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Association of Protein C23 with Rapidly Labeled Nucleolar RNA†

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ABSTRACT: The association of nucleolar phosphoprotein C23 with preribosomal ribonucleoprotein (RNP) particles was examined in Novikoff hepatoma nucleoli. RNA was labeled with [^{3}H]uridine for various times in cell suspensions, and RNP particles were extracted from isolated nucleoli and fractionated by sucrose gradient ultracentrifugation. The majority of protein C23 cosedimented with fractions containing rapidly labeled RNA (RL fraction). To determine whether there was a direct association of RNA with protein C23, the RL fraction was exposed to ultraviolet (UV) light (254 nm) for short periods of time. After 2 min of exposure there was a 50% decrease in C23 as measured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analyses, with no significant further decrease at longer times. When UV-treated fractions were subjected to phenol/chloroform extractions, as much as 30% of the labeled RNA was found in the phenol (protein) layer, indicating that RNA became cross-linked to protein. Similarly, there was an increase in protein C23 extracted into the water layer after irradiation. By SDS–PAGE analyses the cross-linked species migrated more slowly than protein C23, appearing as a smear detected either by [^{3}H]uridine radioactivity or by anti-C23 antibody. With anti-C23 antibodies, up to 25% of the labeled RNA was precipitated from the RL fraction. Dot-blot hybridizations, using cloned rDNA fragments as probes, indicated that the RNA in the RL fraction and the immunoprecipitated RNA contained sequences from 18S and 28S ribosomal RNA. These studies, along with other published work that shows the presence of C23 in fibrillar regions, suggest that protein C23 is associated with nascent preribosomal RNA and that it may be involved in the early stages of preribosomal RNP particle formation.

The nucleolus is the subnuclear organelle where preribosomal RNA is synthesized and ribosome assembly begins (Busch & Smetana, 1970). Ultrastructural studies have shown well-defined regions of the nucleolus, many of which can be related to aspects of cell physiology. For example, fibrillar centers appear as areas of relatively low electron density in transmission electron microscopy and are surrounded by a dense fibrillar component. The fibrillar centers are the apparent location of the genes for preribosomal DNA (Arroua et al., 1982) and RNA polymerase I (Scheer & Rose, 1984; Jordan, 1984). The surrounding dense fibrillar components contain newly synthesized preribosomal RNA with proteins attached (Goessens & Lepoint, 1979; Mirre & Stahl, 1981). The granular components, which are usually distributed throughout the nucleolus, contain the 15-nm preribosomal particles (Jordan, 1984). These ribonucleoprotein (RNP) components consist of preribosomal RNA at various stages of processing associated with unique sets of ribosomal and nonribosomal proteins.

A major nucleolar protein, C23 (also called nucleolin or the 100-kDa nucleolar protein), has a number of unique properties that suggest it may participate in the organization of these nucleolar subcomponents: (a) it is predominantly localized to the nucleolus in interphase cells (Olson et al., 1981; Michalik et al., 1981, (b) it binds silver under conditions in which the nucleolus organizer regions of chromosomes specifically stain with silver (Lischwe et al., 1979; Hubbel et al., 1979), (c) it is found at the nucleolus organizer regions of chromosomes (Lischwe et al., 1981), (d) it is a nonribosomal constituent of nucleolar preribosomal particles (Prestayko et al., 1974; Olson et al., 1974), (e) it contains several highly acidic regions (Mamrack et al., 1977, 1979; Rao et al., 1982), and (f) it contains multiple phosphoryl groups (Mamrack et al., 1977, 1979; Rao et al., 1982).

Of the various nucleolar components, the dense fibrillar regions contain the highest concentration of protein C23 as measured by immunoelectron microscopic methods (Escande et al., 1985; Lischwe et al., 1981; Spector et al., 1984). Like protein C23, the dense fibrillar regions have a high affinity for silver (Hernandez-Verdun et al., 1980; Goessens & Lepoint, 1982) or bismuth salts (Gas et al., 1984), suggesting that these or the enclosed fibrillar centers are interphase

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counterparts of the nucleolar organizer regions (NORs) on chromosomes. Furthermore, protein C23 binds DNA with preference to rDNA spacer regions (Olson et al., 1983), suggesting that it recognizes sequences in the vicinity of the dense fibrillar regions. Thus, there is an accumulation of evidence that this protein is closely associated with the nascent RNP complex.

The fibrillar region of the nucleolus was previously isolated as a 10–12S complex by extraction of nucleoli with low ionic strength buffers and sucrose gradient ultracentrifugation of the soluble RNP elements (Daskal et al., 1974). This fraction contained the most rapidly labeled RNA in the gradient, also suggesting that it was a nascent RNP component. In recent studies (Olson et al., 1986) we isolated this slowly sedimenting fraction by a similar procedure (Auger-Buendia & Longuet, 1978) and observed that it was greatly enriched in protein C23. With this observation we undertook the present study to determine whether protein C23 was directly associated with rapidly labeled preribosomal RNA or simply cosedimented as free protein. By cross-linking with ultraviolet light and by precipitation by anti-C23 antibody we have presented evidence for a close association of protein C23 with nascent pre-rRNA transcripts.

