Protein–protein, protein–RNA and protein–lipid interactions of signal-recognition particle components in the hyperthermoacidophilic archaeon *Acidianus ambivalens*

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The signal-recognition particle (SRP) of one of the most acidophilic and hyperthermophilic archaeal cells, *Acidianus ambivalens*, and its putative receptor component, FtsY (prokaryotic SRP receptor), were investigated in detail. *A. ambivalens* Ffh (fifty-four-homologous protein) was shown to be a soluble protein with strong affinity to membranes. In its membrane-residing form, Ffh was extracted from plasma membranes with chaotropic agents like urea, but not with agents diminishing electrostatic interactions. Using unilamellar tetraether phospholipid vesicles, both Ffh and FtsY associate independently from each other in the absence of other factors, suggesting an equilibrium of soluble and membrane-bound protein forms under *in vivo* conditions.The Ffh protein precipitated from cytosolic cell supernatants with anti-Ffh antibodies, together with an 7 S-alike SRP–RNA, suggesting a stable core ribonucleoprotein composed of both components under native conditions. The SRP RNA of *A. ambivalens* depicted a size of about 309 nucleotides like the SRP RNA of the related organism *Sulfolobus acidocaldarius*. A stable heterodimeric complex composed of Ffh and FtsY was absent in cytosolic supernatants, indicating a transiently formed complex during archaeal SRP targeting. The FtsY protein precipitated in cytosolic supernatants with anti-FtsY antisera as a homomeric protein lacking accessory protein components. However, under *in vitro* conditions, recombinantly generated Ffh and FtsY associate in a nucleotide-independent manner, supporting a structural receptor model with two interacting apoproteins.

Key words: *Acidianus ambivalens*, archaeon, Crenarchaea, hyperthermoacidophile, signal-recognition-particle (SRP) components, tetraether phospholipids.

INTRODUCTION

Signal-recognition particles (SRPs) and their receptors in Bacteria and Eukarya are structurally and functionally well characterized [1,2]. In contrast, much less is known from SRP components in the third Domain of life, the Archaea [3]. Considerable interest in these factors has arisen, since conserved SRP components [SRP54/Ffh (fifty-four-homologous protein), SRP 19-homologous protein, SRP RNA and SRα (SRP receptor α)/FtsY (prokaryotic SRP receptor) receptors] have been detected in nearly all archaeal genomes [4]. Their overall structures strongly resemble the bacterial and eukaryal homologues, underlining the evolutionary and biological conservation of SRP-dependent protein targeting in the third Domain (Urkingdom).

Most of the present studies have been addressed to *in vitro* reconstitution of heterologously overexpressed archaeal SRP components and *in-vitro*-transcribed SRP RNA in order to prove the functionality of the reconstituted archaeal particle. To date, several archaeal ribonucleoprotein particles have been reconstituted, one from an acidophilic and hyperthermophilic crenarchaeal cell (*Acidianus ambivalens* [5]), and all others from thermophilic or halophilic euryarchaeal cells (*Archaeoglobus fulgidus* [6], *Pyrococcus furiosus* [7], *Methanococcus jannaschii* [8] and *Haloferax volcanii* [9]). As deduced from these investigations, the minimal structure of the archaeal SRP is composed of SRP54/Ffh, the SRP 19-homologous component and the 7 S-alike SRP RNA. The biological importance of the particle has clearly been shown for *Haloferax volcanii*, in which deletion of the genomic *ffh* gene results in loss of cell viability [10]. The reconstituted particles are active in GTP hydrolysis [5] and binding of heterologous signal peptides [6].

In addition, all examined archaeal organisms encode the SRα/FtsY receptor component [4]. In bacteria and eukarya it fulfils a prominent role in targeting of the SRP–ribosome–nascent-chain-associated complex to the respective biological membrane [1,2]. In contrast with SRα/FtsY, the SRβ receptor subunit is lacking in all archaea as well as in all bacteria examined to date [4]. Examination of all sequenced archaeal *ftsY* genes reveals a high degree of sequence conservation within the C-terminal N- and G-domains compared with their bacterial and eukaryal counterparts [4]. The function of the former protein domain in Mg^{2+}-dependent GTP hydrolysis has been experimentally proven [11]. In contrast with the conserved N- and G-domains, only minor conservation was observed in the A-domain, the hydrophilic N-terminal region of these proteins (note: the A-domain is the acidic domain, the N-domain is the domain at the N-terminal part of the G-domain in SRP GTPases, and the G-domain is the GTP-binding domain). The function of the A domain is less obvious, but, at least in bacteria, positive charge clusters in the A-domain support the binding of the soluble FtsY to anionic phospholipids in the plasma membrane [12]. Despite the structural similarity of archaeal FtsY proteins to their bacterial homologues, there is yet no experimental evidence for a receptor function, a specific interaction with the plasma membrane and the basic binding reaction to the Ffh protein in archaea.

