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Construction of the 'minimal' SRP that interacts with the translating ribosome but not with specific membrane receptors in *Escherichia coli*

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Abstract ***Escherichia coli*** **signal recognition particle (SRP) consists of 4.5S RNA and Ffh protein. In contrast to eukaryotes, it remains unclear whether translation arrest takes place in prokaryotic cells. To study this problem we constructed a fusion of the M domain of Ffh protein with a cleavable affinity tag. This mutant Ffh, in a complex with 4.5S RNA, can bind signal peptide at the translating ribosome but is unable to bind the membrane. This SRP–ribosome complex should accumulate in the cell if translation is arrested. To test this, the complex was purified from the cells by ultracentrifugation and affinity chromatography. The composition of the complex was analyzed and found to consist of ribosomal RNAs and proteins, the Ffh M domain and 4.5S RNA. The accumulation of this complex in the cell in significant amounts indicated that SRP-mediated translation arrest did occur in bacterial cells.** © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Signal recognition particle; Ribosome; Translation arrest; Ffh; 4.5S RNA

1. Introduction

The signal recognition particle (SRP) mediates protein export and protein insertion into the cellular membrane. In the case of eukaryotes, the SRP interacts with the ribosome and may cause the arrest of translation [1]. However, it is still unclear whether prokaryotic SRP can mediate translation arrest in a similar manner. The prokaryotic SRP has been shown to be unable to cause translation arrest in the eukaryotic extracts [2], but no data relating to the ribosome–SRP interaction in a homologous prokaryotic system are so far available.

In prokaryotes, the SRP consists of only two components, namely 4.5S RNA [3] and Ffh protein [4], whereby the 4.5S RNA is a much shorter homologue of eukaryotic 7S SRP RNA. Both SRP components are essential for cell viability [5,6]. The lethality, caused by the absence of 4.5S RNA, could be suppressed by several mutations in rRNA [7], but it is not known which ribosomal components are involved in the interaction with SRP. Furthermore, the mechanism of elongation arrest is still obscure. However, up to now attempts to investigate translating ribosome–SRP complex have only been made in an eukaryotic in vitro translation system [2]. Here we describe a mutant 'minimal' *Escherichia coli* SRP which contains only 4.5S RNA and the M domain of Ffh protein fused with affinity tag necessary for the purification of ribosome–SRP particles from living *E. coli* cells. Such 'minimal' SRP will bind the ribosome translating signal peptide but not to membrane receptor. If SRP-mediated translation arrest does exist in *E. coli*, SRP–ribosomal complex would accumulate in the cell and thus can be isolated and characterized. We have shown the accumulation of such a complex in cells, isolated it and confirmed its composition. This finding favors the existence of the translation arrest in prokaryotes.

2. Materials and methods

2.1. Materials

T4 polynucleotide kinase, T4 DNA polymerase and T4 DNA ligase were purchased from Roche, Germany. *Pfu* DNA polymerase was purchased from Promega, USA. IgG Sepharose 6 fast flow was purchased from Pharmacia Biotech, Sweden. Nitrocellulose membrane Hybond-N+ was purchased from Amersham, UK.

2.2. Expression of Ffh M domain and isolation of the ribosome–Ffh M–4.5S RNA complex

A fragment encoding the TEV protease cleavage site and ZZ domain was excised from the plasmid pYM10 [8] by digestion with *Eco*RI (followed by T4 DNA polymerase treatment) and *Sal*I, and cloned to the vector pET33b+ (Novagen) linearized with *Xho*I, T4 DNA polymerase and *Sal*I. The plasmid obtained was named pET-TEV-ZZ. The gene encoding the Ffh M domain was amplified from total *E. coli* DNA by *Pfu* polymerase using the primers GCGGCCATGGCTATTCGTCACATCACTGGC and CCAGGCCTCGAGGCGACCAGGGAAGCCTGGGGG. The product was cleaved by *Nco*I and *Xho*I endonucleases and cloned into pET-TEV-ZZ cleaved by *Nco*I and *Sal*I. The Ffh M domain expression vector named pET-FfhM-TEV-ZZ was transformed into the *E. coli* strain BL21(DE3). Freshly transformed cells growing in LB broth were treated with 1 mM IPTG at A_{600} 0.40 and allowed to grow for another 2 h. The cells were harvested and lysed by sonication. 10 U of RNase-free DNase I was added to the lysate. After incubation for 5 min at 20°C, the solution was centrifuged for 1 h at 14 000 × *g*. The supernatant, which contains the ribosome fraction, was removed and centrifuged for 16 h at 30 000 × *g* to pellet the ribosomes. The pellet was resuspended in 20 ml of buffer A (25 mM Tris–HCl (pH 7.6), 150 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol) and centrifuged for 18 h at 45 000 × *g* in a 10–30% sucrose gradient. The pellet was resuspended in 2 ml of buffer A. This solution was added to 100 μl of IgG Sepharose 6 fast flow equilibrated in buffer A. After 16 h binding at 4°C the resin was washed three times with 300 μl of buffer A. The complex containing ribosome, Ffh M and 4.5S RNA were cleaved

