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The archaeal exosome localizes to the membrane

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ABSTRACT

We studied the cellular localization of the archaeal exosome, an RNA-processing protein complex containing orthologs of the eukaryotic proteins Rrp41, Rrp42, Rrp4 and Csl4, and an archaea-specific subunit annotated as DnaG. Fractionation of cell-free extracts of *Sulfolobus solfataricus* in sucrose density gradients revealed that DnaG and the active-site comprising subunit Rrp41 are enriched together with surface layer proteins in a yellow colored ring, implicating that the exosome is membrane-bound. In accordance with this assumption, DnaG and Rrp41 were detected at the periphery of the cell by immunofluorescence microscopy. Our finding suggests that RNA processing in Archaea is spatially organized.

Structured summary:
MINT-7891213: *Rrp41* (uniprotkb:Q9UXC2) and *DnaG* (uniprotkb:P95980) *colocalize* (MI:0403) by *cosedimentation in solution* (MI:0028)
MINT-7891235: *Rrp41* (uniprotkb:Q9UXC2), *DnaG* (uniprotkb:P95980) and *SlaA* (uniprotkb:Q2M1E7) *colocalize* (MI:0403) by *cosedimentation through density gradient* (MI:0029)
MINT-7891278: *Rrp41* (uniprotkb:Q9UXC2) and *DnaG* (uniprotkb:P95980) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

1. Introduction

During the last decade it was shown that the spatial organization of many biochemical processes in Bacteria relies on the subcellular localization of macromolecules. Many RNA-related proteins and macromolecular complexes have a specific localization. While ribosomes and RNA chaperons are localized around the nucleoids when transcription takes place in *Bacillus subtilis* [1,2], the RNA-degrading protein complexes in *Escherichia coli* [3–5] and *B. subtilis* [6], and ribonuclease R in *Caulobacter crescentus* [7] are at the membrane. Like bacteria, archaea are prokaryotic microorganisms without organelles, but nothing is known about the spatial organization of RNA-processing activities in archaeal cells. In this work the localization of the RNA exosome in the hyperthermophilic and acidophilic archaeon *Sulfolobus solfataricus* was studied.

The essential RNA-processing and RNA-degrading exosome was originally described in Eukarya [8], and a similar complex was found in Archaea, where it is a major RNA-tailing and RNA-degrading enzyme [9,10]. Nine subunits of this complex are organized in a structure with similarity to the nine-subunit core of the eukaryotic exosome and to the bacterial PNPase. It comprises a phosphorolytically active hexameric ring of Rrp41–Rrp42 dimers, on the top of which trimer of Rrp4 and/or Csl4 is bound [10]. The archaeal exosome contains an additional subunit of unknown function, annotated as DnaG [11].

In the compartmentalized eukaryotic cells, specific structures in the cytoplasm are known such as the stress granules and processing bodies, in which RNA is translationally arrested and/or degraded. Processing bodies are involved in si- and mi-RNA mediated gene silencing and in mRNA degradation in 5′–3′ direction. The eukaryotic exosome, which degrades RNA in 3′–5′ direction, was found in the nucleus and in the cytoplasm, but not as a part of processing bodies [8,12]. Although the eukaryotic exosome seems not to have a specific localization, we assumed that its archaeal counterpart may be localized because of the need of prokaryotic cells to spatially organize RNA processing and degradation. We tested this hypothesis and show that the exosome of *S. solfataricus* is localized at the periphery of the cell and co-sediments with membrane-bound surface layer proteins in sucrose density gradients.

Abbreviation: rRNA, ribosomal RNA

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2. Materials and methods

2.1. Fractionation experiments

S. solfataricus P2 was grown as previously described [13]. Cells (470 mg) were resuspended in 1 ml MES buffer containing 20 mM MES (2-(N-morpholino)ethanesulfonic acid), pH 6.5, 0.5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulphonyl fluoride, 2 mM dithiothreitol, and DNase I. After lysis with a French press and centrifugation at 2000×*g* for 10 min at 4 °C, the supernatant (crude extract) was used for fractionations. Ultracentrifugation at 100 000×*g* was performed for 75 min at 4 °C [14], and the S100 fraction was collected. The pellet was washed and resuspended in 1 ml of MES buffer (P100 fraction).

