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Site-specific cleavage of the 40S ribosomal subunit reveals eukaryote-specific ribosomal protein S28 in the subunit head

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ABSTRACT

After resolving the crystal structure of the prokaryotic ribosome, mapping the proteins in the eukaryotic ribosome is a challenging task. We applied RNase H digestion to split the human 40S ribosomal subunit into head and body parts. Mass spectrometry of the proteins in the 40S subunit head revealed the presence of eukaryote-specific ribosomal protein S28e. Recombinant S28e was capable of specific binding to the 3′ major domain of the 18S rRNA ($K_a = 8.0 \pm 0.5 \times 10^9\ M^{-1}$). We conclude that S28e has a binding site on the 18S rRNA within the 40S subunit head.

Structured summary:
MINT-8044084: *S8* (uniprotkb:P62241) and *S19* (uniprotkb:P39019) *colocalize* (MI:0403) by *cosedimentation through density gradient* (MI:0029)
MINT-8044095: *S8* (uniprotkb:P62241), *S19* (uniprotkb:P39019) and *S13* (uniprotkb:P62277) *colocalize* (MI:0403) by *cosedimentation through density gradient* (MI:0029)
MINT-8044024: *S29* (uniprotkb:P62273), *S28* (uniprotkb:P62857), *S21* (uniprotkb:P63220), *S20* (uniprotkb:P60866), *S26* (uniprotkb:P62854), *S25* (uniprotkb:P62851), *S12* (uniprotkb:P25398), *S17* (uniprotkb:P08708), *S19* (uniprotkb:P39019), *S14* (uniprotkb:P62263), *S16* (uniprotkb:P62249) and *S11* (uniprotkb:P62280) *colocalize* (MI:0403) by *cosedimentation through density gradient* (MI:0029)
MINT-8044065: *S29* (uniprotkb:P62273), *S28* (uniprotkb:P62857), *S19* (uniprotkb:P39019), *S14* (uniprotkb:P62263) and *S16* (uniprotkb:P62249) *colocalize* (MI:0403) by *cosedimentation through density gradient* (MI:0029)

1. Introduction

After the atomic structure of prokaryotic ribosome was determined a decade ago [1–4], it became a major goal to determine the structure of a ribosome from eukaryotic species [5]. Eukaryotic ribosomal subunits are much more complex than their bacterial counterparts; they have longer rRNAs and a bigger set of ribosomal proteins, some of which are unique to eukaryotes [6]. Hence, determining the fine structure of the eukaryotic ribosomes by various physical and biochemical methods is a very laborious task.

Knowledge on the RNA–protein architecture of eukaryotic ribosomes and especially on the topography of their proteins is very important in modern molecular biology and molecular medicine, since ribosomal proteins are possible targets for cellular regulatory factors and are used by viruses for ribosome recruitment of their genomic RNAs during host cell infection [5,7]. Moreover, mutations in ribosomal proteins are associated with several diseases and these mutations may significantly reduce the amount of mature ribosomal subunits [8]. One of proteins whose mutations are associated with Diamond-Blackfan anaemia is ribosomal protein S19e [9,10]. The discovery of its location on the 40S ribosomal subunit made it possible to position a part of these mutations in a vicinity of the RNA-binding site in this protein [6]. The authors suggested that these mutations disrupt critical RNA–protein interactions required for incorporation of rpS19e into pre-40S subunits, leading to decreased production of the mature subunits [6]. The position of S19e on the ribosome was established after docking of its crystal structure [11] into the cryo-EM model of the 40S ribosomal subunit [6]. However, this strategy may be hard to apply universally because many ribosomal proteins have very flexible structures and are difficult to crystallize [12].

Nowadays, the positions of many ribosomal proteins in the eukaryotic ribosome are determined on the basis of positions of

Abbreviations: SDS–PAGE, polyacrylamide gel electrophoresis in the presence of SDS

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their counterparts in the 30S and 50S ribosomal subunits [6,13,14]. However, the 40S ribosomal subunit has at least 13 eukaryote-specific proteins, whose positions have not been determined yet [6]. Here, we present data showing that eukaryote-specific ribosomal protein S28e is in the head of the 40S subunit. We used an approach based on splitting of the small ribosomal subunit to sub-particles by RNase H hydrolysis [15], modifying the procedure substantially. We demonstrate that recombinant human S28e has a high affinity for RNA corresponding to the 3′ major domain of 18S rRNA, which is a scaffold of the small subunit head. Comparison of our findings with published data suggests that the position of S28e on the 40S subunit head is in the region opposite to the platform.

2. Materials and methods

2.1. RNase H hydrolysis and sucrose gradient centrifugation

40S ribosome subunits from human placenta were obtained as in [16]. Ribosomal subunits (100 pmol) in 40 μl of buffer A (20 mM Tris–HCl, pH 7.5, 120 mM KCl and 10 mM $MgCl_2$) were incubated with 15-fold excess of the oligodeoxyribonucleotides 18S-1207 (5′-CGTCAATTCCTTTA-3′) and 18S-1693 (5′-GACGGGC GGTGTGTAC-3′) and 10 μl (50 units) of RNase H (Fermentas) at 37 °C for 48 h. After the incubation, the reaction mixture was loaded onto sucrose gradient 10–30% in buffer A and centrifuged in SW40 rotor for 17 h at 30,000 rpm and 4 °C. Gradient was fractionated through a flow cuvette of microspectrophotometer Milichrom (Econova, Russia).