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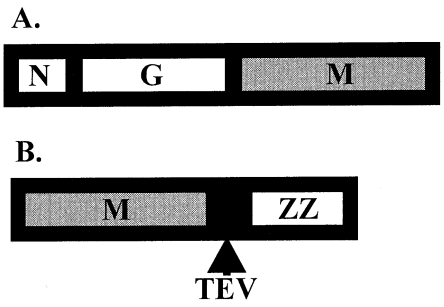

Fig. 1. Domain organization of the Ffh protein. A: The natural Ffh protein from *E. coli*. B: The artificial construct of Ffh M domain used in this study.

from the resin by treatment with 10 U of recombinant TEV protease in 100 μl of buffer A for 3 h at 4°C.

2.3. Northern blot hybridization

RNA was separated by electrophoresis in a 1.5% agarose gel. After electroblotting, a nitrocellulose membrane Hybond-N+ was hybridized with 5′-[^{32}P]TGCATGCATGGTGGGGGCCCTGCCAGC oligonucleotide, which is complementary to the 3′-end of 4.5S RNA. An in vitro transcript of the 4.5S RNA served as a control for the Northern blot analysis.

3. Results and discussion

The Ffh protein (abbreviated from 54 homologues) consists of three domains [9], N, G, and M, listed from the N-terminus (Fig. 1A). The G domain is known to interact with the FtsY receptor in the plasma membrane, thus mediating the steps after translation arrest [10]. The M domain alone has been shown to be sufficient to interact with 4.5S RNA and the signal peptide [11], thus coordinating all SRP activities related to the binding of SRP to the translating ribosome. A ribosome–SRP complex exists only transiently in the cell, just until attachment to the receptor on the membrane has taken place. To prevent the interaction of ribosome–SRP complex with the receptor we decided to express a truncated Ffh gene that encodes only the M domain of the Ffh. We assumed that this truncated protein would form a 'minimal' SRP particle and interact with the ribosome. However, the complex of Ffh M domain, 4.5S RNA and the translating ribosome should not be able to proceed to the further steps in the protein export pathway, and should thus accumulate in the cell.

In order to facilitate the purification of the complex by affinity chromatography we introduced a *Staphylococcus aureus* protein A ZZ domain at the C-terminus of the recombi-

