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Identification and characterization of a hexameric form of nucleolar phosphoprotein B23

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Under native purification conditions, an oligomeric form (M_r = 230 000) and monomeric form (37 000) of protein B23 were purified by affinity chromatography. Both forms were identified by Western blot immunoassay and ELISA. The molecular weight of the oligomeric form of protein B23 was estimated to be 230 000 with a Stoke's radius and a sedimentation coefficient of 51 Å and 10 S, respectively. The oligomer (230 kDa) of protein B23 was dissociated into monomers (37 kDa) by treatment with 7 M urea. Quantitation of the monomer by gel scanning densitometry indicated that the oligomeric form of protein B23 is a hexamer containing four α and two β monomers (37 kDa). A trace amount of nucleic acids (amounting to less than 3% of the total mass) was detected in the affinity-purified oligomers of protein B23. Protein B23 may be a structural element which is involved in ribosome transport or assembly in the nucleus.

Introduction

The nucleolus is the site of rRNA synthesis and ribosome assembly and processing [1]. Many studies have been done on the biosynthesis and metabolism of rRNA and r-proteins [2–9]. In mammalian cells, ribosome assembly begins in the nucleolus with the association of newly synthesized 45 S RNA (pre-rRNA) and specific ribosomal proteins and nucleolar proteins. Nucleolar proteins are recycled in the nucleolus, and the matured ribosomal subunits are transported to the cytoplasm.

The role of nucleolar proteins in ribosome biosynthesis is largely unknown [10–16], and the mechanism of ribosome transport is not well defined [17]. Protein B23 is one of the major nucleolar phosphoproteins identified in Novikoff hepatoma cells [18]. The quantity of protein B23 in nucleoli increases with increased cell growth, as observed in hypertrophic rat liver [19] and tumor cells [20]. Immunoelectron microscopic studies indicate that protein B23 is localized in the granular regions of nucleoli [21]. These results suggest that protein B23 is associated with pre-ribosomal particles, and may be involved in the maturation of ribosomal subunits. Recent studies [22–24] indicated that whenever the synthesis (or the processing) of 45 S rRNA is inhibited by antibiotics (actinomycin D, toyocamycin, high doses of α-amanitin or luzopeptins), protein B23 translocates from the nucleolus to the nucleoplasm. To further understand the function of protein B23 and its relationship to ribosome synthesis, protein B23 has been purified under more native condi-

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; ELISA, enzyme-linked immunosorbent assay; ABTS, 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid), diammonium salt; CMPS, *p*-chloromercuriphenylsulfonic acid.

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tions. Under these conditions, an oligomeric form of protein B23 was identified.

Materials and Methods

Culture materials

Minimum essential medium (Eagle's salt), fetal calf serum, glutamine and penicillin-streptomycin solutions were purchased from Grand Island Biological Co., Grand Island, NY. Petri dishes and flasks were purchased from the Corning Glass Co., Corning, NY. Fluorescent antibody microslides were obtained from Dickinson & Co., Oxnard, CA.

Antibodies

The monoclonal antibody to protein B23 was produced by in vitro fusion techniques [25]. Antibodies were collected from the hybridoma cell culture medium and purified by ammonium sulfate precipitation.

The second antibodies used in the Western immunoblot and immunofluorescence studies were affinity-purified rabbit anti-mouse and fluorescein-conjugated affinity-purified goat anti-mouse IgG, respectively. They were purchased from Cooper Biomedical, Inc., Malvern, PA.

Chemicals

EM-grade formaldehyde solutions were purchased from Polysciences, Inc., Warrington, PA. ABTS was purchased from Boehringer-Mannheim, F.R.G. Nonidet P-40 was purchased from Sigma, St. Louis, MO. Other chemicals were reagent grade.

Cells

HeLa S-3 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum in a 5% CO_2 humidified incubator at 37 °C.