SRP components other than those mentioned above have not yet been detected in archaeal cells. The objectives of the present study are therefore focused on: (a) the composition of the SRP and

Abbreviations used: Biotin-NHS-ester, D-biotinoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester; Ffh, fifty-four-homologous protein; FtsY, prokaryotic SRP (signal-recognition particle) receptor; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate; SR, SRP receptor; TBE, Tris/borate/EDTA buffer; TBS, Tris-buffered saline; TUVs, tetraether phospholipid unilamellar vesicles.

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its receptor (SR) in cytosolic supernatants, (b) the intrinsic *in vitro* interaction of heterologously expressed Ffh and FtsY, and (c) the *in vitro* interaction of Ffh and FtsY with tetraether phospholipid unilamellar vesicles (TUVs) of the hyperthermophilic crenarcheon *A. ambivalens*.

EXPERIMENTAL

Archaeal cultures, protein and RNA preparation, and *in vitro* transcription of 7 S RNA

A. ambivalens (DSM 3772) [13,14] and *Sulfolobus* acidocaldarius (DSM 639) were obtained from the DSMZ [Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Micro-organisms and Cell Cultures), Braunschweig, Germany]. *A. ambivalens* was cultivated at 78 °C and initial pH of 2.5 on elementary sulphur under aerobic conditions in the presence of CO_2 and yeast extract as sole carbon sources. Protein fractions containing cytosolic supernatants and plasma membranes have been isolated after low-speed centrifugation and ultracentrifugation of ultrasonified cells at pH 5.5 [15]. Preparations of recombinant *A. ambivalens* Ffh and FtsY protein were described in detail [5,11]. The *S. acidocaldarius* SRP RNA *in vitro* transcription was performed with plasmid pSs7S [16] as described in [5]. Polyclonal antisera have been produced as antigenic sources using lacZ′/Ffh-ΔC, lacking the C domain in Ffh, and lacZ′/FtsY-(Δ1–34), lacking part of the A domain in FtsY, according to the procedures described in [5,11].

Total cellular RNA fractions of *A. ambivalens* and *S. acidocaldarius* were prepared from cell lysates using the Trizol reagent as described by the manufacturer (Gibco BRL). *In-vitro*-transcribed *S. acidocaldarius* SRP RNA and the cellular RNA fractions were hybridized with two oligodeoxynucleotides. Probe 1 is reverse-complementary to nucleotides 290–309 at the 3′end of the *S. acidocaldarius* 7 S RNA (5′ ATG GTC AGC TCC CCT ATG CC 3′). Probe 2 is reverse-complementary to nucleotides 192–209 in helix 8 of *S. acidocaldarius* 7 S RNA (5′ TCC CTT CCG GGC CTG GCC 3′). The genetic information encoding this archaeal RNA was originally described as genomic DNA derived from the species *S. solfataricus* [16]. However, its gene sequence (GenBank® accession X17239) is 100 % identical with the respective *S. acidocaldarius* sequence (L. Cheng and R. Garrett, personal communication) and only 69 % identical with the respective sequence of *S. solfataricus* strain P2 (GenBank® accession no. Y08257) [17]. Therefore we assigned the *in vitro* transcript used in the present study as *S. acidocaldarius* RNA. RNA samples were subjected to urea/PAGE using 6 %-polyacrylamide slab gels (0.75 mm thick, 10 cm × 8 cm; Hoefer Mighty Small Electrophoresis Unit from Amersham Biosciences) in TBE (Tris/borate/EDTA; 89 mM Tris base/89 mM boric acid/2 mM EDTA, pH 8.0) buffer for 2 h at 10 mA. Non-radioactive Northern blotting was performed in a submarine electrophoresis chamber in 40 mM Tris/acetate/2 mM EDTA buffer, pH 8.0, overnight at room temperature using nylon membranes (Hybond-N+; Amersham Biosciences). The blots were hybridized with 20 pmol/ml 3′-digoxigenin-11-dUTP-labelled probe at 42–43 °C according to procedures described elsewhere [18,19].

Extraction of membrane-bound *A. ambivalens* Ffh

Membrane fractions were prepared as described in [15]. Portions (0.26 mg) of *A. ambivalens* membrane protein were incubated in, separately, water (control), 50 mM NaCl, 1 M NaCl, 4 M urea and 0.2 M Na_2CO_3 at 4 °C. The samples were centrifuged for 30 min at room temperature in an air-driven ultracentrifuge (100 000 ***g***; Beckman Airfuge). Supernatants were carefully removed. Resulting pellet fractions were washed in 50 mM Tris/HCl, pH 7.5, and again centrifuged at 100 000 ***g***. Supernatants were discarded and the membranous pellet fractions resuspended in Tris buffer, pH 7.5. Soluble extracts derived after the ultracentrifugation step and the insoluble pellet fractions after the second ultracentrifugation were subjected to SDS/PAGE and Western blotting.