Materials and Methods

Materials. All chemicals were purchased from Sigma with the following exceptions. Tris,[1] SDS, and sucrose were purchased from Bethesda Research Laboratories, [^{3}H]uridine was from New England Nuclear, acrylamide was from Bio-Rad, and media components such as minimal essential media (MEM), calf serum, L-glutamine, and penicillin/streptomycin were purchased from Gibco. Instant nonfat dry milk was a product of the Carnation Co. ^{125}I-Labeled protein A was generously supplied by Dr. J. D. Dignam, Staphylococcus A was a gift from Dr. C. Lobb, and ^{3}H-labeled vesicular stomatitis virus (VSV) RNA was provided by Dr. D. Sittman. Nitrocellulose was purchased from Schleicher and Schuell. Organic reagents came from Baker. All glassware and plasticware were either autoclaved for 20 min or baked overnight at 200 °C. The water was treated for 2–18 h with 1.3% diethyl pyrocarbonate and autoclaved before use.

Cells and Cell Fractions. Novikoff hepatoma ascites cells were maintained in male Sprague–Dawley rats, which were bred in The University of Mississippi Medical Center Animal Facilities. Cells were harvested 6 days after transplantation. Nucleoli were isolated from the Novikoff cells by using the magnesium/sucrose method of Rothblum et al. (1977). Isolated nucleoli were stored at −80 °C in a buffer containing 50% glycerol, 10 mM Tris, 0.1 mM PMSF, and 0.1 mM EPNP (pH 8.0).

To label the RNA in cells with [^{3}H]uridine, the cells were first removed from the peritoneum of the rats with a sterile syringe. The cells were washed 3 times with 10 volumes of an isotonic buffer composed of 130 mM NaCl, 10 mM Tris, 9 mM $MgCl_2$, and 5 mM KCl (pH 7.4), centrifuging in a Sorvall RC 3B centrifuge for 10 min at 4500 rpm after each wash. Cells were then suspended in 2 volumes of isotonic buffer and added to the culture media to a final concentration of 3×10^6/mL. The culture medium was composed of 1 × MEM, 10% calf serum, 2 mM L-glutamine, 50 units of penicillin G, 50 μg of streptomycin, 0.21% sodium bicarbonate, and 1 mCi of [5,6-^{3}H]uridine per 100 mL of media. The cells were incubated at 37 °C, shaking for 1, 4, and 9 h. The 4-h incubation became the standard incubation time. Nucleoli were isolated as previously described.

The nucleoli were extracted according to the procedure of Auger-Buendia and Longuet (1978) by homogenizing nucleoli at a concentration of 20 A_{260} units/mL of a buffer containing 10 mM Tris and 10 mM NaCl (pH 8.0). After this suspension was allowed to stand for 20 min at 15 °C, 20 μg/mL leupeptin and 200 units/mL RNasin (Promega Biotec) were added. The mixture was centrifuged at 10000*g* for 10 min and the supernatant used for sucrose gradient ultracentrifugation. The nucleolar extract was applied in 0.4-mL aliquots to linear sucrose gradients (5–30%) containing 10 mM Tris and 10 mM NaCl (pH 8.0). Samples were centrifuged at 40000 rpm for 2 h in a Beckman SW41 rotor. The gradients were fractionated into 0.4-mL fractions with an ISCO 185 fractionator. Labeled fractions were counted after addition of 5 mL of Aquasol II in a Beckman liquid scintillation counter.

Electrophoresis. Electrophoresis of protein samples was carried out in vertical slabs (14 cm × 14 cm) by using a discontinuous gel system, according to the method of Laemmli (1979). Gradient fractions to be run on the gel system were prepared by either of two methods. The first method is essentially that of Valenzuela et al. (1976) where samples were precipitated in the presence of 20 μg of poly(adenylic acid) by 10% TCA and then washed twice in ethanol–ether (1:1 v/v) and once with ether. After ether evaporation the precipitates were dissolved in sample buffer and boiled for 3 min before application to the gel. The maximum volume of the samples was 40 μL. In the second method, fractions from the gradient were directly added to the 3× sample buffer, boiled for 3 min, and applied to the gel. After staining with Coomassie Blue and destaining, gels were scanned with a Transidyne densitometer at 650 nm to obtain relative staining intensities. For detection of tritium the gels were first soaked in 50% methanol for 10 min, then soaked for 20 min in 0.25 M sodium salicylate, and then subjected to autoradiography on Kodak XAR-5 film for 14 days at −70 °C.

