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Biochemical Analysis of a 5S rRNA-Associated Sub-Particle from Trypsinized Eukaryotic Ribosomes[1]

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On limited trypsinization, eukaryotic ribosomes released sub-particles that comprised a 5S rRNA molecule and two peptides (a 32 kDa and a 14 kDa). By tryptic finger-printing and amino-terminal sequence analysis, these two peptides were determined to be derived from large subunit ribosomal protein L5 (rpL5). The 32 kDa peptide represents the rpL5 protein minus the amino terminal eight residues and the carboxyl terminal ends (approximately 21 residues), whereas the 14 kDa peptide comprised near the amino-terminal region. The time course of ribosome trypsinization revealed that the two peptides were released kinetically. The indicated that the amino and carboxyl terminal ends of rpL5 were the first to be hydrolyzed, suggesting that the two ends of the rpL5 protein were exposed on the surface of ribosomes. Exposure of the carboxyl-terminal end was confirmed by use of an anti-L5c antibody raised against the carboxyl terminal region of rpL5. The kinetic data also revealed that the nearby amino terminal region of rpL5 (represented by the 14 kDa peptide) was the last part of rpL5 to be hydrolyzed, which was considered to be the 5S rRNA binding site.

Key words: ribosome, ribosomal protein, 5S rRNA, RNA-protein complex (RNP), trypsinization.

A eukaryotic 5S rRNA-ribosomal protein complex (5S rRNP) can be spilt from monomers or large subunits of eukaryotic ribosomes by treatment with EDTA (*1*) or other (*2, 3*). This makes it an attractive model system for the study of RNA-protein interactions and ribosomal assembly. 5S rRNP has been localized the nucleoplasm as well as the cytosol fraction of eukaryotic cells (*6-8*). It has also been considered to be the precursor for ribosome assembly in mammalian cells (*4, 5*). The structural details of how protein rpL5 and 5S rRNA interact to form the 5S rRNP complex have been limited due to the lack of an appropriate method for reconstituting the complex. Instead, most of the structure information was provided through alternative biochemical analyses. The RNA foot printing (*9*) and other (*4, 10, 11*) methods have been successfully used for elucidating the region of 5S rRNA that interacts with rpL5. Besides, the region of 5S rRNA that is protected by the rpL5 protein has been well defined, and the nucleotide bases that interact with the rpL5 protein have been determined at the single base level (*11*). In contrast, little is known about the structure of the rpL5 protein that is involved in the binding of 5S rRNA. The available information was mostly obtained through studies on yeast 5S rRNA binding protein YL1 (a homologue of mammalian rpL5) (*12-16*). Data have indicated that the carboxyl terminus of YL1 is important for binding to 5S rRNA. However, recent studies have revealed that mammalian 5S rRNA binds at the amino terminal region of rpL5 (*17, 18*), although yeast YL1 and mammalian rpL5 exhibit greater than 80% similarity in their primary structures (*19, 20*), and the structures of yeast 5S rRNA and mammalian 5S rRNA are the same (*21*). In this study, we identified a sub-particle of the 5S rRNP complex that arose on limited tryptic digestion of eukaryotic 80S ribosomes. The properties of this complex were determined by means of biochemical and immunological assays. Throughout the examinations, the region of rpL5 that responded to bind with 5S rRNA was derived. The region of rpL5 that is exposed on the surface of ribosomes is also revealed.

MATERIALS AND METHODS

Preparation of Ribosomes and Ribosomal Proteins from Rat Liver—Eukaryotic ribosomes (80S) were prepared as described elsewhere (*22*). In brief, 5 g of rat liver tissue was homogenized in 50 ml of Buffer A (20 mM Tris- HCl, pH 7.4; 50 mM KCl; 12.5 mM $MgCl_2$; and 6 mM β-mercaptoethanol) containing 0.25 M sucrose. The homogenates were centrifuged in a Sorvall SS-34 rotor at 8,500 rpm for 15 min at 0°C. The post-nuclear supernatants were filtered through 4 layers of cheese cloth. A 15% solution (w/v) of sodium deoxycholate (DOC) in water was added to the supernatants, while being stirred on ice, until the final concentration of DOC reached 1.5% (w/v). Ten-milliliter aliquots were distributed into Beckman Ti60 centrifuge

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Abbreviations: RNP, ribonucleic protein complex; rRNA, ribosomal ribonucleic acid; rpL5, ribosomal protein L5; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetracetate.

tubes and then under layered with 5 ml of 1.0 M sucrose in Buffer A. Ribosomes were sedimented by centrifugation at 60,000 rpm at 4°C for 2 h. The ribosomal pellets were stored at −70°C.