Sucrose density gradients (15–70% sucrose) in MES buffer and in MES buffer containing 500 mM ammonium chloride and 10 mM magnesium acetate (salt-containing gradient, [15]) were used. One milli litre of the crude extract, the S100 or the P100 fraction was layered onto an 10 ml gradient and centrifuged in a SW41Ti rotor at 4 °C for 24 h at 100 000×*g*. Fractions were collected from top to bottom.

2.2. Protein and RNA methods

Western blot was performed using sera against Rrp41 or DnaG [9,11], anti-rabbit IgG conjugated with peroxidase (Pierce), and the Lumi-Light Western blotting substrate (Roche Diagnostics GmbH). SlaA and SlaB were isolated as described [16]. Briefly, 40 mg cells were incubated in sodium lauroylsarcosine-containing buffer at 45 °C and centrifuged. The white layer at the bottom of the tube was removed, incubated at 45 °C in Triton X-100 containing buffer, centrifuged and the pellet was resuspended in water. Samples were incubated for 2 h at 45 °C in 10 mM sodium carbonate prior to SDS–PAGE analysis.

RNA was isolated from 100 μl of the indicated fractions using TRIzol (Invitrogen); 1/5 of the precipitated RNA was analysed by dot blot hybridisation at 56 °C with the 23S ribosomal RNA (rRNA)-specific oligonucleotide 5′-GGTTCAAAGGCAGCGTGTGC-3′ or at 50 °C with the 16S rRNA-specific oligonucleotide 5′-TGTAACTATGCAGCCG-3′, which were 5′-end labelled with gamma ^{32}P-ATP. Membranes were washed with 2 × SSC, 0.01% SDS; signals were detected using a molecular imager (BioRad).

2.3. Immuno-fluorescence labelling and microscopy

Cells grown to an $OD_{600} \sim 0.4–0.5$ were cryoimmobilized by high-pressure freezing [17,18]. The experiments were performed three times, in two of the experiments the K11M resin was used for embedding of the cells, in the third experiment the HM20 resin was used. Ultrathin sections (50 nm) were labelled as described [19] using first the DnaG- or Rpr41-specific antibody. In a second step, bound rabbit antibodies were labelled with goat anti-rabbit antibodies conjugated to the indocarbocyanine fluorophore Cy3 (Dianova, Germany). Mounted samples were imaged in an Axiophot light microscope (Zeiss, Germany) at 100-fold magnification, with a F-View II CCD camera and an X-Cite 120 fluorescence source (EXFO LSI, Canada).

3. Results

3.1. Rrp41 and DnaG are in the membrane fraction

After ultracentrifugation of crude extracts at 100 000×g to separate membrane (pellet, P100) and cytoplasmic (supernatant, S100) proteins, DnaG was detected only in the P100 fraction, while approximately 2/3 of Rrp41 were detected in the P100 fraction and 1/3 in the S100 fraction. Both ribosomal subunits were also found in the P100 fraction (Supplementary Fig. 1), in agreement with the previously described co-sedimentation of Rrp41 with ribosomal subunits in glycerol density gradients [9]. To clarify whether the sedimentation behavior of the exosome is due to its interaction with membranes or ribosomal subunits, sucrose density gradients were applied.