Ultraviolet Cross-Linking. Fractions 2–5 of the sucrose gradient were pooled and designated the RL (rapidly labeled) fraction of the gradient. The RL fraction was subjected to ultraviolet irradiation at 254.5 nm by a Rayonet photochemical reactor (Model RMR-500) to induce cross-linking of protein to RNA. Typically, the RL fraction was divided into 100-μL aliquots that were exposed for various times from 30 s to 10 min. Control aliquots were placed in the reactor but were shielded from the UV light by aluminum foil. Irradiated samples were either subjected to electrophoresis or to chloroform/phenol extraction as described by van Eekelen and van Venrooij (1981). Essentially, chloroform/phenol (1:1 v/v) was added to an equal volume of the irradiated samples, and the samples were vortexed and centrifuged for 10 min at 10000*g*. The aqueous layer was removed, and the organic layer was washed twice with a buffer containing 50 mM Tris, 100 mM NaCl, and 5 mM EDTA (pH 7.4). All aqueous layers were pooled. Aliquots from both the aqueous and organic layers were counted as previously described. The aqueous layers were

[1] Abbreviations: DNase I, deoxyribonuclease I; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; DTT, dithiothreitol; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; MEM, minimum Eagle's essential medium; PCA, perchloric acid; PMSF, phenylmethanesufonyl fluoride; RNasin, ribonuclease inhibitor; NOR, nucleolar organizer region; RNase A, ribonuclease A; RNP, ribonucleoprotein; SDS, sodium dodecyl sulfate; SSC, standard saline citrate containing 0.15 M NaCl and 0.017 M sodium citrate (pH 7.0); TCA, trichloroacetic acid; RL fraction, gradient fraction containing the most rapidly labeled nucleolar RNA; rDNA, DNA derived from a gene for preribosomal 45S RNA; VSV, vesicular stomatitis virus; UV, ultraviolet; kDa, kilodalton; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

also assayed for the presence of protein C23 after application to nitrocellulose paper using a Minifold apparatus and then immunoblotting as described below.

Immunochemical Methods. Anti-C23 antibodies were produced in rabbits as previously described (Olson et al., 1981) with electrophoretically purified protein C23. A "western blotting" technique was used to identify C23 and degradation products in various experiments. Briefly, the gel was placed on a nitrocellulose filter in a Bio-Rad transblot cell and electrotransferred at 30 V for 17 h in a transfer buffer of 0.025 M Tris, 0.192 M glycine, and 20% methanol at pH 8.3. After transfer, the nitrocellulose was washed for 4 h in a "milk buffer" composed of 0.05 M Tris (pH 7.4), 0.15 M NaCl, 0.1% Triton X-100, 5% instant nonfat dry milk, and 10 drops antifoaming agent (Johnson et al., 1984). The filter was then placed in a Seal-A-Meal bag containing anti-C23 in 5 mL of milk buffer (1:5000 dilution), agitated overnight, and then washed for 4 h with three changes of milk buffer. The ^{125}I-labeled protein A (300 μL) was added to 5 mL of milk buffer without the filter in the Seal-A-Meal bag and incubated with gentle agitation for 4 h. The filter was then washed as above, dried, and autoradiographed with Kodak XAR-5 X-ray film.

Immunoprecipitation of RL Fraction. To 100 μL of the RL fraction were added 20 μg of leupeptin and 10 units of RNasin, along with varying concentrations of anti-C23. Samples were allowed to stand for 1 h at 0–4 °C at which point 100 μL of *Staphylococcus* A (1 g/mL in NET buffer) was added, and the mixture was allowed to stand for 10 min on ice. The precipitate was washed 3 times in NET buffer (0.15 M NaCl, 0.05 M Tris, 0.005 M EDTA, 0.02% EGTA, 0.05% NP-40, pH 7.4), and the supernatants were pooled. The precipitates were solubilized in 1% SDS and centrifuged, and the supernatants were saved. Aliquots from the washes and precipitates were counted as described above.

RNA–DNA Hybridization. The RL and RNP fractions and the 1% SDS-solubilized immunoprecipitates of the RL fraction were subjected to chloroform/phenol extraction and ethanol precipitation (see above). The RNA was dissolved in 250 μL of 20 × SSC and 250 μL of 15% formaldehyde. The concentration of RNA was determined by absorbance at 260 nm and adjusted to 0.01 μg/μL. Various concentrations (0.01–1.0 μg) of the samples were added to nitrocellulose filters according to the method of Cheley & Anderson (1984). Once the RNA was added and the filter dried, it was probed with ribosomal DNA plasmids pDF8 and pDF4 that were previously ^{32}P-labeled by nick translation (Olson et al., 1983). Essentially, the filter was soaked for 30 min in 10 × SSC, washed for 3 h at 37 °C in 4 × SSC, 30% formamide, 10 × Denhardts, and 0.1% SDS, then prehybridized for 3 h at 37 °C in the same buffer, and finally hybridized with the buffer containing 10^6 cpm of the labeled plasmids, for 48 h. After the hybridization was completed, the filter was rinsed in prehybridization buffer with three changes for 1 h at 37 °C and then 2 × SSC and 70% ethanol. The nitrocellulose filter was dried and autoradiographed on Kodak XAR-5 film.