Isolation of HeLa cell nucleoli

Nuclei and nucleoli were isolated from HeLa cells using the Nonidet P-40 method, as described by Chan et al. [26]. Cells were suspended in 20 vol. of RSB buffer (0.01 M Tris-HCl/0.02 M NaCl/1.5 mM $MgCl_2$ (pH 7.2)) for 30 min and then centrifuged at $630 \times g$ for 8 min. Swollen cells were resuspended in 20 vol. of RSB buffer containing 0.5% Nonidet P-40. The cells were homogenized with a Dounce homogenizer (10 up and down strokes), and the crude nuclei were collected by centrifugation at $630 \times g$ for 8 min. The nuclei were resuspended in 10 vol. of 0.25 M sucrose/10 mM $MgCl_2$, and underlayered with an equal volume of 0.88 M sucrose/0.05 mM $MgCl_2$. After centrifugation at $1700 \times g$ for 10 min, the nuclei were resuspended in 10 vol. of 0.34 M sucrose/0.05 mM $MgCl_2$. The suspension was sonicated for 1 min, underlayered with an equal vol. of 0.88 M sucrose/0.05 mM $MgCl_2$, and centrifuged at $3000 \times g$ for 18 min. The supernatant (nucleoplasm) and the pellet (nucleoli) were collected separately.

Extraction of protein B23 under native conditions

The HeLa nucleoli were extracted (stirred at 10 °C) with 10 mM Tris-HCl (pH 7.5)/0.5 mM $MgCl_2$/1 mM PMSF/1 mM leupeptin/1 mM CMPS for 15 min and centrifuged at $27000 \times g$ for 20 min. The supernatant (Tris extract of HeLa nucleoli), containing the partially purified protein B23, was applied to an immunoaffinity column for further purification.

Immunoaffinity chromatography

The monoclonal antibody to protein B23 was coupled to CNBr-activated Sepharose 4B. The Tris extract of HeLa nucleoli was mixed with B23 antibody-Sepharose overnight at 4 °C by tumbling end-over-end. The unbound proteins were removed from the column by washing with 20 bed vol. of 10 mM Tris/0.5 mM $MgCl_2$/1 mM PMSF/1 mM leupeptin/1 mM CMPS, and the bound protein B23 was eluted with 4 M $MgCl_2$. All fractions, including the unbound, the washed fraction, and the eluted samples were analyzed for protein B23 by ELISA and immunoblot assays. A control column, made by coupling pre-immune serum to Sepharose 4B, was also incubated with the Tris extract of HeLa nucleoli, washed, and eluted similarly.

Molecular sieve chromatography

An Ultrogel AcA-22 column (0.8 × 45 cm) was equilibrated with 10 mM Tris-HCl (pH 7.5)/0.5 mM $MgCl_2$/100 μg/ml bovine serum albumin.

Affinity-purified protein B23 in the above buffer was applied to the column and eluted in 0.25 ml fractions at a flow rate of 10 ml/h. The fractions containing protein B23 were identified by ELISA analysis. A gel filtration calibration kit (Pharmacia Fine Chemicals), which included aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa), was used to establish molecular weights.

Determination of the sedimentation coefficient of the complex form of protein B23 by sucrose density gradient centrifugation

The immunoaffinity-purified native protein B23 was layered on a sucrose density gradient (5–20% in 10 mM Tris-HCl (pH 7.5)/0.5 mM $MgCl_2$/1 mM PMSF/1 mM CMPS/1 mM leupeptin) and centrifuged at 80000 × *g* for 24 h at 4°C in an SW28 rotor. Fractions of 0.6 ml were collected and assayed for ELISA activity. Lactate dehydrogenase and catalase were run as standards in another tube.

ELISA procedures

The proteins were bound to microtiter plates overnight at 4°C. Blocking of the unbound sites was done with 3.0% bovine serum albumin/0.05% Tween 20/phosphate-buffered saline (pH 7.5), and 10% chicken serum to reduce background. The primary antibody was anti-protein B23 monoclonal antibody (diluted 1:25000). The second antibody was a peroxidase-labeled goat anti-mouse antibody. Color was developed after the addition of 0.02% H_2O_2 and 300 μM ABTS. The reaction was terminated in 30 min with the addition of 1 M NaF. The absorbance was read at 415 nm on an ELISA reader.

Polyacrylamide gel electrophoresis

One-dimensional SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [27]. Samples were mixed with an equal volume of SDS sample buffer before application to an 8% SDS-polyacrylamide gel. For two-dimensional gel analysis, the samples were incorporated into isoelectrofocusing gels, and electrophoresis was performed as described by Chan et al. [28].