The total ribosomal proteins were extracted from 80S ribosomes with 67% acetic acid in 10 mM Tris-HCl containing 33 mM magnesium acetate.

Preparation of Sub-Particles and 5S rRNP Complexes from 80S Ribosomes—The 5S rRNP complexes were prepared from 80S ribosomes by EDTA treatment (*1*). The preparation of sub-particles was carried out by mild treatment of 20 A_{260} units of 80S ribosomes with 1 μM trypsin in TKM buffer at 37°C for a predetermined time. At the end of the treatment, the reaction was terminated by adding immobilized soybean trypsin inhibitor (resin bound soybean trypsin inhibitor; Pierer Chemical). The reaction mixtures were analyzed by 8% non-denatural gel electrophoresis. The positions of sub-particles and RNP complexes were visualized by silver staining. In this study, the sub-particles and 5S rRNP complexes were isolated from the gels and then subjected to biochemical analysis.

Northern Blotting—The excised gel containing RNP complexes was first crushed in a solution comprising 40 mM ammonium acetate and 1 mM EDTA, and then the RNA was extracted by the hot phenol-SDS procedure. The RNA was run on a 2% agarose gel in TBE buffer and then immobilized on nitrocellulose paper. Northern hybridization was carried out exclusively with the ^{32}P-labeled sense strand of 5S rDNA. The probe was obtained by means of the polymerase chain reaction from a plasmid (pGEM-T-$5S_H$) that contained human 5S rDNA prepared in our laboratory. The probe was made with radioactive [α-^{32}P]-ATP during PCR amplification.

Radioiodination of Protein in Polyacrylamide Gel Slices—The radioiodination of RNP complexes in non-denatural gel was performed by an established procedure (*23*). Briefly, gel slices containing RNP complexes were placed in siliconized test tubes, washed extensively with 25% (v/v) isopropanol and then with 10% (v/v) methanol, and finally dried by vacuum desiccation. The following components were added sequentially to each dried gel slice: 20 ml 0.5 M sodium phosphate buffer, pH 7.5, 300 mCi sodium ^{125}I, and 5 μl chloramine T (1 mg/ml; Sigma). The gel slices were allowed to absorb the liquid for 30-45 min, and then 1 ml of sodium bisulfite (1 mg/ml) was added to terminate the reactions. After 15 min, the slices were removed and placed in individual nylon pouches, and then allowed to dialyze for at least 24 h against four one-liter changes of 10% (v/v) methanol. At the end of the dialysis, each gel slice was removed, placed in a siliconized tube, dried by vacuum desiccation and then crushed into powder. Proteins were extracted by adding a denatural solution comprising 400 mM Tris-HCl, pH 8.0, and 1% SDS. The extracted proteins were analyzed by 15% polyacrylamide gel electrophoresis in the presence of SDS and autoradiography.

Peptide Mapping of Radioiodinated Proteins—To each tube of iodinated gel powder, 1 ml of 50 μg/ml TPCK-trypsin (Worthington) in 50 mM ammonium bicarbonate was added, followed by incubation for 24 h at 37°C. At the end of the incubation, the supernatant was removed and lyophilized. The trypsin-digested sample was dissolved in 20 μl of the first dimension running solution (1-D solution), comprising acetic acid, formic acid, and water (15:5:80), and then spotted onto 20×20 cm cellulose thin layer plate (Polygram cel 300). Electrophoresis was carried out for 90 min at 400 V in a flat bed electrophoresis apparatus maintained at 4-10°C with the 1-D solution. After the electrophoresis, the plate was dried and then subjected to the second dimensional chromatography in a solution of butanol, pyridine, acetic acid, and water (32.5:25:5:20). Following the chromatography, the plate was dried and autoradiographed.