Results

In previous studies (Olson et al., 1986) it was noted that protein C23 sedimented near the top of the gradient in sucrose gradient separations of nucleolar RNP extracts. It was also observed from earlier published work (Daskal et al., 1974; Bachellerie et al., 1975) that a similar fraction contained rapidly labeled nucleolar RNA. To determine whether protein C23 was directly associated with this rapidly labeled (RL) fraction, preliminary studies were done to optimize extraction and gradient conditions. The method of Auger-Buendia and

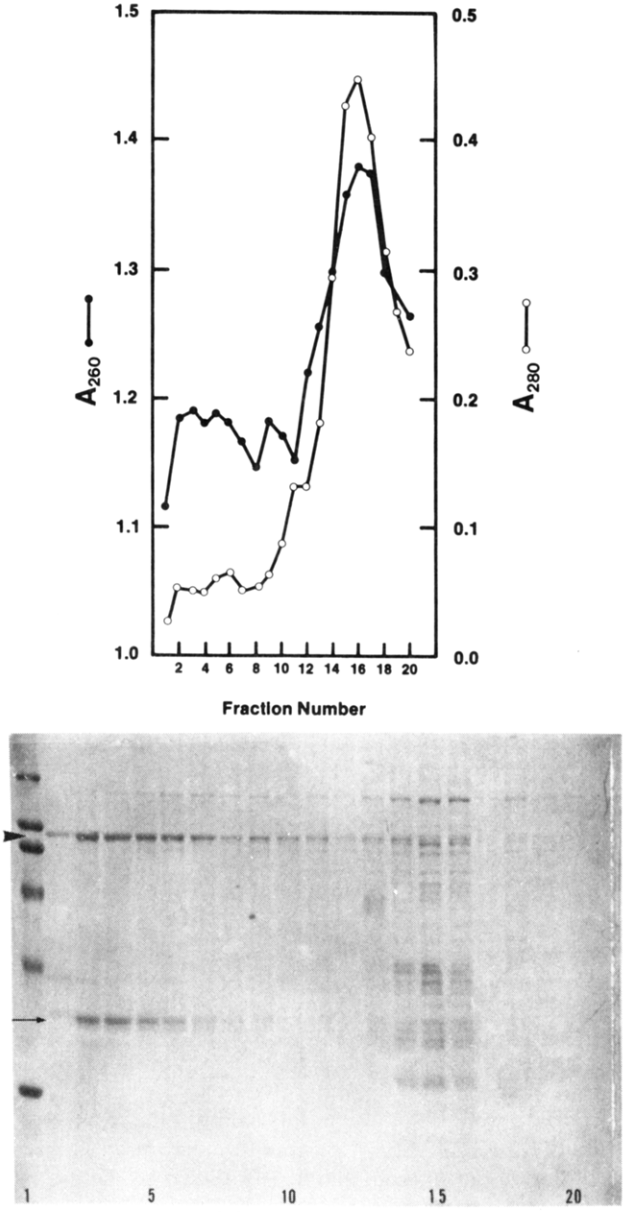

FIGURE 1: Fractionation of nucleolar RNP extract by sucrose density gradient ultracentrifugation. Nucleolar extract was subjected to centrifugation on a 5–30% sucrose gradient. (A) Absorbance profile at 260 and 280 nm. Tubes are numbered from the top. (B) SDS–PAGE analysis of sucrose gradient fractions on 10% Laemmli-type polyacrylamide gel. Lane 1 contains molecular weight standards consisting of (top to bottom) myosin (M_r 205 000), β-galactosidase (M_r 116 000), phosphorylase b (M_r 97 400), bovine albumin (M_r 66 000), ovalbumin (M_r 45 000), and carbonic anhydrase (M_r 29 000). Lanes 2–29 contain gradient fractions (top to bottom): large arrow, protein C23; small arrow, protein B23.

Longuet (1978) was the most satisfactory procedure for extraction and minimizing degradation of protein C23. With a 5–30% sucrose gradient (Figure 1A), the majority of protein C23 sedimented near the top of the gradient (Figure 1B). It was also noted that in addition to C23 the only other major polypeptide present was B23. The 60S–80S RNP fraction appeared at tubes 13–15, which corresponds to the abrupt change in protein pattern seen by SDS gel electrophoresis (Figure 1B).

To determine where the rapidly labeled RNA sedimented in the gradient, cells were first grown in the presence of [^{3}H]uridine before isolation and extraction of nucleoli. Parts A, B, and C of Figure 2 show the gradient profiles of labeled RNA after cells were incubated 1, 4, and 9 h, respectively.