Since the membrane of *S. solfataricus* is bound to the glycosylated surface (S-) layer proteins SlaA (120 kDa) and SlaB (60 kDa), membranes can be expected in heavy fractions in sucrose density gradients. The S-layer proteins build the paracrystalline proteinaceous cell wall anchored in the membrane of *S. solfataricus* [16]. After fractionation of extracts through 15–70% sucrose density gradients, a yellow colored ring with whitish particles was visible near the bottom of the tube (Fig. 1A). We assumed that membranes were enriched in this ring, because purified lipids from *S. solfataricus* are yellow [V.R. and S.V.A, unpublished], and the S-layer is opaque whitish [16].

The gradient was separated in 11 fractions. The protein and RNA content of the fractions was monitored at 280 and 260 nm, respectively (Fig. 1B). Peaks were detected in fractions 2 and 3 (contain tRNAs (not shown) and S100 proteins, Supplementary Fig. 2), 5–7 (contain 30S and 50S ribosomal subunits, Supplementary Fig. 3), and 9, which contains a major part of the yellow ring. DnaG was present in fractions containing ribosomal subunits and in the yellow ring fractions 9 and 10. Rrp41 was detected in the same fractions like DnaG, and in addition, in the fractions 3 and 4, in agreement with its detection in S100 (Fig. 1C, see also Supplementary Figs. 1 and 2). To confirm that membranes were enriched in the yellow ring, we analyzed it for the presence of S-layer proteins. SlaA and SlaB were isolated and used as positive controls in SDS–PAGE analysis of gradient fractions 6 and 9. As expected, SlaA was clearly detectable in fraction 9 but not in fraction 6 (Fig. 1D). The identity of SlaA in fraction 9 was confirmed by mass spectrometry (G. Lochnit, University of Giessen; Supplementary Fig. 4).

Thus, the exosome was detected in fractions containing membranes and ribosomal subunits when the gradient was prepared with MES buffer without salt. This buffer was used previously for analysis of trans-membrane domain containing proteins of *S. solfataricus* [14]. However, hydrophobic interactions of membranes with membrane-associated proteins lacking trans-membrane domains are disturbed under low-salt conditions [20]. Since the subunits of the exosome do not harbor trans-membrane domains, the detection of an interaction between the membrane and the exosome may depend on the presence of salt in the buffer. To verify this, a 500 mM ammonium chloride-containing sucrose density gradient was used.

The gradient was separated in 22 fractions, where the yellow ring was collected in fractions 19 and 20. According to the absorption at 280 and 260 nm, protein and RNA peaks largely overlap (Fig. 2A). The peaks of the 30S and 50S ribosomal subunits were detected in the fractions 11 and 14, respectively (Fig. 2B). DnaG was detectable in the fractions 12–21 of the gradient (Fig. 2C). Its amount increased from fraction 12 to fraction 19, and the highest amount was present in the yellow ring fractions 19 and 20. The distribution of Rrp41 in fractions 12–21 was similar (Fig. 2C), in agreement with the tight interaction of DnaG and Rrp41 in the exosome [11]. The distribution of the exosome in the gradient was clearly different from that of the ribosomal subunits: its amount gradually increased from fraction 12 to fraction 19 and did not parallel the ribosomal peaks in fractions 11 and 14. Most importantly, the distribution of the exosome paralleled that of the S-layer proteins: the amount of SlaA also gradually increased from fraction 14 to fraction 19, and the highest amount of SlaA was in fractions 19