Time Course of Trypinization and Analysis of Truncated Peptides—80S ribosomes were treated with trypsin (as described above) for different time intervals (0, 5, 10, 15, and 30 min). After the treatment, the ribosomes were analyzed by 8% non-denatural gel electrophoresis. Gel corresponding to the positions of sub-particles was excised and radioiodinated. Proteins extracted from the radioiodinated sub-particles were analyzed on a 15% polyacrylamide gel containing SDS. In each case trypsinization treatment was started with an equivalent of 30 A_{260} units of 80S ribosomes. As a control, 5S rRNP was prepared from EDTA-treated 80S (10 A_{260}) by the same procedures.

Immunological Dot Blotting Assay for Ribosomes—The immuno dot blotting assay was used to examine the surface properties of rpL5 on ribosomal complexes. Anti-L5c antibodies were obtained by immunizing a rabbit with a synthetic peptide comprising the last 21 residues of the carboxyl terminal end of rpL5. The authenticity of the anti-L5c antiserum has been determined. It reacts positively with rpL5 and the synthetic carboxyl terminal peptide of rpL5. Ribosome complexes of 80S, trypsinized 80S, EDTA-treated 80S, and *Escherichia coli* 70S, along with total proteins from 80S and trypsinized 80S, were dotted on nitrocellulose paper. Their immuno-cross reactivity to anti-L5c antibodies was examined according to the standard procedure and detected with non-radioactive AP-Biotin-Avidin chromogen (Bio-Rad, USA).

RESULTS AND DISCUSSION

Identification of Constituents of the Sub-Particles from 80S Ribosomes—When rat liver 80S ribosomes were mildly treated with trypsin for 15 min, a sub-particle that appeared in a nondenatural gel as a band at a position between those of the 5S rRNP complex and 5S rRNA was observed (Fig. 1). The molecular nature of the sub-particle was first examined by the protein-radioiodination method. This method was established for the detection of proteins in a polyacrylamide gel band (*23*). Gel that contained the 5S rRNP complex or the sub-particles was excised and radioiodinated. Autoradiography after SDS polyacrylamide gel electrophoresis revealed that the 5S rRNP complex gives a single radioactive peptide that corresponds exactly to the 5S rRNA binding protein, rpL5 (*1, 3, 19*) (Fig. 2A), whereas the sub-particle yields two radioiodinated peptides with approximate molecular weights of 32 and 14 kDa (Fig. 2A).

The possibility of the ribosomal RNA moiety being contained in the sub-particle was next examined by means of the Northern blotting procedure. RNA was extracted from gel containing sub-particles and 5S rRNP complexes and then hybridized with ^{32}P-labeled anti-sense 5S DNA probe. The results showed that the sub-particles gave two

hybridized bands (Fig. 2B), both bands being due to 5S rRNA. The major one corresponds to the fast moving form of 5S rRNA, and the minor one to the slow moving form. Both the fast and slow moving 5S rRNAs were also found in the gel containing the 5S rRNP complex (Fig. 2B). Two forms for 5S rRNA under certain electrophoresis conditions have been reported (personal communication from Dr. P.W. Huber). The results confirm that the sub-particle released from trypsinized ribosomes is a 5S rRNA associated ribonucleic-protein complex in nature.

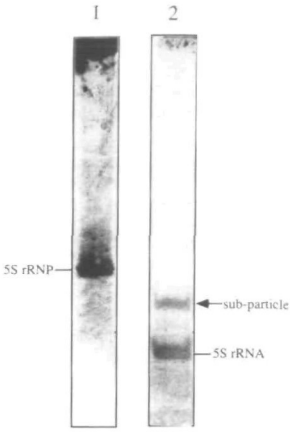

Fig. 1. **Analysis of the RNP complex by non-denatural polyacrylamide gel electrophoresis.** Analysis was carried out in a 8% non-denatural polyacrylamide gel in TBE buffer, pH 8.0, at a constant 100 V for 100 min. Lane 1, 10 A_{260} units of 80S ribosomes treated with EDTA, and lane 2, 30 A_{260} units of 80S ribosomes trypsinized for 15 min. The bands were visualized by silver staining. The positions of 5S rRNP and 5S rRNA are indicated. The sub-particle is indicated by an arrow.