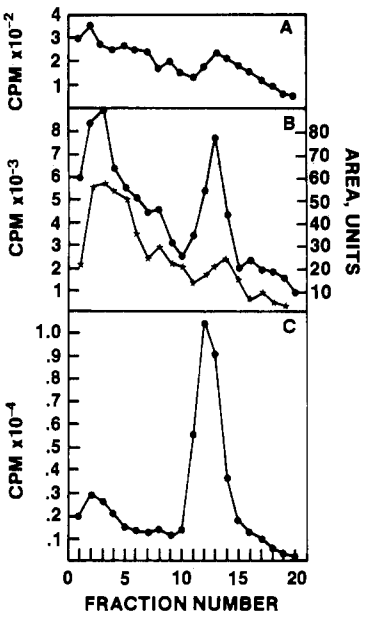

FIGURE 2: [^{3}H]Uridine incorporation into nucleolar RNP extract. (A–C) [^{3}H]Uridine radioactivity (●) of gradient fractions was determined by scintillation counting. Each gradient contained 10 A_{260} units of extracted nucleoli from cells that had been incubated for 1 (A), 4 (B), and 9 h (C). Panel B also contains the relative protein C23 concentrations (★) determined by densitometric tracings from the gel in Figure 1B.

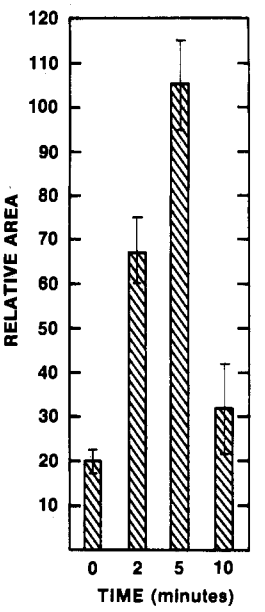

FIGURE 3: Change of protein partitioning after UV irradition and phenol/chloroform extraction. Samples of the RL fractions were exposed to UV light and then extracted with phenol/chloroform. Aliquots of the water layer were absorbed onto nitrocellulose filters and reacted with anti-C23 antibody, followed by detection with radioiodinated protein A. Densitometric scans of the exposed film were plotted in relative units vs. time of irradiation. Error bars indicate standard deviations from three separate determinations. In other experiments not shown (see text) up to 30% of the RNA (as [^{3}H]-uridine) also moves into the phenol layer after UV irradiation.

It was noted that at shorter times of labeling (1 and 4 h) the major peak of radioactivity appeared near the top of the gradient. However, after 9 h of labeling, the peak of radioactivity had shifted to the 60S–80S region of the gradient (Figure 2C). When the relative concentration of C23 determined by densitometric scans of the gel was plotted with the radioactivity pattern after 4 h of labeling, C23 concentration followed the same general pattern as did the radioactivity of the rapidly labeled peak (Figure 2B). This fraction was termed the RL fraction.

To determine whether protein C23 was directly associated with RNA, the RL fraction was subjected to cross-linking by ultraviolet light for varying lengths of time. Initial experiments showed that, by electrophoretic analysis, protein C23 concentration decreased as the time of exposure to UV light increased (data not shown). After 2 min of exposure about 50% of C23 remained, with no significant changes with longer exposure times. The other major protein in the fraction, B23, did not decrease significantly after irradiation.

In additional experiments to determine if the loss of protein C23 was due to direct association with RNA, the RL fraction was extracted with chloroform/phenol after UV cross-linking. In this case there was an increase in the percentage of labeled RNA extracted into the phenol (protein) layer after cross-linking, reaching a plateau of approximately 30% of the total cpm retained in the phenol fraction after 2 min of exposure. Ribonuclease treatment after cross-linking reversed this phenomenon. Additional controls were done in which exogenous ^{3}H-labeled VSV RNA was added to the unlabeled RL fraction before exposure to UV light or simply irradiated in the absence of the RL fraction. There was no change in the partitioning of the VSV RNA after irradiation. This suggested that there was no rearrangement of the protein RNA complexes during irradiation and that irradiation of RNA alone did not affect the partitioning.

The aqueous or RNA phase was also assayed for the presence of C23 by using the immunoblotting technique described under Methods. Figure 3 shows that the C23 signal in the aqueous phase increased after 2 min of UV exposure until about 5 min, after which time it decreased. The decrease at 10 min was presumably due to degradation induced by the elevated temperature of the reaction. When preimmune serum was substituted for immune serum, the blot intensities of irradiated samples were similar to those of the unexposed samples, indicating that the detecting system was selective for protein C23. Furthermore, when the experiment was repeated with purified protein C23, there was no change in the partitioning of C23 between the aqueous and phenol phases (data not shown).