Protein labelling and immunoprecipitation

A 17 ml portion of *Acidianus* cytosol (protein concentration 12 mg/ml), prepared as described in [15], was dialysed twice against 1 litre of 1 × PBS buffer/1 mM $MgCl_2$/10 units/ml RNasin (Promega) at 4 °C overnight. The dialysis membrane had a molecular-mass cut-off of 12–14 kDa. Dialysed cytosol (7 ml) was centrifuged for 15 min (10 000 ***g***) and supplemented with protease-inhibitor cocktail (Roche) and 10 units/ml RNasin. The cytosolic protein fraction was labelled with 155 μl of Biotin-NHS-ester (D-biotinoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester; stock 20 mg/ml in water-free DMSO; Roche) for 4 h under pH (7–7.5) control, with slight stirring, at room temperature. The labelling reaction was stopped by dialysing the biotinylated cytosol three times against 1 litre of 1 × TBS (Tris-bufffered saline; 80 g/l NaCl/3.8 g/l KCl/2 g/l Na_2HPO_4/30 g/l Tris base, pH 7.5)/1 mM $MgCl_2$ at 4 °C.

For the immunoprecipitation sample, 40 μl of 25-fold-concentrated protease-inhibitor cocktail (Roche), 10 μl of 10 % (v/v) Nonidet P40, 10 units of RNasin, 10 μl of 100 mg/ml BSA and 50 μl of Sepharose CL-2B (Amersham Pharmacia; 1:1 slurry in 50 mM Tris/HCl, pH 7.0) were added to 1 ml of biotinylated cytosol. The samples were incubated for 15 min at room temperature and centrifuged (see below) for 5 min. Supernatants were carefully removed, then incubated with 40 μl of the respective preimmune serum and again incubated for 2 h at room temperature. After this time, antibodies of the preimmune serum with unspecifically bound protein were removed by adding 100 μl of Protein A–Sepharose CL-4B (1:1 slurry in 50 mM Tris/HCl, pH 7.0). Samples were incubated for 1 h and centrifuged for 30 s. The supernatant was then subjected to immunoprecipitation by adding 20 μl of anti-Ffh or anti-FtsY. Instead of antiserum, 20 μl of preimmune serum or 1 × TBS buffer were used in controls. The samples were slightly agitated on a rocking platform for 2 h. The immunocomplexes which formed were incubated with 50 μl of Protein A–Sepharose CL-4B, and rocked for 1 h. After centrifugation for 15 s the supernatants were removed. The resulting Protein A–Sepharose-bound immunocomplexes were centrifuged and washed four times with 1 × TBS, 0.02 % Nonidet P40, 1 mM $MgCl_2$, 50 mM NaCl and 1 ml of wash volume for each step. The last washing step was performed with 50 mM Tris/HCl, pH 7.0. The resulting pellets were incubated for 1 min at 95 °C in SDS/PAGE sample buffer, cooled on ice, and centrifuged for 2 min. Supernatants were collected with a Hamilton syringe. All steps were performed at room temperature, and centrifugations were carried out in a Biofuge (Biofuge 13; Heraeus; no ***g***-conversion factor available) at 13 000 rev./min. Supernatants to be discarded after washing steps were sucked off with a syringe connected to vacuum.

Protein was blotted from vertical SDS/PAGE slab gels [15 % (w/v) total acrylamide/1 % bisacrylamide cross-linker; 0.7 mm thick; 18 mm × 16 mm) on PVDF membranes (Immobilon-P; Millipore), applying the tank-blotting procedure in transfer buffer [25 mM Tris/190 mM glycine (pH 8.3)/20 % (v/v) methanol/0.02 % SDS] for 2–2.5 h at 4 °C (200 mA, 30 V).

Low-molecular-mass proteins were separated on a tripartite discontinuous gel system [20] (separating gel: total acrylamide 17.5 %; cross-linker 1.9 %; spacer gel: total acrylamide 16 %; cross-linker 1.2 %; upper collecting gel: total acrylamide 4.2 %; cross-linker 0.5 %). Biotinylated marker proteins ranging from 105 to 6.5 kDa were purchased from New England Biolabs. Blots were submerged in 1 × PBS at 4 °C. Biotinylated proteins were detected on blots after development with streptavidin–peroxidase using the protocol of the BM chemiluminescence kit (Roche). The chemiluminescence signals were detected on X-ray films using exposure times of 5, 15, 30 and 60 s.