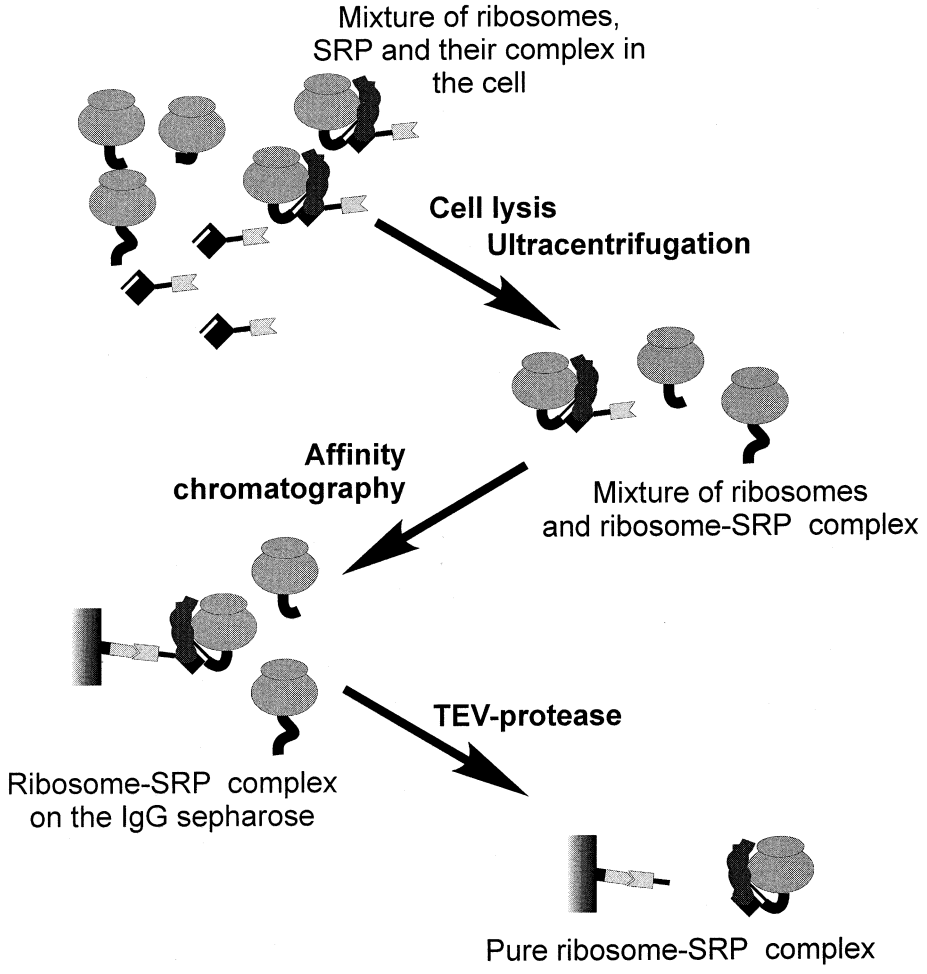

Fig. 2. Purification procedure of the ribosome complex with SRP from cells expressing the Ffh M domain fusion.