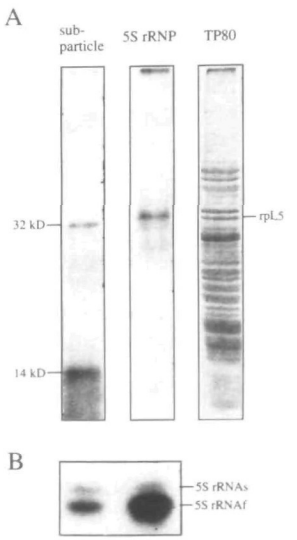

Fig. 2. **Determination of the molecular moieties of sub-particles extracted from gel slices.** Panel A shows autoradiography after SDS polyacrylamide gel electrophoresis of sub-particles or 5S rRNP complexes. Radioiodination was performed as described in the text. *Lane TP80, Coomassie blue staining of the co-electrophoretic pattern* of total proteins extracted (40 μg) from rat liver 80S ribosomes. 32 and 14 kDa indicate approximate molecular weights of 32,000 and 14,000 daltons, respectively. The position of rpL5 is also indicated. Panel B shows the results of Northern blotting of RNA extracted from gel containing to sub-particles and 5S rRNP complexes. The RNA was separated in a 2% agarose gel in TBE buffer and then transferred to nitrocellulose paper for Northern hybridization. A full-length human [^{32}P]5S rDNA was used as the probe. The slow and fast moving forms of 5S rRNA are denoted as 5S rRNAs and 5S rRNAf, respectively.

The peptides of 32 and 14 kDa are speculated to be derived from ribosomal protein L5 (rpL5) because rpL5 is known to associate with the 5S rRNA molecule (*1-3*). Thus, the radioiodinated tryptic mapping of rpL5 was performed for comparison with 32 and 14 kDa peptides. A tryptic peptide map that comprises sixteen tyrosine-containing peptides is expected if one counts the available tryptic peptide containing possible iodinated tyrosine residues from rpL5 (*19*). Evidently, the radioiodinated rpL5 was derived from the 5S rRNP complex gave the expected number of tyrosine-containing tryptic peptides (Fig. 3). Under the same experimental conditions, the radioiodinated tryptic map of the 32 kDa peptide coincides with that of rpL5 (Fig. 3), with one minor exception (Fig. 3, right; indicated by an arrow). Moreover, the map of the 14 kDa peptide contains ten tryptic radioactive spots and coordinately matches that of rpL5 too (Fig. 3). Analysis of the tryptic maps suggested that the two peptides were indeed derived from rpL5 through trypsinization. With the evidence that both 5S rRNA and rpL5 are moieties of the sub-particle, the sub-particle can therefore be considered