The above experiments established that when exposed to UV light protein C23 disappeared from the RL fraction, that RNA was cross-linked to protein, and that there was an increase in the amount of C23 entering the aqueous phase after phenol extraction. To determine if protein C23 was cross-linked to RNA, a cross-linked species needed to be identified. In doing this, it was necessary to use shorter times of exposure to UV light to minimize the degradation of protein C23. Because the cross-linked species did not appear as a single band, but was distributed over a broad molecular weight range, it was also necessary to use the more sensitive immunoblotting technique to detect the RNA–C23 complex. Both the RL fraction and controls containing purified C23 were exposed to UV light for 0, 30, 60, 90, 120, 300, and 600 s and then analyzed by SDS–polyacrylamide gel electrophoresis. The gels were immunoblotted, and a duplicate gel of the [^{3}H]uridine-labeled RL fraction was fluorographed. When the purified C23 was analyzed by the immunoblotting technique (Figure 4A), protein C23 appeared to be partially degraded with lower molecular weight immunoreactive species increasing in proportion to the time of exposure. No material higher in molecular weight than protein C23 was visible, indicating the absence of protein–protein cross-links. In contrast to this, in the RL fraction (Figure 4B) there was an appearance of higher molecular weight species believed to be RNA cross-linked to

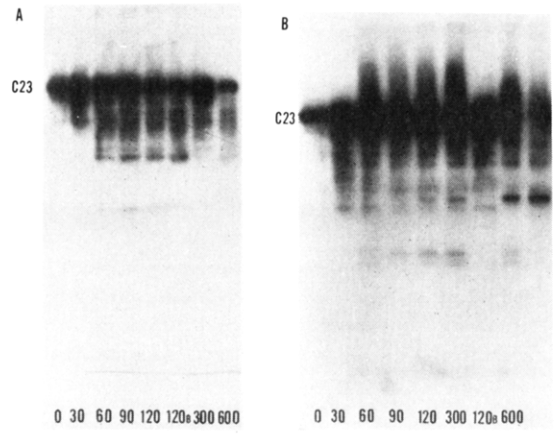

FIGURE 4: Identification of cross-linked species after UV irradiation. (A) SDS–polyacrylamide gel electropheretogram of purified protein C23 exposed to UV light for 0, 30, 60, 90, 120, 120 blank, 300, and 600 s blotted onto nitrocellulose filters and probed with anti-C23 antibody. (B) Immunoblot of the RL fraction exposed to UV light for the same intervals as in panel A.

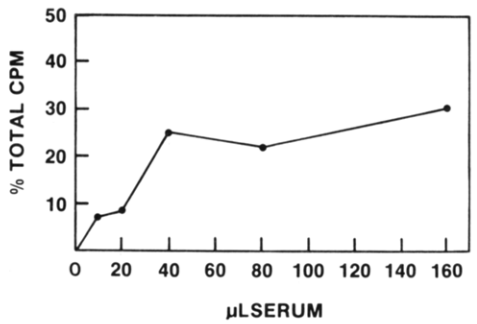

FIGURE 5: Immunoprecipitation of labeled RNA by anti-C23 antibody. Aliquots of the RL fraction were reacted with varying amounts of anti-C23 antiserum and precipitated with *Staphylococcus* A. The precipitates were washed and counted in a scintillation counter.

protein C23 in addition to the bands representing degradation products of the protein. Similar gels were run with [^{3}H]-uridine-labeled material and then subjected to fluorography to determine if there was labeled RNA associated with this new species. As with the immunoblotted gels, there was smearing of signal above C23 in samples that were exposed to UV light (data not shown). In contrast, in control samples not exposed to irradiation little or no ^{3}H radioactivity entered the gel. Thus, upon exposure to UV light, protein C23 appears to be cross-linked to RNA in the RL fraction to produce a mixture of products higher in molecular weight than protein C23.

To confirm that protein C23 was associated with RNA in the RL fraction, anti-C23 antibody was used to precipitate the protein to determine whether labeled RNA was also precipitated. Titration of the protein was accomplished by adding increasing concentrations of antibody to a set concentration of the protein. Figure 5, which demonstrates that approximately 25% of the labeled RNA was precipitated by the antibody at saturating levels, confirms that RNA is associated with the protein. Preimmune serum did not precipitate a significant amount of radioactivity.

To determine whether the RNA precipitated by the anti-C23 antibody contained ribosomal RNA sequences, the precipitated RNA was bound to nitrocellulose filters and probed with cloned sequences of a gene for preribosomal RNA. The probes used were plasmids pDF8 (exclusively from the 18S portion of the gene) and pDF4 (containing most of the 28S plus internal transcribed spacers and 5.8S RNA sequences),