Immunoblot (Western) procedures

Proteins were fractionated by SDS-polyacrylamide gel electrophoresis and subsequently transferred electrophoretically to nitrocellulose papers (Schleicher & Schuell type BA-85, 0.45 μm) at 10 V for 16 h or at 50 V for 1 h (high molecular weight proteins could not be transferred efficiently using the latter conditions). The nitrocellulose paper was soaked in blocking solution (3% bovine serum albumin/0.9% NaCl/10 mM Tris-HCl (pH 7.5)/10% chicken serum) overnight. The paper was then incubated with protein B23 antibody in Nonidet P-40 buffer (0.015 M NaCl/5 mM EDTA/5 mM Tris-HCl (pH 7.5)/0.05% Nonidet P-40/0.25% gelatin) for 2 h at 25°C. The antibody was removed by two washes with Nonidet P-40 buffer (25 ml, 10 min each wash). The paper was subsequently incubated with the second antibody (rabbit anti-mouse IgG), washed (2 ×), incubated with ^{125}I-protein A solution, and washed (2 ×). The paper was finally washed twelve times (25 ml, 10 min each) with sarkosyl buffer (1 M NaCl/5 mM EDTA/5 mM Tris-HCl (pH 7.5)/0.4% sarkosyl/0.25% gelatin). The dried nitrocellulose paper was then exposed to Kodak X-ray film (XRP-5).

Determination of protein

Protein concentration was determined by the Bio-Rad protein assay [29]. Bovine serum albumin was used as the standard.

Results

Purification of native protein B23

Fig. 1 shows the isolation scheme for native protein B23. The HeLa cell nucleoli were suspended in 10 mM Tris-HCl/0.5 mM $MgCl_2$/1 mM PMSF/1 mM leupeptin/1 mM CMPS, and were magnetically stirred for 15 min at 10°C. The suspension was then centrifuged at 27000 × *g* for 20 min. The supernatant (Tris extract) was then loaded onto a protein B23 immunoaffinity column. The elution profile is shown in Fig. 2. Protein B23 bound to the immunoaffinity column but not to the control column. The recovery of protein B23 ELISA activity after the affinity chromatography was about 50%. The proteins from the total Tris extract before and after affinity chro-

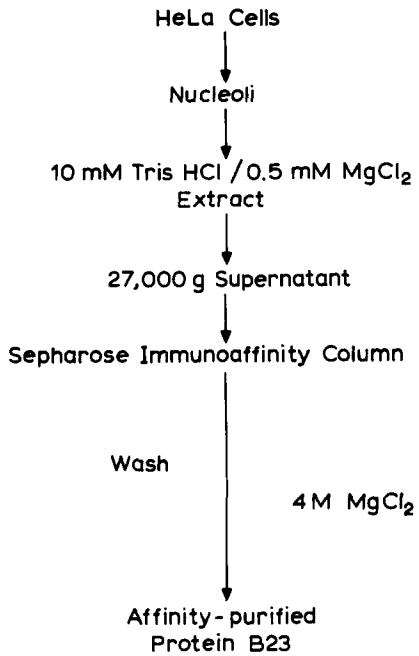

Fig. 1. Isolation scheme for native protein B23.

matography were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis. About 40 protein bands were observed in the total Tris extract of HeLa nucleoli (Fig. 3, lane B). A high molecular weight band (arrow), which was later

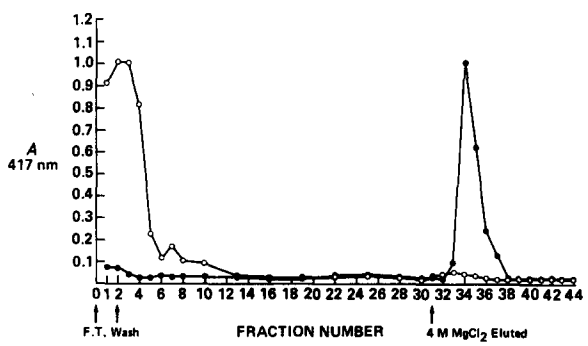

Fig. 2. Purification of native protein B23 by immunoaffinity chromatography. The Tris extract of HeLa nucleoli was collected and applied to the protein B23 immunoaffinity column (●) or to the pre-immune control column (○). Antigen was eluted with 4 M $MgCl_2$ in the Tris buffer (see Materials and Methods). 2-ml fractions were collected and assayed for protein B23 ELISA activity.