Co-immunoprecipitation of Ffh and SRP RNA was principally the same as described above. Pellets containing the immunocomplexes after the last wash step were incubated in 100 μl of 0.5 % SDS/10 mM EDTA containing 10 μg of proteinase K for 30 min at 55 °C. Supernatants were extracted with chloroform/phenol and the aqueous phase was subjected to ethanol precipitation as described elsewhere [21]. RNA precipitation was completed after 2 h at − 20 °C, and the resulting RNA pellets were redissolved in RNase-free water. RNA samples were subjected to urea/PAGE as described above. After electrophoresis the gel was stained with TBE buffer containing ethidium bromide for 10 min and then destained.

A modified biotinylation/immunoprecipitation procedure was followed for the co-precipitation of recombinant Ffh and FtsY. Recombinant *A. ambivalens* Ffh (2.4 μM) and *A. ambivalens* FtsY (2.9 μM) were incubated either alone or together in 1 × PBS/1 mM p[NH]ppG (guanosine 5′-[β,γ-imido]triphosphate)/1 mM $MgCl_2$, pH 7.4. These samples were incubated for 30 min at room temperature. Biotin-NHS-ester was added with a 4- and 20-fold molar excess over protein and the samples were incubated for another 30 min. The biotinylation reaction was stopped by adding 50 mM Tris, pH 7.0, and the samples were incubated for a further 45 min. The 200 μl samples were further dialysed three times against 50 mM Tris/HCl, pH 7.0, in small dialysis tubes at room temperature. After dialysis the samples were supplied with 1.2 mM p[NH]ppG and 1 mM $MgCl_2$ and were incubated overnight at 4 °C. After addition of 800 μl of 1 × TBS/0.5 % Triton X-100/1 mg/ml BSA, 50 μl of Sepharose CL4B [1:1 (v/v) slurry in 50 mM Tris, pH 7.0], 2.5 μl of anti-FtsY or anti-Ffh antiserum was added to the preincubated supernatants as indicated in Figure 7 (below). The samples were incubated for 2 h at room temperature with slight agitation. A 50 μl portion of Protein A–Sepharose CL6B [1:1 (v:v) slurry] was applied to precipitate the immunocomplexes. The resulting pellets after 1 h incubation were washed four times with 1 × TBS/0.5 % Triton X-100/1 mg/ml BSA, twice in the same buffer without BSA, and 50 mM Tris/HCl, pH 7.0, in the final step. Pellets were handled as described above prior to Western blotting and chemiluminescence detection.

TUV preparation and flotation assay

A 1.5 g portion of freeze-dried *S. acidocaldarius* cells was extracted under reflux in 400 ml of chloroform/methanol (1:1, v/v) for 12 h. Organic solvent was evaporated for 2 h at 60–80 °C under partial vacuum of 70–80 kPa (700–800 mbar) and resuspended in 20 ml of methanol/water (1:1, v/v) in an ultrasonification water bath for 15 min. A 3 ml portion of the raw extract was ultrasonified with a microtip for 15 min at 0 °C (Branson sonifier; 40 W; 30 % duty cycle) under a nitrogen atmosphere. A 1 ml portion of the raw extract was applied to C_{18} reversed-phase HPLC column (Vydac) and lipids were eluted with the following step gradient: methanol/water (1:1 v/v), chloroform/methanol/water (2:5:2, by vol.) and chloroform/methanol/water (65:25:4 by vol.). Phosphate determinations revealed the middle fraction as the main phospholipid-containing fraction (S2). The organic solvent in S2 was evaporated and the remaining lipid film was vortex-mixed in 100 mM Tris/HCl, pH 7.0. Unilamellar vesicles were generated following the procedure referred in [22]. The pressure-filtration method was performed with a lipid extruder (Liposofast; Avestin Europe G.m.b.H., Mannheim, Germany) containing polycarbonate membranes with a pore size of 100 nm.

In vitro reconstitution of recombinant produced *A. ambivalens* Ffh and FtsY with TUVs was assayed with flotation experiments in ultracentrifugation density gradients. *A. ambivalens* FtsY, Ffh and control proteins were mixed with TUV and resuspended in 40 % Optiprep (Nycomed)/110 mM sucrose/44 mM NaCl/44 mM imidazole, pH 8.0. This fraction corresponds to the bottom (b) of the gradient prior to ultracentrifugation. The b-fraction was overlaid with 30 % Optiprep/62.5 mM sucrose/50 mM imidazole/50 mM Tricine (pH 7.5)/56 mM NaCl corresponding to the middle fraction (m). The top fraction (t) contained 50 mM Tricine (pH 7.5)/100 mM NaCl. The volume ratio b/m/t was 1:0.63:1.5. After 5 h ultracentrifugation in a fixed angle rotor (100 000 ***g***; TFT 65, Sorvall) at room temperature, the resulting gradient was separated into four fractions from top to bottom in a volume ratio of 1:1.29:1.29:0.88. Proteins in these fractions were precipitated with 10 % (w/v) trichloroacetic acid, neutralized, and subjected to SDS/PAGE and Western blotting. Proteins were quantified by recording signal intensities after chemiluminescence detection of immunodecorated Ffh and FtsY protein with a charge-coupled-device (CCD) camera.