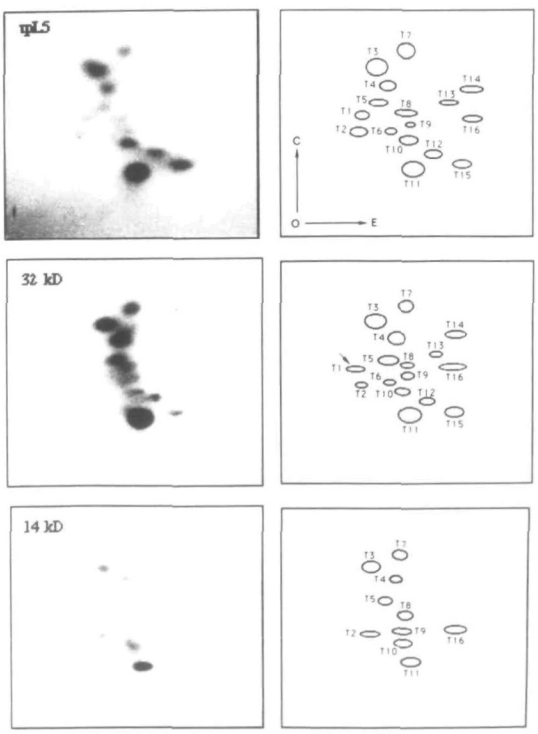

Fig. 3. ***Radioiodinated tryptic peptide mapping of rpL5, and the 32 and 14 kDa peptides.*** Two dimensional tryptic finger-printing of radioiodinated polypeptides was performed as described in the text. On the left are autoradiographic maps of the radioiodinated tyrosine containing tryptic peptide of rpL5, and the 32 and 14 kDa peptides. On the right are schematic representations of the corresponding patterns. The peptides are named T_1 through T_{16} for comparison. The directions of electrophoresis (E) and chromatography (C) on the thin-layer cellulose plate are indicated by arrows at the origin, O, in the top right panel.

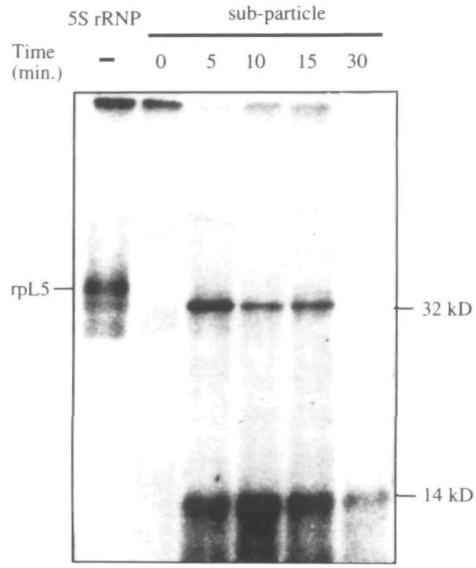

Fig. 4. **The time course on the release of 32 and 14 kDa peptides from trypsinized 80S ribosomes.** The detection of radioactive polypeptides released from trypsinized 80S ribosomes was performed essentially as described in the text. Each lane carried the equivalent of 30 A_{260} units of 80S ribosomes, trypsinized for 0, 5, 10, 15, of 30 min, as indicated at the top of the figure. Sub-particles were subsequently isolated from the gel and radioiodinated. The positions of radioiodinated peptides were visualized by autoradiography of the 15% polyacrylamide gel electrophoresis gel containing SDS. As a control 5S rRNP was prepared from EDTA-treated 80S by the same procedures. The positions of protein rpL5, and the 32 and 14 kDa peptides are indicated.

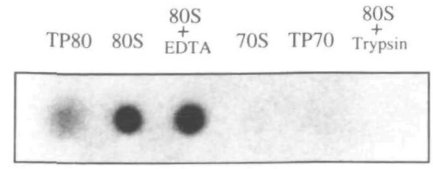

Fig. 5. **Analysis of the surface exposure of rpL5 on ribosomes with an anti-L5c antiserum by immuno dot blotting.** Rat ribosomes (80S), EDTA-treated rat ribosomes (80S + EDTA), trypsinized rat ribosomes (80S + trypsin), *E. coli* ribosomes (70S), total proteins of rat ribosome 80S (TP80), and total proteins of *E. coli* 70S ribosomes (TP70) were immobilized on nitrocellulose paper (NC paper) and then incubated with the anti-L5c serum (diluted 1:100 in PBS) for 2 h, followed by non-radioactive AP-Biotin-Avidin chromogen detection (Bio-Rad, USA). The amounts of sample applied on the NC paper were 2 A_{260} units for ribosomes, and 15 μg for total protein extracts of 80S and 70S.

to be a truncated form of the 5S rRNP complex.