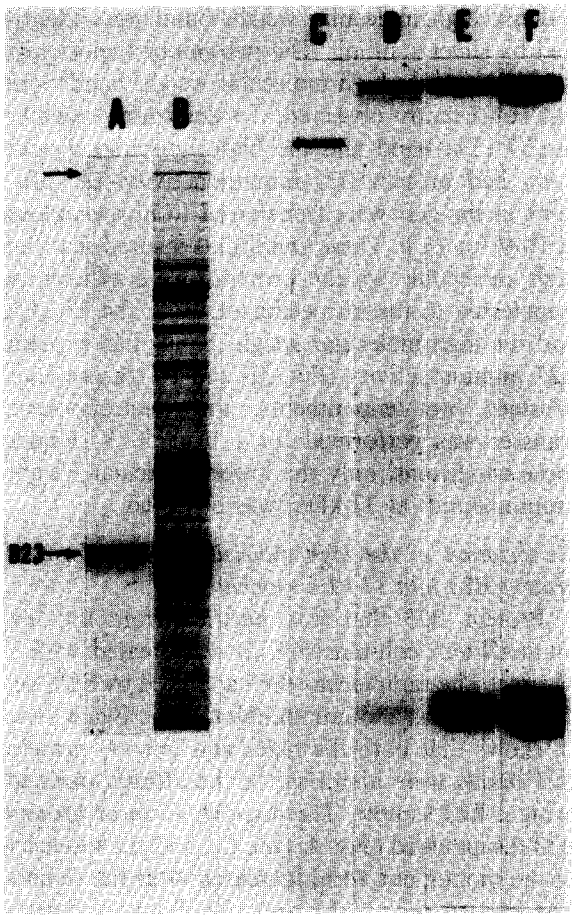

Fig. 3. Characterization of native protein B23 by SDS gel electrophoresis and immunoblot analysis. Lane A, purified protein B23 from Novikoff hepatoma cells. Lane B, the Tris extract of HeLa nucleoli. Lane C, proteins eluted from the pre-immune control column. A protein band with a molecular weight of 160000 was observed. The identity of this band is not presently known. Lane D, proteins eluted from the protein B23 immunoaffinity column. A dense, high molecular weight band and a protein B23 band (37 kDa) were observed. Lanes A–D, gels were fixed and silver-stained. Lane F, immunoblot of the Tris extract of HeLa nucleoli. Lane E, immunoblot of proteins eluted from immunoaffinity column. Two dense immunobands corresponding to protein B23 and the high molecular weight form of protein B23 were observed in both lanes E and F.

identified to be the hexamer of protein B23, was observed. Fig. 3, lane D, shows the proteins specifically bound to the affinity column. A weak band corresponding to protein B23 (37 kDa) and

a dense high molecular weight band were identified by silver staining. The two minor bands just below the dense high molecular weight band were not consistently observed. To determine whether the 37 kDa band and the high molecular weight band had protein B23 immunoactivity, the proteins in the gels were transferred to nitrocellulose at 10 V for 16 h. These conditions insured that the high molecular weight proteins were efficiently transferred to the nitrocellulose. Both the 37 kDa and the high molecular weight bands had protein B23 immunoactivity (Fig. 3E and F). In previous Western blot experiments, the electrophoretic transfer was performed for 1 h at 50 V. Under these conditions, only the lower molecular weight immunoband (at 37 kDa) was observed.

Dissociation of the high molecular weight form of protein B23 into 37 kDa monomers

Protein B23 that was purified from the immunoaffinity column (Fig. 2) was treated with 7 M urea. The sample was then analyzed by SDS gel electrophoresis and immunoblot assay. Fig. 4, lane B, shows that both the high- and low-M_r protein B23 bands were identified in the affinity-purified protein B23 sample. The high-M_r form of protein B23 disappeared (Fig. 4, lane C), and the intensity of the monomeric form increased after the sample was treated with 7 M urea. To further confirm that the high-M_r form was dissociated into monomers, the high-M_r band of protein B23 (Fig. 4, lane B) was excised and electrophoretically eluted from the polyacrylamide gel. The sample was then treated with 7 M urea and analyzed by one-dimensional gel electrophoresis (Fig. 4, lane D). The high-M_r form of protein B23 was not observed. Instead, the monomers (α and β) appeared in the one-dimensional gel. The α and β monomers are present in an approximate 2:1 ratio, as measured by densitometric scanning.