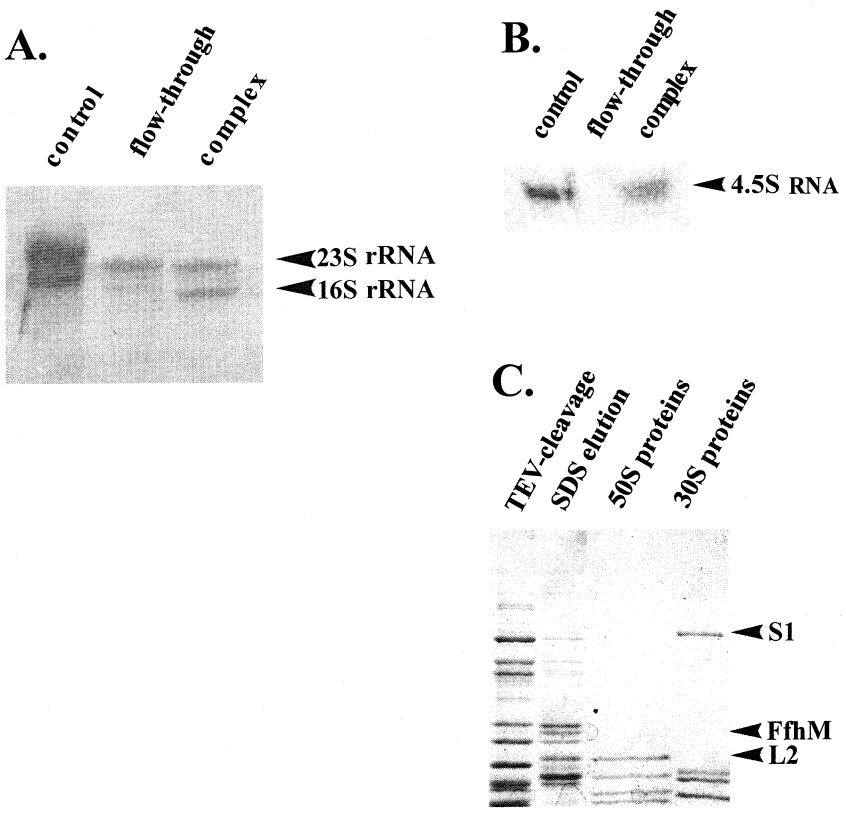

Fig. 3. Verification of the composition of the SRP–ribosome complex. A: rRNAs isolated from the complex by denaturing 4% PAGE. Isolated large ribosomal RNAs are shown in the control lane. 'Complex' and 'flow-through' correspond to the material bound and not bound to IgG Sepharose, respectively. B: Northern blot analysis for the presence of 4.5S RNA in the isolated complex. The control is in vitro transcribed 4.5S rRNA. 'Complex' and 'flow-through' correspond to the material bound and not bound to IgG Sepharose, as in 'A'. C: Protein composition of the complex, as detected by SDS protein gel electrophoresis. 30S and 50S indicate lanes with ribosomal proteins from the small and large ribosomal subunits, respectively. 'SDS elution' denotes the material eluted from the IgG Sepharose by SDS treatment; the band corresponding to Ffh M–ZZ fusion is indicated. TEV cleavage corresponds to the material released from the IgG Sepharose by TEV protease cleavage. Notice the absence of the Ffh M–ZZ fusion product, which is cleaved by TEV treatment.

nant protein. In addition to the ZZ tag, the recombinant protein carries a His6 sequence C-terminal to the ZZ domain. Further, the affinity tag was separated from the Ffh M domain by a peptide cleavable by TEV protease (Fig. 1B). No additional cleavage sites for TEV protease exist either in the Ffh M domain, or in any of the ribosomal proteins or elongation factors. Thus, the recombinant protein used for in vivo incorporation into the ribosome–SRP complex consists of the Ffh M domain, the TEV cleavage site and the *S. aureus* protein A ZZ domain.

Recombinant protein expression was induced in BL21(DE3) cells by 1 mM IPTG for 2 h. In order to test whether the minimal SRP is formed, can such an SRP interact with translation ribosome and is the complex of minimal SRP with the ribosome really accumulated in the cell, the isolation of SRP–ribosomal complex has been performed. For that purpose firstly the ribosomal fraction was prepared from the cells by a two step centrifugation procedure (Fig. 2) which contains SRP–ribosomal complex. After application of the mixture of ribosomes and ribosome–SRP complex to IgG Sepharose and subsequent washes, the ribosome–SRP complex was eluted by TEV protease cleavage. The isolated ribosome–SRP complex in this system constitutes about 2–5% of the total ribosomes. In a control experiment without Ffh M domain–ZZ fusion gene expression neither detectable binding of ribosomes to IgG Sepharose nor TEV protease-assisted elution was detected.

To prove that we do have isolated the minimal SRP–ribosomal complex the composition of the eluted fraction was studied by several methods. The presence of both 16S and 23S rRNA was checked by a denaturing 4% PAGE gel (Fig. 3A). 4.5S RNA was indistinguishable from the 5S rRNA in this system, so its presence in the complex was determined by Northern blot analysis (Fig. 3B). A protein SDS gel showed the presence of equimolar amounts of ribosomal proteins and the Ffh M domain–ZZ fusion product, cleavable by TEV protease (Fig. 3C).

The very existence of translational arrest by SRP in prokaryotes has so far seemed questionable, since the prokaryotic SRP lacks the SRP9/14 proteins, which are necessary for elongation arrest in eukaryotes [12]. The fact that the 'minimal' SRP–ribosomal complex is accumulated in the cell provides an indication for the translational arrest in prokaryotes. In order to confirm the translation arrest by 'minimal' SRP, we performed a similar purification of the ribosome–SRP complex for the full-length His-tagged Ffh protein. Compared side-by-side, an expression of truncated Ffh protein, lacking the domains responsible for membrane receptor binding, caused an accumulation of ribosome–SRP complex which was an order of magnitude more than that caused by the

correspondent expression of the full-length Ffh. If there is no translation arrest, no accumulation of the ribosome–Ffh M domain–4.5S RNA should be observed. This experiment indicates for the first time that in prokaryotes the SRP arrests the ribosomal elongation cycle until membrane receptor binding has taken place.

The possibility to isolate the SRP–ribosomal complex with arrested translation offers a unique opportunity to investigate the precise mechanism of elongation arrest by structure probing and cryo-electron microscopy of the complex, both of which are now in progress.

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