The Kinetics Releasing Two rpL5 Peptides during the Trypsinization of Ribosomes and the Alignment of These Two Peptides on rpL5—The presence of two peptides in the sub-particle obtained on kinetic trypsinization of ribosomes was observed (Fig. 4). After 5 min ribosome trypsinization, ribosomes released sub-particles containing both peptides but predominantly the 32 kDa one (Fig. 4). As the time of ribosome trypsinization increased, the amount of the 14 kDa peptide increased (time, 15 min) and eventually it became the sole peptide (time, 30 min). If the trypsinization of ribosomes was performed for over 45 min, neither the sub-particle nor the rpL5 peptides were detected (results not shown). The kinetic appearance of two rpL5 peptides reflects the topological arrangement of rpL5 on ribosome particles. First, a region of rpL5 of approximately 3 kDa in size (the difference between molecular weight of the 32 kDa peptide and that of the native rpL5) is expected to be exposed on the surface of ribosomes because it is susceptible for hydrolysis at the beginning of ribosome trypsinization. Second, the increasing amount of the 14 kDa peptide and the decreasing amount of the 32 kDa peptide as trypsinization progressed indicated that a region of 14 kDa in size is protected by the 5S rRNA molecule. The locations of 32 kDa and 14 kDa peptides in the structure of rpL5 are therefore of interest.

The locations of 32 and 14 kDa peptides were separately determined by automated sequencing by the PVDF membrane method. Both peptides had the same amino terminal sequence, that is, Asn-Lys-Ala-Tyr-Phe-. The determined sequence is aligned at the ninth position of the amino terminal end, which is preceded by a lysine residue (Lys^8) (*19*). An approximate molecular weight of 0.9 kDa was estimated for the first eight residues of the amino terminal end, which can not cover the molecular weight difference of 3 kDa between the native rpL5 and the 32 kDa peptide. Therefore, it is inevitable that the carboxyl terminal end has to be cleaved off to obtain the 32 kDa peptide from rpL5. Of the 296 residues of rpL5, 64 are basic (22 mol percentage) (*19, 20*). Most of the basic amino acids occur in groups of 3 or 4 consecutive residues, and there are two regions in which they are concentrated: 20 of 54 near the amino-terminus (positions 5-58) and 19 of 44 near the carboxyl terminus (positions 240-283) (*19, 20*). Thus, both ends could be hydrolyzed by trypsin if they were unprotected, and the production of a 32 kDa derivative is therefore expected. In fact, when an antibody raised against the carboxyl-terminal end comprising the last 21 residues was used to examine the cross-reactivity with different ribosome particles by means of immunological dot blotting, the antibody only cross-reacted with 80S or EDTA-treated 80S, *i.e.* not with trypsinized 80S or *E. coli* 70S (Fig. 5). The results indicated that the carboxyl end of rpL5 was on the surface of ribosomes. Interestingly, the last 21 residues at the carboxyl terminal end are characteristic of an amphipathic structure and are suggested to play a role to bridge the 5S rRNP into the pre-mature ribosome previously (*15, 19*). The observation of *in vivo* lethality of yeast cells that had the carboxyl-terminal residues of ribosomal protein YL1 (a homologue of rpL5) deleted supported this suggestion (*14*).

The 14 kDa peptide is starts tentatively at the ninth position, an Asn residue, and extends to residue Arg^{111} of rpL5. The aligned region was deduced because it is approximately 14 kDa in molecular mass and covers nine iodinated tryptic peptides that coordinately match the data in the iodinated tryptic map of the 14 kDa peptide. According to the alignment, the 14 kDa peptide should theoretically carry nine iodinated tryptic peptides, however, the 14 kDa peptide actually gave ten spots on the tryptic map (Fig. 4). The additional spot is a common phenomenon in ribosomal protein biochemistry; it might be caused by the incomplete digestion by trypsin of the cluster of lysine and arginine residues (*24*). The determined region coincided with the finding made previously through a combined approach of

genetic cloning and affinity chromatography (*18*). This region was not susceptible to digestion on trypsinization in our kinetic study, suggesting that it is protected by the 5S rRNA molecule.

Limited proteolysis has been performed to identify an exposed peptide area on intact ribosomes in studies on prokaryotic ribosomes previously (*25, 26*). In this study, we successfully used this approach to reveal the regional structure of eukaryotic rpL5 that is exposed on the surface of ribosomal particles and that binds to 5S rRNA.

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