In other experiments, it was observed that the high molecular weight form of protein B23 could be partially dissociated by boiling it in SDS sample buffer (62 mM Tris-HCl (pH 6.8)/5% mercaptoethanol/2.3% SDS) for 5 min or by storing it in the sample buffer for longer than 24 h.

Detection of the high-M_r form of protein B23 in HeLa cellular, nuclear and nucleolar extracts

In order to ascertain that the high-M_r protein

Fig. 4. Dissociation of the oligomer of protein B23 into monomers by treatment with 7 M urea. Lane A, purified protein B23 from Novikoff hepatoma cells. Lane B, proteins eluted from the affinity column without urea treatment. Lane C, proteins eluted from the affinity column and treated with 7 M urea. Lane D, the high-M_r band of protein B23 (lane B) was excised, electrophoretically eluted and treated with 7 M urea.

B23 was not an artificial aggregate generated by exposure to magnesium ions or other chemicals during purification, whole cells (no purification steps), and nuclei or nucleoli of HeLa cells were dissolved immediately after isolation in SDS sample buffer and subjected to SDS gel electrophoresis. A Western immunoblot assay shows that both immunobands, corresponding to 37 kDa and 230 kDa, were identified in the total cellular (Fig. 5),

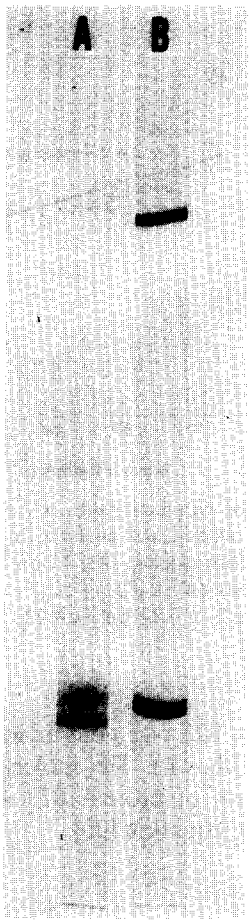

Fig. 5. Detection of the high-M_r form of protein B23 in HeLa cell extract. Lane A, purified protein B23 from Novikoff hepatoma cells. Lane B, total proteins of HeLa cells were separated on 8% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose sheets and reacted with anti-B23 antibody. Two immunobands corresponding to the high-M_r form and monomeric form of protein B23 were observed.

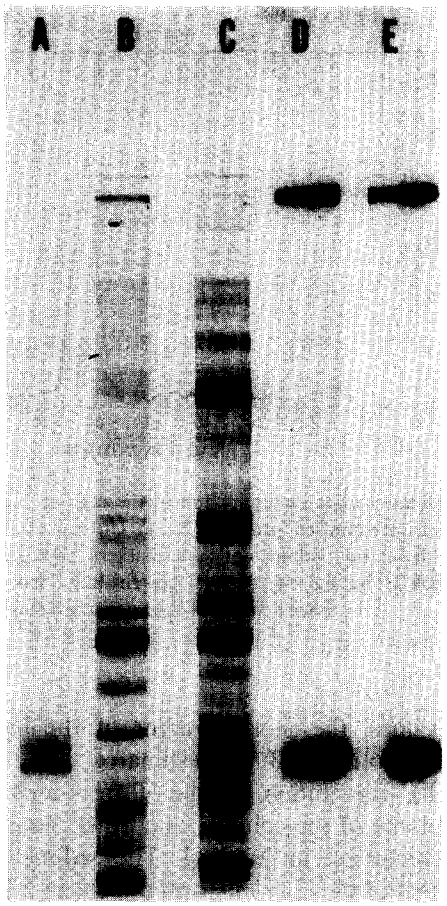

Fig. 6. Detection of the high-M_r form of protein B23 in total nuclear and nucleolar extracts. Lane A, purified protein B23 from Novikoff hepatoma cells. Lane B, proteins from HeLa nuclei. Lane C, proteins from HeLa nucleoli. Lanes A–C, gels were fixed and stained with Coomassie brilliant blue. Lanes D and E, immunoblot analysis of proteins from HeLa nuclei (lane D) and from HeLa nucleoli (lane E). Two immunobands corresponding to protein B23 and the high-M_r form of protein B23 were observed in both lanes.

the nuclear, and the nucleolar extracts (Fig. 6D and E).