RESULTS

In vivo localization of *A. ambivalens* Ffh

Anti-Ffh antisera clearly reacted with cellular Ffh in cytosolic as well as plasma-membrane fractions of *A. ambivalens* and with the histidine-tagged recombinant *A. ambivalens* Ffh protein (Figure 1A). The cellular Ffh migrates in both fractions with an apparent molecular mass of 46 kDa (p46). Cellular p46-Ffh has a lower molecular mass than the recombinant protein, owing to the absence of the 3.1 kDa histidine-tagged sequence. The monomeric conformation of *A. ambivalens* Ffh was demonstrated in gel filtration in which the heterologously expressed protein displayed an apparent molecular mass of 46 kDa (results not shown). Preimmune serum cross-reacted only weakly with a multitude of *A. ambivalens* proteins and not with anti-(*Escherichia coli* β-galactosidase) serum (results not shown).

Ffh is an unusual protein which lacks any membrane-spanning domains. To investigate the membrane binding in more detail, membrane fractions were subjected to extraction with different chemical agents (Figure 1B). Compared with the control, membrane-bound Ffh was substantially extracted with 4 M urea, but was resistant to increasing ionic strength or to alkaline conditions. This suggests that most of the membrane-bound Ffh does not interact electrostatically with the membrane, but associates more strongly than suggested for normal peripheral membrane proteins.

In vitro membrane-binding of *A. ambivalens* Ffh and FtsY

Therefore one of the endogenous factors which might play an important role in the archaeal SRP cycle is the archaeal plasma membrane itself, to which the targeting complex must bind in order to contact the integral protein translocase. However, sequences and structures of the archaeal Ffh and FtsY proteins

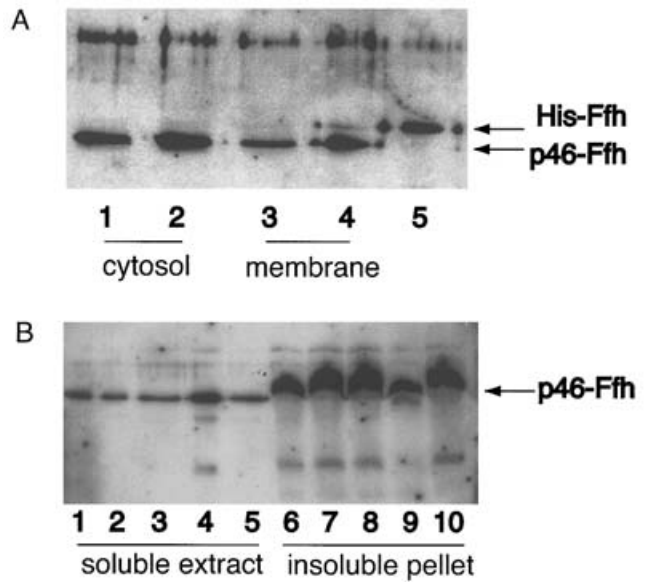

Figure 1 (A) Immunoblot analysis of *A. ambivalens* cellular fractions with α-Ffh and (B) extraction of *A. ambivalens* plasma membranes expressing Ffh

(**A**) Lanes 1 and 2, cytosolic supernatant (20 and 40 μg of protein); lanes 3 and 4, plasma-membrane fraction (20 and 40 μg of protein); lane 5, heterologously expressed His_{10}-tagged *A. ambivalens* Ffh (1 μg). (**B**) Lanes 1–5, soluble extract after first ultracentrifugation; lanes 6–10, insoluble pellet fractions after second ultracentrifugation. Lanes 1 and 6, control; lanes 2 and 7, 50 mM NaCl; lanes 3 and 8, 1 M NaCl; lanes 4 and 9, 4 M urea; lanes 5 and 10, 0.1 M Na_2CO_3.

