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Research paper

Interactions of human ribosomal proteins S16 and S5 with an 18S rRNA fragment containing their binding sites

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ABSTRACT

Human ribosomal proteins S5e and S16e are the homologues of prokaryotic S7p and S9p, respectively. It was shown that S5e and S16e are capable of the specific binding with a rRNA transcript corresponding to the region of human 18S rRNA containing helices H28–30 and H41–43 (3Dm), which is homologous to the region in 16S rRNA containing the entire binding site for S7p and the major part of the site for S9p. We have studied binding of S5e and S16e to 3Dm and demonstrated that while each of them is able to bind to the rRNA transcript independently, their simultaneous binding has a noticeable synergetic effect. Using enzymatic footprinting, we showed that these proteins protect 3Dm against hydrolysis with RNases mainly in the regions homologous to the sites of S7p and S9p binding on the 16S rRNA. At the same time, we found regions that correspond to 16S rRNA fragments distant from the binding sites of the respective homologous prokaryotic proteins. Comparison of these results with the data on 3Dm footprinting in binary complexes with S5e or S16e revealed that each of these proteins affects binding of another one to 3Dm, which is displayed in significant expansion of 3Dm sites protected by the proteins against hydrolysis in the ternary complex.

1. Introduction

The ribosome is a giant ribonucleoprotein responsible for protein synthesis in all living cells. It consists of 3–4 RNA chains and several dozen different ribosomal proteins, and it is composed of two ribosomal subunits, a large and a small subunit (for a review see [1]). Correct assembly of the subunits is absolutely required for translational activity. Prokaryotic ribosomal subunits were shown to possess the ability to self-assemble [2–8]. In other words, they can be assembled *in vitro* into active subunits from the individual ribosomal proteins (native or recombinant) and the rRNAs. The order of protein binding reflects the cooperative and hierarchical nature of subunit assembly. All proteins of the small (30S) ribosomal subunit can be divided into three groups [2,5]. The primary binding proteins can bind to the 16S rRNA independently of the others. Only after binding of these proteins, the proteins of the second group can be incorporated. For binding of the proteins from the third group, at least one protein from the primary and one protein from the second group should be bound to 16S rRNA.

In comparison with the assembly of prokaryotic subunits, the assembly of eukaryotic ribosomal subunits is much more complicated and poorly understood. Studies of ribosomal subunits assembly carried out mainly on yeast showed that *in vivo* dozens of snoRNAs and more than 200 regulatory proteins take part in this process (for a review, see [9]). Obviously, due to the complexity of the process, *in vitro* methods for the reconstitution of active eukaryotic ribosomal subunits have not yet been elaborated. Therefore, an approach based on simulating the structure of individual regions of ribosome with the use of relatively short rRNA fragments and their cognate proteins is one of the few approaches available to study the structure of the eukaryotic ribosome.

Previously, we studied the dissociation of proteins from human 40S ribosomal subunits in LiCl concentration gradients [10]. Four proteins (S7e, S10e, S16e and S19e) were most resistant to dissociation and this could reflect their strong interaction with the 18S rRNA. It was supposed that these proteins are core proteins of the 40S ribosomal subunit. Among these proteins, only S16e has a prokaryotic homologue – S9p [11], which does bind to the 3′ major domain of the 16S rRNA, but prior to this the binding of protein S7p is absolutely essential [12]. Human S5e, a homologue of prokaryotic S7p [11], dissociates from 40S ribosomal subunit more easily than S16e [10] and therefore cannot be considered as a core protein of the subunit. Using footprinting, we have shown earlier which nucleotides in an RNA-transcript containing a part of the 3′ major domain of the 18S rRNA change their accessibilities to RNase probes after binding of the transcript to human S5e [13] or S16e

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[14]. This RNA-transcript corresponds to a sub-domain of the 16S rRNA bearing the larger part of the binding site for S9p and the entire site for S7p [15]. The question whether binding of S16e to the 18S rRNA affects S5e positioning on the RNA, and vice versa, remained unclear.

To examine the mutual effects of S5e and S16e on their binding to the 18S rRNA, we have studied here the joint binding of recombinant S16e and S5e with the same RNA transcript as used earlier [13,14]. First, we test the ability of S16e and S5e to bind alone to the RNA transcript and show that both S16e and S5e have a high affinity for the RNA. We then performed footprinting experiments with the RNA transcript complexed with S16e together with S5e and identified nucleotides whose accessibilities to enzymatic probes changed. The data obtained were compared with the results on footprinting of the same RNA transcript in the complexes with S5e or S16e alone reported earlier [13,14].

2. Materials and methods

2.1. Ribosomal proteins

Recombinant human ribosomal proteins S16e, S5e, S10e and S26e were obtained as described in [16,17].