Characterization of the high-M_r form of protein B23

To estimate the molecular weight of the high-M_r form of protein B23, the Stoke's radius and the sedimentation coefficient were determined by gel filtration (Ultrogel AcA-22) and sucrose density gradient centrifugation. Fig. 7A shows the elution profile of the immunoaffinity-purified protein B23 and the protein markers on the Ultrogel AcA-22 column. Two immunoreactive peaks were observed in the elution profile. The first peak (I) corresponded to the high-M_r form of protein B23. The second peak (II), which was eluted at the column's bed volume, corresponded to the monomer of protein B23 (37 kDa). By a plot of the Stoke's radius of the known proteins against their

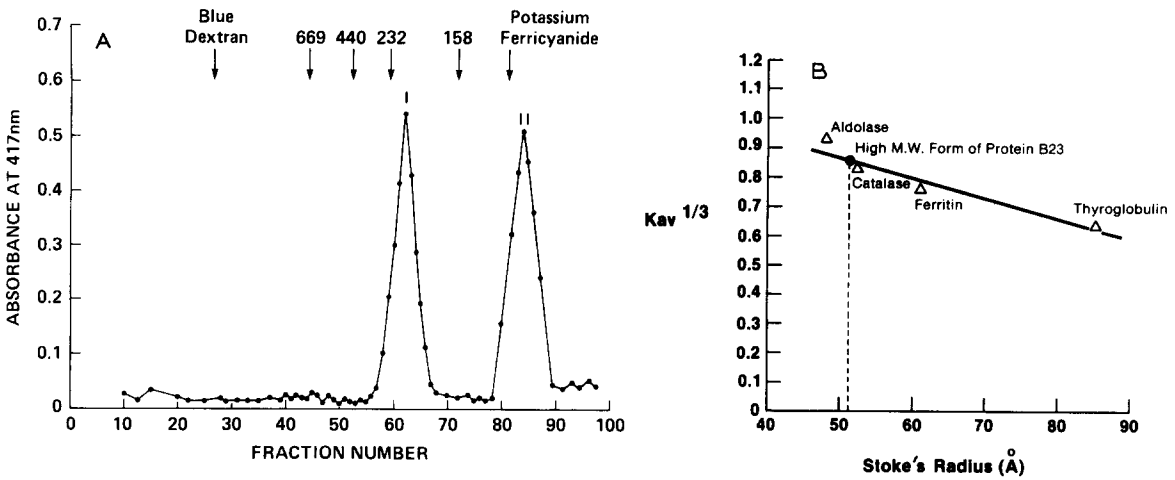

Fig. 7. Stoke's radius determination of the high-M_r form of protein B23 by gel filtration chromatography. (A) Elution profile of immunoaffinity-purified protein B23 on Ultrogel AcA-22. Fractions (0.25 ml) were collected and analyzed by ELISA. Arrows indicate the elution volumes of these markers: thyroglobulin (669 kDa); ferritin (440 kDa); catalase (232 kDa); and aldolase (158 kDa). Peak I, the high-M_r form of protein B23. Peak II, protein B23 monomer (37 kDa). (B) A plot of $K_{av}^{1/3}$ values against the Stoke's radius of the known proteins and the high-M_r form of protein B23 on Ultrogel AcA-22.

$K_{av}^{1/3}$ values, the Stoke's radius of the high-M_r form of protein B23 was estimated to be 51 Å (Fig. 7B). The elution volume of the samples, the void volume of the column, and other parameters are summarized in Table I. The sedimentation coefficient of the high-M_r form of protein B23 was determined by sucrose density gradient centrifugation. By comparison with the sedimentation coefficients of lactate dehydrogenase (7.3 S) and catalase (11.3 S), the sedimentation coefficient of the high-M_r protein B23 was determined to be about 10 S (data not shown). The molecular weight of the high-M_r form of protein B23 was calculated by the following equation [30]:

$$M_r = 6\pi Nas/(1-\bar{u}p)$$

where a = Stoke's radius, s = sedimentation coefficient, $\bar{u}$ = partial specific volume, p = density of the medium, and N = Avogadro's number. As-