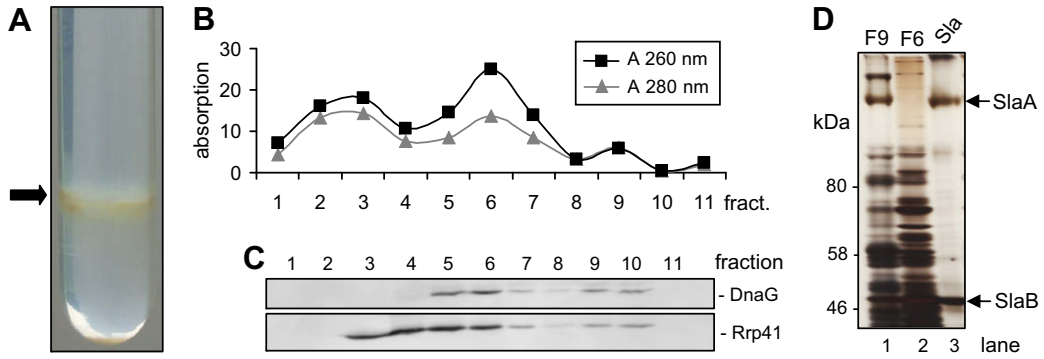

Fig. 1. Fractionation through a 15–70% sucrose density gradient without salt. (A) A photograph showing the bottom half of a tube containing the gradient with a fractionated cell-free extract. The yellow ring with opaque whitish particles is marked with an arrow. (B) Absorption of the gradient fractions at 280 or 260 nm. (C) Western blot analysis of the gradient fractions. The number of the individual fractions is given above the panels. The use of DnaG-specific or Rrp41-specific antibodies is indicated at the right side. (D) Silver stained, 8% SDS–PAA gel showing the protein content of fractions (F) 6 and 9, as indicated on the top, and the isolated S-layer proteins (Sla). M, protein marker. Sla A and Sla B are marked and the molecular size of the marker proteins is indicated.

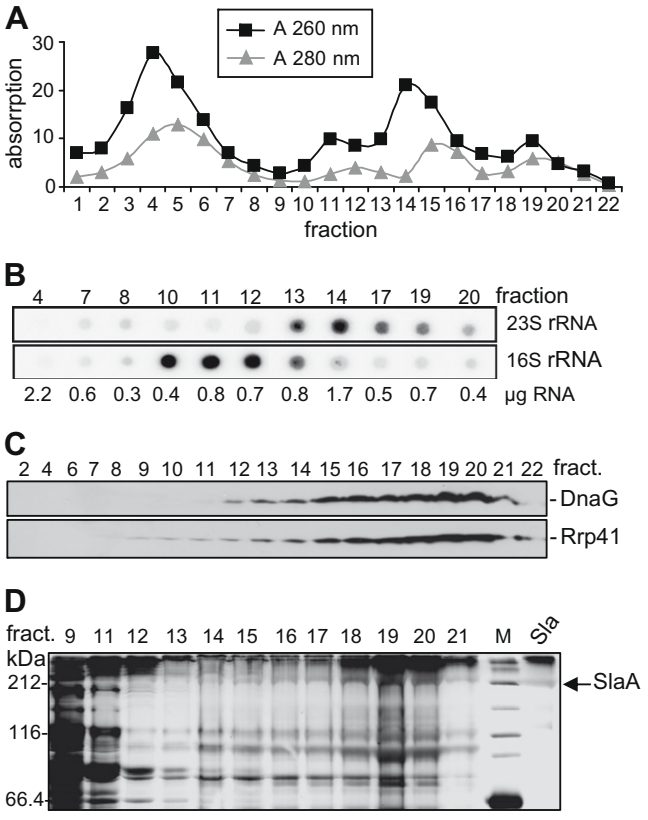

Fig. 2. Fractionation through a salt-containing 15–70% sucrose density gradient. (A) Absorption of the gradient fractions at 280 or 260 nm. (B) Dot blot analysis of RNA isolated from gradient fractions with probes complementary to 16S rRNA and 23S rRNA. Loaded RNA amounts in µg are given below the panels. (C) Western blot analysis of gradient fractions. For descriptions see (C). (D) Silver stained, 12% SDS–PAGE showing the protein content of individual gradient fractions as indicated above the panel. Sla, isolated S-layer proteins; M, protein marker.

and 20 (Fig. 2D). Thus, membranes together with S-layer proteins and exosome were enriched in the yellow ring.