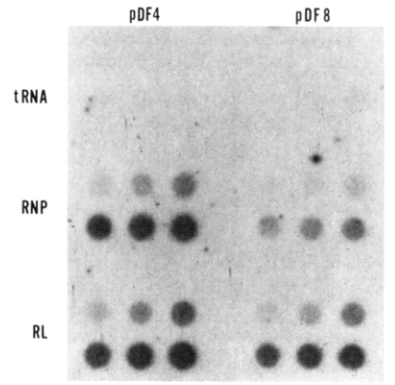

FIGURE 6: Identification of RNA sequences in immunoprecipitated RL fraction. RNA (0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 μg) extracted from the precipitate obtained at slightly above the saturation point (Figure 5) was extracted and subjected to dot-blot analyses. Hybridizations were done with ^{32}P-labeled plasmids pDF8 (containing sequences exclusively from the 18S portion of the 45S RNA gene) and pDF4 (containing most of the 28S region plus internal transcribed spacers and the 5.8S sequence). For comparison, RNA from the 60S–80S RNP fraction (known to contain rRNA sequences) was applied to the filters in the same amounts (RNP). Yeast tRNA was also applied to determine nonspecific background (tRNA).

which were prepared and ^{32}P-labeled by nick translation as previously described (Olson et al., 1983). Figure 6 shows that the immunoprecipitated RNA hybridized with both probes to approximately the same extent. For comparison, equal amounts of RNA from the 60S–80S RNP fraction, which is known to contain ribosomal RNA sequences (Bachellarie et al., 1975), were also hybridized against the rDNA probes. This RNA on a microgram basis contained comparable amounts of the same sequences as did the immunoprecipitated RL fraction. Yeast tRNA was used as a background control, in which case no significant hybridization was seen. The RL fraction was also analyzed by the diphenylamine reaction and found to contain no detectable amounts of DNA, indicating that the hybridization was not due to DNA contamination. Therefore, the RNA associated with C23 appears to contain ribosomal or preribosomal RNA sequences.

Discussion

Although the structure of protein C23 has been studied extensively (Mamrack et al., 1977, 1979; Rao et al., 1982), little is known about how it may function in its proposed nucleolus organizer role. Since the protein is localized to certain regions of the nucleolus, it must recognize and interact with other nucleolar specific macromolecules. In recent years we have attempted to define these interactions as an early step in elucidating the functions of the protein. For example, we found that protein C23 is capable of binding DNA with a preference for the rDNA spacer regions (Olson et al., 1983). However, since the bulk of protein C23 may be extracted from nucleoli at low ionic strength or by ribonuclease, it is unlikely that a major portion of it is tightly bound to DNA (Olson & Thompson, 1983). These studies confirm that, in addition to the possibility of being partially DNA bound, a substantial portion of protein C23 is associated with preribosomal RNA.

Three lines of evidence suggest that protein C23 is associated with nascent preribosomal RNA. First, the majority of protein C23 extracted from nucleoli under conditions of low ionic strength cosediments with the most rapidly labeled RNA peak separated by sucrose gradient ultracentrifugation. Second, RNA in the RL fraction was cross-linked to protein C23 by UV light. Third, the labeled RNA was precipitated by anti-C23 antibody and found to contain sequences from 18S and 28S ribosomal RNA.

The cross-linked species could be identified by electrophoresis as smears, higher in molecular weight than protein C23. These presumably consist of protein C23 with RNA covalently attached. However, since the molecular weight of this material is much smaller than the sum of protein C23 and ribosomal RNA molecular weights, it is likely that this smear represents partially degraded RNA attached to the protein. It is also unlikely that very high molecular weight species would enter the gel, and efforts to run this material on other gel systems have failed. Thus, we cannot precisely identify the RNA by the molecular weight of the cross-linked species.

On the other hand, it was possible to analyze the antibody-precipitated RNA by dot-blot hybridizations, and the RNA was indeed shown to contain 18S and 28S RNA sequences. Because the cloned rDNA fragments used for hybridization spanned internal transcribed spacers as well as the final expressed RNA products, it was not possible to determine whether the complete 45S molecule was precipitated. Thus, the current evidence for the immunoprecipitated RNA being the nascent precursor is that it is the most rapidly labeled species. In addition, it is also not possible to say that the precipitated RNA is the same as the cross-linked RNA, although it is unlikely that they are different species.

The presumed association of protein C23 with nascent preribosomal RNP particles supports previous studies indicating that the protein is largely localized to dense fibrillar regions of the nucleolus (Escande et al., 1985; Lischwe et al., 1981; Spector et al., 1984). However, this is the first indication that there is a direct association with RNA. The implication of this is that C23 is one of the first proteins seen by the newly synthesized RNA. That the putative role as a nucleolus organizer molecule is dependent on RNA binding is supported by recent studies, which suggest that RNP particles themselves organize nucleolar subcomponents into a three-dimensional structure (Labhart et al., 1984; Scheer & Rose, 1984; Wachtler et al., 1984) and that rDNA transcription is necessary for NOR formation (Dhar et al., 1985). Precisely how this is done may only be speculated on at this point. A domain of protein C23 may initially bind DNA, and as preribosomal RNA is synthesized this becomes bound to another domain of the protein. Some of the negatively charged regions of the molecule could interact with histones to condense the chromatin components. Other negatively charged regions may interact with ribosomal proteins to begin the assembly process.

