Ser	13
Glx	26
Pro	7
Gly	13
Ala	10
Cys/2	2*
Val	11
Met	7*
Ile	5
Leu	18
Tyr	4
Phe	4
Lys	12
His	7
Arg	4
Total	171
M_r	19,000
Amino-terminus	blocked
Carboxyl-terminus	Leu

*Performic acid oxidation

Discussion

The phosphoprotein 19 kD/pI 4.5 was purified to homogeneity with respect to two-dimensional PAGE and carboxyl-terminal sequence. In addition, preliminary peptide mapping experiments indicate the presence of approximately 15 tryptic peptides in good agreement with the amino acid composition. The relatively high incorporation of ^{35}S-methionine into this protein is probably due to the high content of this amino acid in its composition. The acidic/basic amino acid residue ratio is consistent with the low isoelectric point.

Within the nucleolus, the fibrillar region is the site of rDNA transcription and under the conditions used in this study, its components are in the slowly sedimenting region of the sucrose density gradient (35). This fraction contains several phosphoproteins including B23, C23 and RNA polymerase I

(12,14,35). Further studies are needed to determine whether the 19 kD/pI 4.5 phosphoprotein is directly involved in the function of nucleolar transcription. Preliminary studies with NII kinase, an enzyme which phosphorylates subunits of RNA polymerase I *in vitro* (36,37), indicate that the 19 kD/pI 4.5 protein can serve as substrate *in vitro*.

Acknowledgments

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