3.2. Immunofluorescence detection of Rrp41 and DnaG

The above results suggest that the exosome of *S. solfataricus* is localized at the cytoplasmic membrane. To obtain additional evidence, we decided to detect the exosomal subunits Rrp41 and DnaG by immunofluorescence using thin section cuts of *S. solfataricus* cells and the polyclonal antibodies directed against each of these proteins. Fig. 3 shows that both DnaG and Rrp41 are detectable at the periphery of the cell. This is consistent with a membrane localization of the two proteins and supports the data of the fractionation experiments.

4. Discussion

Rrp41 was not detected in monomeric form previously and was co-purified in stoichiometric amounts with Rrp4 and Csl4, indicating that the hexamer of Rrp41 and Rrp42 is not existing separately in vivo [9,11]. Therefore we assume that Rrp41 represents the nine-subunit exosome. Since depletion of Rrp41 from the cell-free extract is paralleled by depletion of DnaG, and identical protein complexes are coimmunoprecipitated by DnaG-specific antibodies and Rrp41-specific antibodies [11], we assume that DnaG represents the DnaG-containing exosome. Based on our results, we propose that a minor amount of the nine-subunit exosome is present in the cytoplasm, while the vast majority of the nine-subunit exosome is localized together with DnaG at the membrane.

The mechanisms responsible for the specific subcellular localization of the archaeal exosome remain to be elucidated. However, our data show that hydrophobic interactions are responsible for the co-sedimentation of the exosome with membranes, since this co-sedimentation is observed at high ionic strength. In gradients without salt, the major part of the exosome does not co-sediment with membranes but with ribosomal subunits. The physiological relevance of this co-sedimentation was not investigated, but it explains the detection of the exosome in the P100 fraction of a cell-free extract prepared in buffer without salt. High-salt buffer, containing 500 mM ammonium acetate, disturbs weak non-hydrophobic interactions and was previously used to purify ribosomal subunits of *S. solfataricus* [15]. The same study reports that the ribosomes of this organism dissociate into subunits upon cell lysis [15], explaining the lack of 70S ribosomes or polysomes in our gradients.

The salt dependence of the co-sedimentation of the exosome with membranes suggests that this protein complex is membrane-associated or interacts with a still unknown membrane protein via hydrophobic surfaces, but is not strongly bound to the membrane. Since DnaG was detected exclusively in the membrane fraction under high salt conditions and since it was not possible to obtain soluble recombinant DnaG and DnaG-containing complexes previously [11], we think that the subcellular localization of the exosome depends on DnaG. Indeed, *S. solfataricus* DnaG was identified previously in the Tris-insoluble fraction containing membrane-associated proteins [21].