After a transient existence in the fibrillar region, the nascent transcripts and associated proteins move into the granular elements of the nucleolus, which are more mature 60S–80S RNP particles (Busch & Smetana, 1970; Daskal et al., 1974). Protein C23 was also observed in the 60S–80S region of the gradient, although at a much lower concentration (Figure 1B). Presumably, protein C23 moves with the nascent particles into the granular regions as suggested by this observation. The ultimate fate of the protein is unclear, although degradation products of C23 are found in this fraction (Bourbon et al., 1983; A. H. Herrera and M. O. J. Olson, unpublished results).

In summary, these studies further support the hypothesis that protein C23 functions as a nucleolus organizer by engaging in multiple interactions with nucleolar components and by participating in the ribosome assembly process. The precise nature of the interactions and the dynamics of the system have yet to be defined.

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Radiation Inactivation Analysis of Influenza Virus Reveals Different Target Sizes for Fusion, Leakage, and Neuraminidase Activities

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ABSTRACT: The size of the functional units responsible for several activities carried out by the influenza virus envelope glycoproteins was determined by radiation inactivation analysis. Neuraminidase activity, which resides in the glycoprotein NA, was inactivated exponentially with an increasing radiation dose, yielding a target size of 94 ± 5 kilodaltons (kDa), in reasonable agreement with that of the disulfide-bonded dimer (120 kDa). All the other activities studied are properties of the HA glycoprotein and were normalized to the known molecular weight of the neuraminidase dimer. Virus-induced fusion activity was measured by two phospholipid dilution assays: (i) relief of energy transfer between *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (N-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-dioleoyl-L-α-phosphatidylethanolamine (N-Rh-PE) in target liposomes and (ii) relief of self-quenching of N-Rh-PE in target liposomes. Radiation inactivation of fusion activity proceeded exponentially with radiation dose, yielding normalized target sizes of 68 ± 6 kDa by assay i and 70 ± 4 kDa by assay ii. These values are close to the molecular weight of a single disulfide-bonded (HA_1 + HA_2) unit (75 kDa), the "monomer" of the HA trimer. A single monomer is thus inactivated by each radiation event, and each monomer (or some part of it) constitutes a minimal functional unit capable of mediating fusion. Virus-induced leakage of calcein from target liposomes and virus-induced leakage of hemoglobin from erythrocytes (hemolysis) both showed more complex inactivation behavior: a pronounced shoulder was present in both inactivation curves, followed by a steep drop in activity at higher radiation levels. These curves showed that virus-induced leakage is mediated by a larger and more complex functional unit than is required for the prerequisite fusion process, for which the minimum functional unit is a single monomer.

Influenza is an enveloped virus that carries two transmembrane glycoproteins called HA and NA in its lipid membrane (Nayak, 1977). The predominant one, HA, is a trimer consisting of three identical subunits of ca. 75 kDa held together by noncovalent interactions. Each subunit consists of two disulfide-linked polypeptide chains, HA_1 and HA_2, of ca. 50 and 25 kDa, respectively. The X-ray crystal structure of the extracellular portion of HA, comprising over 90% of the molecule, has been determined to 3-Å resolution (Wilson et al., 1981). The second influenza glycoprotein, NA, is a tetramer of subunits of ca. 60 kDa. The X-ray crystal structure of its external portion has been determined to 2.9-Å resolution (Varghese et al., 1983). About 90% of total influenza glycoprotein is HA, and 10% is NA (Nayak, 1977).

HA is the sole influenza protein to catalyze fusion reactions, which the virus undergoes readily at pHs below 6.0 (White et al., 1983). Fusion of the viral envelope with a cellular membrane at low pH is an essential step in the viral infectious cycle and is a necessary prerequisite of virus-induced membrane leakage reactions (White et al., 1983; Lenard & Miller, 1983). HA also functions to bind influenza virions to target cells, through a sialic acid binding site on each monomer (Wilson et al., 1981; White et al., 1983). Binding is independent of the fusion function, since specific proteolytic activity of the HA precursor, HA_0, is absolutely required for fusion (and hence for leakage), but not for hemagglutination (White et al., 1983). NA possesses a readily measurable neuraminidase activity, which may be important in multicycle infections (Nayak, 1977).

An important unanswered question is how many HA molecules constitute a minimum functional unit for fusion. This paper reports results from a radiation inactivation study carried out to answer this question. Radiation inactivation has been successfully used to determine the functional target size and, from this, the molecular weight of a variety of both soluble and membrane-bound enzymes in a frozen, hydrated state (Kempner & Schlegel, 1979; Jung, 1984). In this paper we report different target sizes for the inactivation of two HA-catalyzed functions: a surprisingly small target size is calculated for fusion, while leakage assays reveal a much larger and more complex functional unit. A convenient internal reference for these determinations was provided by measuring the target size for the neuraminidase activity of NA.

Materials and Methods

Influenza Virus. Strain APR/8/34 was grown in 10- to 11-day-old embryonated eggs and purified on 5–40% potassium tartrate gradients.

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