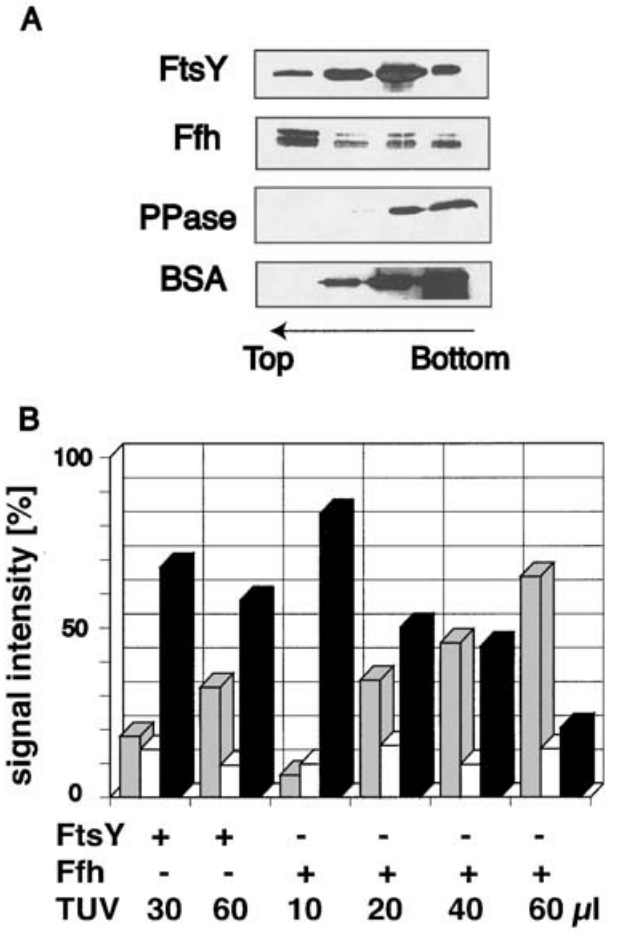

Figure 2 Binding of Ffh and FtsY to TUVs

(**A**) Flotation efficiency of heterologously expressed FtsY and Ffh in density gradients containing TUVs. A 60 μg portion of FtsY, 20 μg of Ffh, 20 μg of BSA (first control), 18 μg of pyrophosphatase (second control) and 60 μg of tetraether phospholipids were resuspended in buffer having the highest density. The gradient was further fractionated into four fractions analysed by SDS/PAGE. (**B**) Flotation efficiencies of Ffh and FtsY depend on TUV concentration. TUV concentration was varied from 36 to 72 μg of phospholipid. The gradients were separated into three fractions after ultracentrifugation, subjected to SDS/PAGE, and further analysed by immunoblots with anti-Ffh or anti-FtsY serum. Grey bars, top fraction; white bars, middle fraction; black bars, bottom fraction.

do not suggest any membrane-binding domains. Furthermore, an SRβ-like integral receptor component is absent in archaea. However, as shown above (Figure 1A), anti-Ffh antibodies detect Ffh (p46) in the plasma-membrane fraction. Also the FtsY protein of *A. ambivalens* has been discovered in small amounts in membranes using anti-FtsY antibodies [11].

To address the quality of membrane binding for both components, the intrinsic affinites of heterologously expressed *A. ambivalens* Ffh and FtsY to TUVs were investigated by *in vitro* reconstitution assays (Figure 2). The reconstitution was qualitatively tested using the flotation efficiency of liposome-bound protein in density gradients during ultracentrifugation. Both proteins floated to the top of the gradient, indicating their association to phospholipid vesicles (Figure 2A, first and second row), but remain in the bottom fraction in the absence of vesicles. Controls with cytosolic proteins such as the *S. acidocaldarius* cytosolic pyrophosphatase or BSA were not detected in the top fraction with the lowest density (Figure 2A, third and fourth row). The flotation efficiency of both Ffh and FtsY clearly increased when the vesicle concentration was raised (Figure 2B). Ffh protein tends to float with a stronger efficiency than FtsY, because higher amounts of floated Ffh were detected using the same concentrations of vesicles (Figure 2B). In contrast, binding of Ffh to vesicles did not depend on the presence of *in vitro* transcribed SRP RNA or GTP (results not shown).

Immunoprecipitations of Ffh, SRP RNA and FtsY from *A. ambivalens* cytosolic cell supernatants

The biotinylated *A. ambivalens* cytosolic fraction was further subjected to immunoprecipitation with the highly specific anti-Ffh antiserum. The protein pattern obtained was compared with controls with preimmune antisera and buffer lacking any antibody (Figure 3A). The pattern obtained clearly displayed the p46-Ffh protein (upper white arrow in lanes 3 and 4) as the major band when compared with controls (lanes 1, 2, 5 and 6). Some less intense, but reproducible, signals in several experiments were observed in the lower-molecular-mass range between 20.5 and 6.5 kDa of the SDS/polyacrylamide gel (lower arrow in lanes 3 and 4). This co-immunoprecipitation suggests further low-molecular-mass proteins which might be integral parts of the archaeal particle. Since proteins of different molecular masses were transferred to different extents in the Western-blotting procedures, various transfer times were applied (results not shown). In all the cases examined, no protein bands in the high-molecular-mass range above the Ffh protein were observed, indicating the absence of SRP68/72-homologous archaeal components.