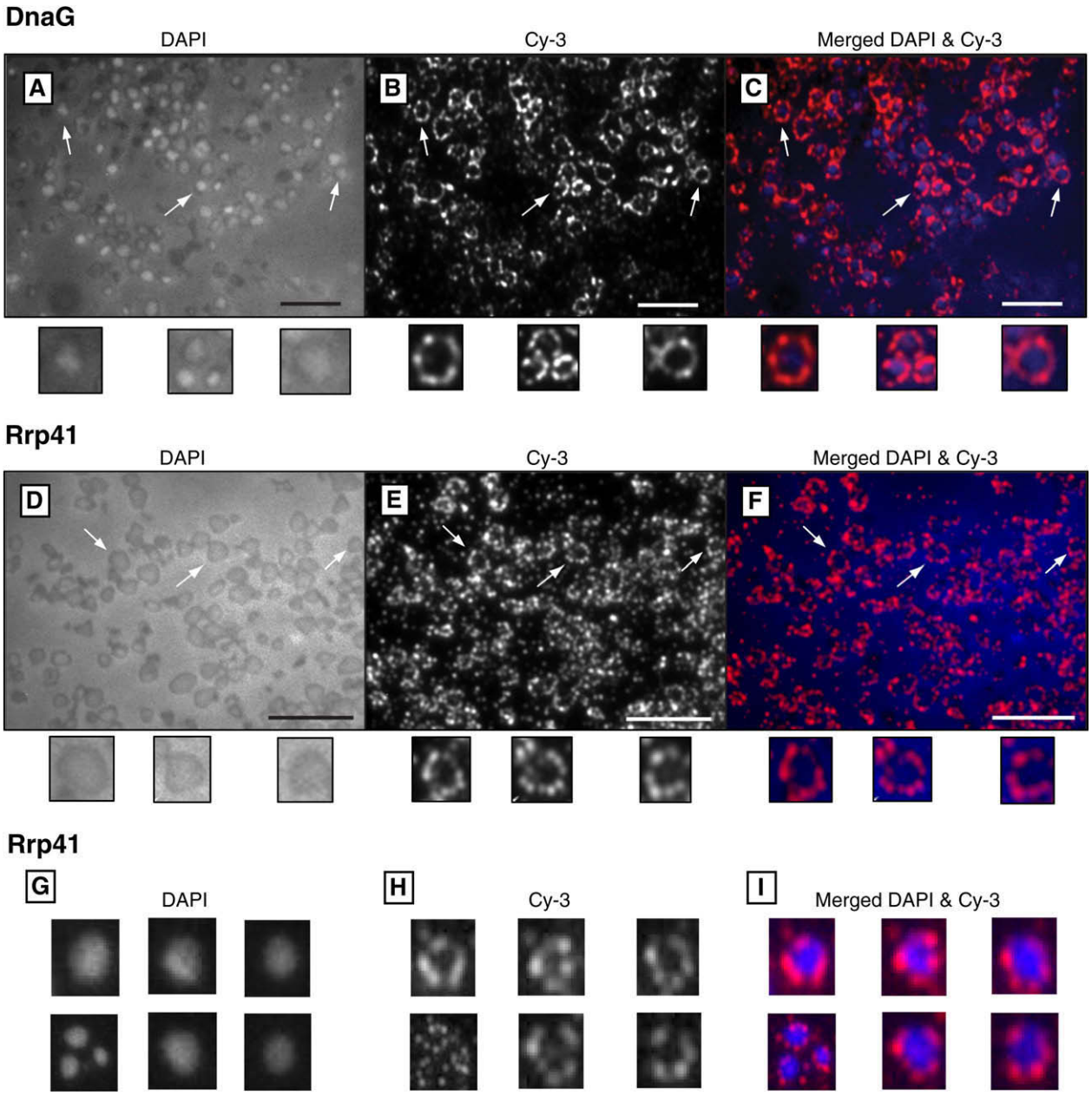

Fig. 3. Cellular localization of the *S. solfataricus* DnaG and Rrp41 proteins by immunolabelling. (A–C) Localization of DnaG, cells were embedded in K11M resin. (D–F) Localization of Rrp41, the embedding resin was K11M. (G–I) Localization of Rrp41, HM20 resin was used for the embedding of the cells. Ultra-thin sections of exponentially growing cells were stained with the DNA-staining 4′,6′-diamidino-2-phenylindole (A, D and G) and immuno-labelled with fluorescent Cy-3-tagged antibodies targeted against DnaG (B) and Rrp41 (E and H). Panels C, F and I show the respective merges. Scale bar: 5 μm. Both proteins exhibit a distinct ring-shaped distribution, located almost exclusively at the membrane periphery of the cells. The arrows in panels A to F refer to selected cells imaged simultaneously with both methods; magnification of these cells is presented. In panels G, H and I, only cell magnifications are shown (see also Supplementary Fig. 5).

In conclusion, the localization of the archaeal exosome at the cell periphery and its co-sedimentation with membranes suggests that the membrane is involved in the spatial organization of RNA processing in the third domain of life. Together with reports on membrane localization of bacterial RNA-degrading proteins [3–6], our data support the view that the cytoplasmic membrane plays an important role in the compartmentalization of RNA-related events in prokaryotic cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.05.013.

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