2.2. Gel electrophoretic analysis of RNA and proteins. Western blotting

Nucleotide material from the sucrose gradient fractions was precipitated with 2 vol of ice-cold ethanol and dissolved in water. RNA was isolated by phenol deproteination as described elsewhere and analyzed by 4% denaturing PAGE. Proteins were analyzed by 14% polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE) following the dissociation of the 40S subunit sub-particles into RNA and proteins by incubation in 2% SDS containing 5 mM EDTA at 37 °C for 15 min. Rabbit antisera and specific antibodies against human ribosomal proteins S8e, S13e and S19e (obtained by S. Vladimirov) and anti-rabbit HRP (Sigma) were used for ribosomal protein identification by Western blotting.

2.3. MALDI-TOF mass spectrometry

Prior to mass spectrometry, the 40S ribosomal subunits and sub-particles were incubated in 0.1% TFA at 37 °C for 10 min. A saturated solution of sinapinic acid in 50% aqueous acetonitrile containing 0.1% TFA was used as a matrix. Mass spectra were recorded using an autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a pulsed N_2 laser (337 nm) in a positive linear mode. The final spectra were obtained by accumulating about 1700 single laser shot spectra using flexControl 2.4.41.0 software.

2.4. Synthesis of recombinant human ribosomal protein S28

A DNA fragment corresponding to S28e mRNA was obtained by conventional PCR with the use of the primers S28F 5′-GAGGAGC TCATATGGACACCAGCCGTGTGCAG-3′ and S28R 5′-GAGAGGATCC TCAGCGCAACCTCCGGGCTT-3′ and total cDNA from human placenta as a template. After BamH I and Nde I digestion, the fragment was cloned into pET-15b (Novagen) digested at the same sites. Recombinant S28e was obtained in BL21(DE3) *Escherichia coli* strain transformed with the resulting plasmid. The cell culture was grown at 20 °C in LB medium to an OD_{600} of 0.5 and protein expression was induced with 0.5 mM IPTG. After growth for 3 h, cells were precipitated, resuspended in a buffer containing 20 mM Hepes–KOH, pH 7.6, 20 mM KCl, 5 mM 2-mercaptoethanol and 2 mM PMSF, incubated with 100 μg/mL of lysozyme for 15 min at 30 °C, and sonicated on ice at 44 MHz. Recombinant protein from soluble fraction was purified on Ni^{2+} – NTA agarose resin (Invitrogen) as described [17]. Protein concentration was calculated by method of Bradford.

2.5. RNA transcription and nitrocellulose binding assay

A DNA template for T7 transcription was obtained by PCR using primers 18S3DF 5′-TAATACGACTCACTATAGGGACGGAAGGGCAC-CACC-3′ and 18S3DR 5′-GTGTACAAAGGGCAGGGA-3′ and the plasmid pAM18T7-2 containing human 18S rRNA gene. Labeled with ^{32}P RNA was transcribed as in [18]. RNA–protein binding was performed in buffer (20 mM Tris–HCl, pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$ and 0.25 mM spermidine) by incubation of components at 25 °C for 1 h. Nitrocellulose binding assays were done as described in [19]. Fragment of S13e pre-mRNA was obtained as [20].

3. Results and discussion

3.1. Cleavage of the 40S ribosomal subunit into the head and body with RNase H

The head of the 30S ribosomal subunit is formed by the 3′ major domain of 16S rRNA and ribosomal proteins bound to it [2–4,21,22]. This RNA domain folds separately [23,24] and practically does not interact with the other rRNA domains [2–4]. This domain can be detached from the rest of the16S rRNA by cleavage of the RNA strands between helices H27/H28 and H28/H44 [15]. We designed oligonucleotides complementary to corresponding regions in human 18S rRNA and used these to target RNase H digestion to the 18S rRNA in 40S subunits. Since 18S rRNA strands might be inaccessible to complementary oligonucleotides, we tested whether long-term incubation of the 40S subunits under digestion conditions might be required. We found that digestion of the 18S rRNA in the 40S ribosomal subunits reached about 20% after 2 days incubation at 37 °C (Fig. 1A). With longer incubations, spontaneous degradation of the 40S subunits becomes very significant. To isolate the 40S subunit head parts, the reaction mixture was subjected to sucrose gradient centrifugation (Fig. 1B) and sub-particles with sedimentation coefficients of 10–15S were precipitated. The RNA isolated from these sub-particles migrated in denaturing PAGE as a single band corresponding to a length of about 480 nt that coincided with the length of the 3′ major domain (Fig. 1D). Thus, we conclude that isolated particles are the head parts of the 40S subunits.

3.2. The protein content of the 40S ribosomal subunit head

Gel-electrophoretic analysis of the proteins isolated from the 40S subunit head fractions showed that they contained a significantly smaller set of proteins than that in the original 40S ribosomal subunits (Fig. 1C). Moreover, analysis of the proteins isolated from the sucrose gradient fractions containing a mix of the unhydrolyzed 40S ribosomal subunits and the beheaded particles revealed that they are also depleted of some proteins (Fig. 1C). Since SDS–PAGE does not resolve reliably all ribosomal proteins, western blotting and mass spectrometry were applied. Immunostaining of three ribosomal proteins (Fig. 2E) showed that eukaryote-specific ribosomal protein S19e was present in both the unhydrolysed 40S subunits and the 40S subunit head fractions,