In order to achieve better resolution in the low-molecular-mass range, modified Tricine/gel systems were used (Figure 3B). Two additional bands with lower intensities than the Ffh protein band

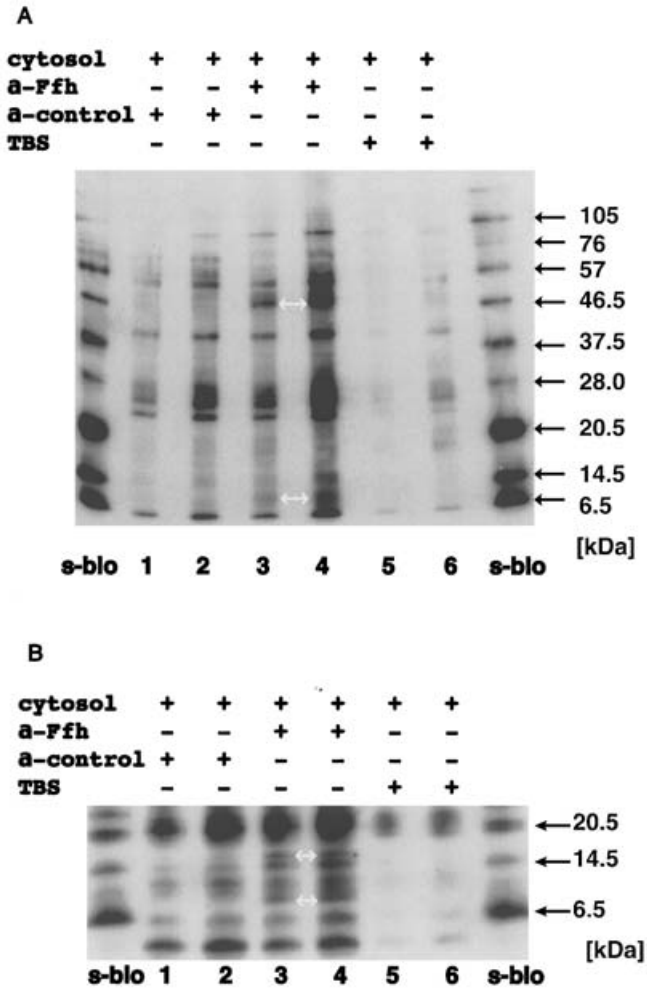

Figure 3 Immunoprecipitation of *A. ambivalens* Ffh with α-Ffh

Precipitation was performed with preimmune antisera (lane 1 and 2), with α-Ffh (lanes 3 and 4) and buffer without antibodies (lanes 5 and 6). Immunoprecipitates were subjected to normal SDS/PAGE (**A**) or to the discontinuous Tricine gel system (**B**). Only the range of molecular masses between 6.5 and 20.5 kDa of the gel is displayed in (**B**). A volume of 0.5 and 1 μl in (**A**) and 1 and 2 μl in (**B**) of the immunoprecipitate was applied to gel electrophoresis. White arrows indicate the protein that was precipitated with α-Ffh, but not in controls. s-bio, biotinylated marker proteins.

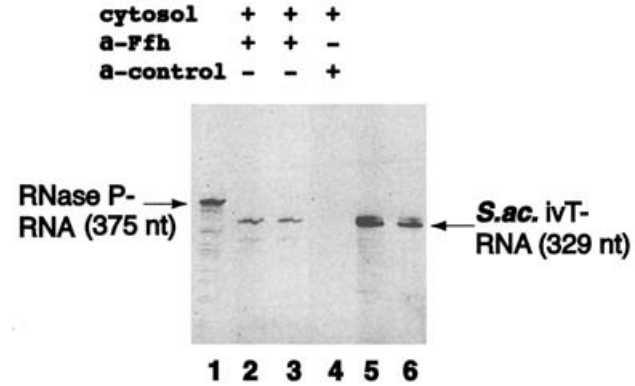

Figure 4 Co-immunoprecipitation of Ffh with SRP RNA

RNA of the co-immunoprecipitation assay with *A. ambivalens* cytosol was subjected to denaturing urea/PAGE. Lane 1, 0.1 μg of RNase P-RNA (an RNA moiety from the *E. coli* ribozyme acting as a size standard); lanes 2 and 3, co-precipitated RNA with α-Ffh; lane 4, precipitation with preimmune serum; lanes 5 and 6, 50 ng of SRP RNA of *S. acidocaldarius* transcribed *in vitro* ['***S.ac.*** ivT-RNA (329 nt)'].