TABLE I

PARAMETERS OF THE KNOWN PROTEINS AND THE HIGH-M_r FORM OF PROTEIN B23 FROM AcA-22 GEL FILTRATION CHROMATOGRAPHY IN 10 mM TRIS-HCl/0.5 mM $MgCl_2$ BUFFER

Protein	Molecular weight	Log M_r	V_e (ml)	$\frac{V_e}{V_0}$	$K_{av} = \frac{V_e - V_0}{V_t - V_0}$	$K_{av}^{1/3}$	Stoke's radius (Å)
Aldolase	158000	5.20	18.0	2.57	0.82	0.93	48.1
High-M_r form of protein B23			15.3	2.18	0.61	0.85	51.0
Catalase	232000	5.37	14.8	2.11	0.57	0.83	52.2
Ferritin	440000	5.64	13.0	1.86	0.44	0.76	61.0
Thyroglobulin	669000	5.83	10.5	1.50	0.26	0.64	85.0
Blue dextran	2000000		7 (V_0)	1.00			

TABLE II

CHARACTERIZATION OF THE HEXAMERIC FORM OF PROTEIN B23

Stoke's radius	51.0 Å
Sedimentation coefficient	10 S
Molecular weight	230000
Chemical composition	
protein	97%
nucleic acid	< 3%
Subunits	4 α and 2 β monomers of protein B23
Estimated number of hexamers per pre-rRNP	2

suming a partial specific volume of 0.725, which is the average of most known proteins, the molecular weight of the high-M_r form of protein B23 was calculated to be 230000. Since the molecular weight of the monomer of protein B23 is 37000, the results suggest that the high-M_r forms of protein B23 may be a hexamer. Previous experiments showed that the ratio of the α and β forms in the high-M_r protein B23 is 2 to 1 (Fig. 4). Accordingly, the high-M_r form of protein B23 is composed of four α and two β monomers. Table II summarizes the results of the characterization of the high-M_r protein B23.

Discussion

Protein B23 was previously isolated from Novikoff hepatoma cells with urea [31]. The protein may have been denatured under these conditions. In order to preserve its nativeness and understand its function, attempts were made to purify protein B23 without urea. Under 'no urea' conditions, a high-M_r form ($M_r = 230000$) of protein B23 was identified. Since this high-M_r form could be detected in cellular (Fig. 5), nuclear, and nucleolar extracts (Fig. 6) and in pre-rRNP particles (data not shown), it is not likely that the high-M_r form of protein B23 is an artificial aggregate produced during purification. This high-M_r form was also identified by gel filtration (Fig. 7) and sucrose density centrifugation.

Protein B23 is associated with pre-RNPs [16,21], and our previous studies suggested that protein B23 could interact with the spacer region of 45 S rRNA [23]. However, the fact that only a trace amount of nucleic acid (3%) was observed in the affinity-purified protein B23 sample (Table II) suggests that protein B23 is not covalently bound to RNA. It is possible that protein B23 is weakly bound to RNAs, and that this association is disrupted during the isolation procedure. Analysis of the affinity-purified protein B23 sample with SDS gel electrophoresis revealed no other protein except protein B23. It is speculated that the hexameric form of protein B23 may be a structural element which associates with pre-RNP particles. Our previous studies reported that there are approximately $6 \cdot 10^6$ molecules of monomeric protein B23/HeLa cell [26].

The high-M_r form of protein B23 consists of both α and β monomers (in a 2:1 ratio), while only the α monomer is observed in the absence of urea treatment (Fig. 4, lane B). Carbamylation of protein B23 is not likely because: (i) freshly prepared ultra-pure urea solutions were used, and (ii) dissociation of the oligomer to α and β monomers was also observed when 'no urea' conditions were employed. These observations indicate that there may be two pools of protein B23 (the α monomer and α,β hexamer) in the nucleus with possibly different functions. Recent studies indicated that protein B23 was bound to an RNA-containing structure in the nucleoplasm (unpublished data). Whether protein B23 is involved in the transport of large ribosomal subunits through the internal nuclear matrix network [13] remains to be investigated.

Acknowledgments

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