2.2. Construction of DNA templates and synthesis of the RNA fragments

A fragment of 18S rRNA 1203–1236/1521–1698 was produced by *in vitro* transcription from synthetic double-stranded DNA utilizing the T7 RNA polymerase [18]. A DNA template for synthesis of the fragment was constructed with two successive PCRs. In the first step, a plasmid pAM18T7-2 containing the full-length human 18S rRNA gene was used as template in amplification with the forward primer 1 (5′-AAGGGCACCACCAGGAGTGGAGCCTGCGGATCCACGCGCGCTAC-3′) whose 5′- and 3′-terminal sequences (underlined) correspond to regions 1208–1236 and 1521–1532 of human 18S rRNA, respectively, and the reverse primer 3 (5′-GTGTACAAAGGGCAGGGA-3′), which is complementary to the region 1681–1698 of the 18S rRNA. The product of the PCR amplification was used as a template in the second PCR. The second amplification step was performed with the forward primer 2 (5′-TAATACGACTCACTATAGGGACGGAAGGGCACCACC-3′) whose 5′-terminal sequence corresponds to the T7 RNA polymerase promoter and whose 3′-terminal sequence corresponds to the region 1203–1219 (underlined) of the 18S rRNA, and the reverse primer 3. The resulting DNA template was used to synthesize the RNA transcript designated as 3Dm. DNA template for synthesis of the RNA transcript (designated as 3DM) corresponding to the full-size 3′ major domain of the 18S rRNA (region 1203–1698) was constructed by PCR using primers 2 and 3 and plasmid pAM18T7-2. Non-labeled 3Dm, and 3Dm labeled with ^{32}P to a lower extent (specific activity below 5000 cpm/pmol) were obtained as described previously [19]. The 3Dm and 3DM fragments with a high specific activity (50,000–100,000 cpm/pmol) were synthesized as in [20]. The resulting full-length RNAs were purified by denaturing gel electrophoresis with subsequent excision of the desired product and elution of the RNA with buffer (0.3 M NaOAc, pH 5.5, 0.05% SDS and 1 mM EDTA). RNA transcripts corresponding to U2 snRNA and a fragment of the central domain of human 18S rRNA were obtained similarly. A DNA template for the U2 snRNA synthesis was a kind gift from I. Eperon (University of Leicester, UK); a template for synthesis of a fragment of the central domain of the 18S rRNA was obtained by PCR using primers 5′-TAATACGACTCACTATAGGGTTGGAAGAGGGACGGCCGGG-3′ and 5′-GATTAATGAAAACATTCTT-3′.

2.3. Nitrocellulose filter binding assay

Binding of proteins to the RNA transcript was carried out in 10 μl of binding buffer (20 mM HEPES-KOH, pH 7.0, 250 mM KCl and 4 mM $MgCl_2$) containing 0.05% Triton X-100 and 0.1% BSA at 20 °C for 15 min. The protein concentration in the reaction mixtures varied from 0.1 nM to 1 μM; the RNA concentration was from 5 to 10 pM. Since the RNA concentration was much less than that of a protein, the apparent association constant could be evaluated as the reciprocal value of the protein concentration, at which half of the RNA is bound [21]. The reaction mixtures were filtered through nitrocellulose filters with a pore diameter 0.45 μm (Millipore). The radioactivity retained on the filters was measured using a Molecular Imager FX Pro (Bio-Rad). Treatment of data, plotting adsorption isotherms and calculations of apparent association constants from an equation of specific binding of a ligand at one site was carried out using program Prism 5 (GraphPad). In the cases of competitive binding, RNA transcripts obtained by T7 transcription were used, they corresponded to either U2 snRNA or fragment 680–684/919–1026 of the human 18S rRNA.

2.4. Enzymatic footprinting

Enzymatic footprinting were carried out according to a standard protocol [22]. The 3Dm fragment was 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase or 3′-end labeled with [5′-^{32}P]pCp and T4 RNA ligase and purified by PAGE on an 8% denaturing gel. To obtain the RNA–protein complex, the components (0.05 pmol of 3Dm fragment, 2.5 pmol of S16e and 2.5 pmol of S5e) were incubated in 10 μl of binding buffer containing 5 μg of *Escherichia coli* total tRNA at 22 °C for 15 min. For enzymatic hydrolysis, the mixture was supplemented with 1–3 units of RNase T1 (Boehringer) or T2 (Sigma) and incubated at 22 °C for 15 min. The reactions were stopped by adding an equal volume of water-saturated phenol. All controls without probes were done under the corresponding conditions. The RNA was purified by phenol deproteinization. The hydrolysis product was resolved by denaturing PAGE on a 12% gel; the gels were dried and autoradiographed.

3. Results

3.1. Choice and construction of the model RNA for S16e and S5e binding and measurement of K_a

Since the positions of S16e and S5e on the 40S ribosomal subunit have not been identified at atomic resolution, the choice of the 18S rRNA fragments was based on knowledge of the spatial structures of homologous prokaryotic proteins, S9p and S7p, and their position on the 16S rRNA within the 30S ribosomal subunit of *Thermus thermophilus* obtained from X-ray crystallography [15]. This study revealed that the spatial structure of S9p presents a globular α+β sandwich with a long unstructured C-terminal tail. One edge of the sandwich contacts the 3′ major domain of the 16S rRNA in the area of helices H38–40, whereas the opposite edge and tail are located close to the other part of this domain formed by helices H28–30, H41–43 and their junctions. These parts of the 3′ major domain are located in different independent sub-domains distant from each other on the secondary structure map, but nucleotides contacting S9p are located largely in the part including helices H28–30 and H41–43. The secondary structure of the region in the small subunit RNA is conserved in pro- and eukaryotes [23]. This region in the 16S rRNA also contains the entire binding site for S7p. Therefore, to study binding of S16e and S5e to 18S rRNA, we chose the fragment 1203–1236/1521–1698 formed by helices