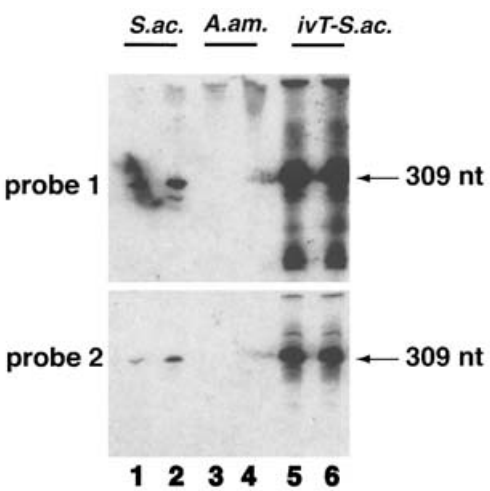

Figure 5 Northern-blot analysis of *A. ambivalens* and *S. acidocaldarius* cellular RNA

Lanes 1 and 2, 2 and 10 μg of *S. acidocaldarius* total RNA; lanes 3 and 4, 2 and 10 μg of *A. ambivalens* total RNA; lanes 5 and 6: 1 μg of 7 S RNA *of S. acidocaldarius* transcribed *in vitro*. Probes 1 and 2 are *S. acidocaldarius* SRP RNA-specific digoxigenin-labelled oligonucleotides.

appeared as prominent signals absent in the controls: one in the range between 14.5 and 20.5 kDa, the other in the range between 6.5 and 14.5 kDa (white arrows). The upper of the two bands of yet unknown function and sequence may correspond to the homologous archaeal SRP 19 protein.

In order to prove the integrity and the presence of RNA in this cellular particle, a modified immunoprecipitation procedure was used which allows the detection of cellular SRP RNA. *A. ambivalens* RNA co-precipitates with the *A. ambivalens* Ffh from cytosolic supernatants, suggesting a strong binding between both components under cellular conditions (Figure 4). The *A. ambivalens* SRP RNA appears to encompass approximately the same size as the *S. acidocaldarius* SRP RNA transcribed *in vitro* which was used as a control. As deduced from the referred sequence [16], the *S. acidocaldarius* SRP RNA contains 309 nucleotides. However, although almost identical in size, the two RNAs display sequence differences, since two total match oligonucleotide probes specific for the archaeal-specific helix 1 and the conserved helix 8 in *S. acidocaldarius* 7 S RNA hybridized with an RNA in the cellular *S. acidocaldarius* RNA fraction with about the same size as the *in vitro* transcript, but did not anneal to any RNA in the total RNA fraction of *A. ambivalens* cells (Figure 5). Taken together, the results demonstrated *in vivo* stable ribonucleoprotein complexes composed of Ffh and 7 S RNA for the first time in the crenarchaeal subdomain. Secondly, the genome-derived sequence of archaeal-type helix 1 is transcribed under cellular conditions and therefore is present in the mature archaeal RNA.

The same set of experiments was performed using anti-(*A. ambivalens* FtsY) antibodies (Figure 6). In contrast with anti-Ffh, the anti-FtsY-specific antibody precipitated only the FtsY component (white arrow in lanes 3 and 4). Thus the archaeal

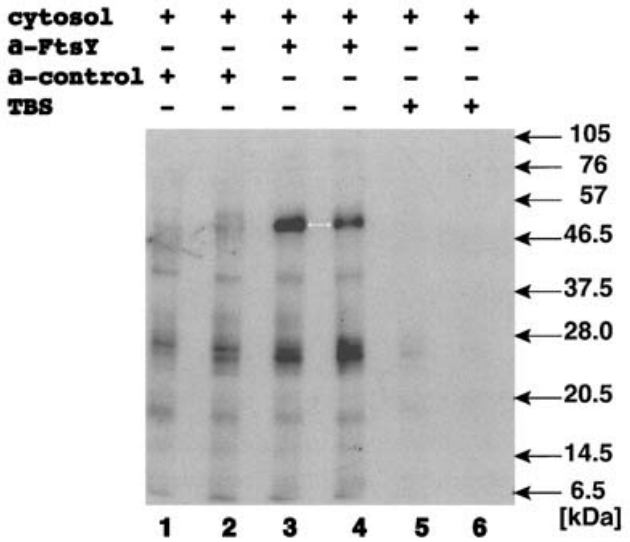

Figure 6 Immunoprecipitation of *A. ambivalens* FtsY with α-FtsY

A volume of 2 μl of the immunoprecipitated sample was subjected to SDS/PAGE. Lanes 1 and 2, control precipitation with preimmune antisera; lane 3 and 4, precipitation with α-FtsY; lanes 5 and 6, control precipitation without any antibody. The white arrow indicates protein which was precipitated with α-FtsY, but not in controls.

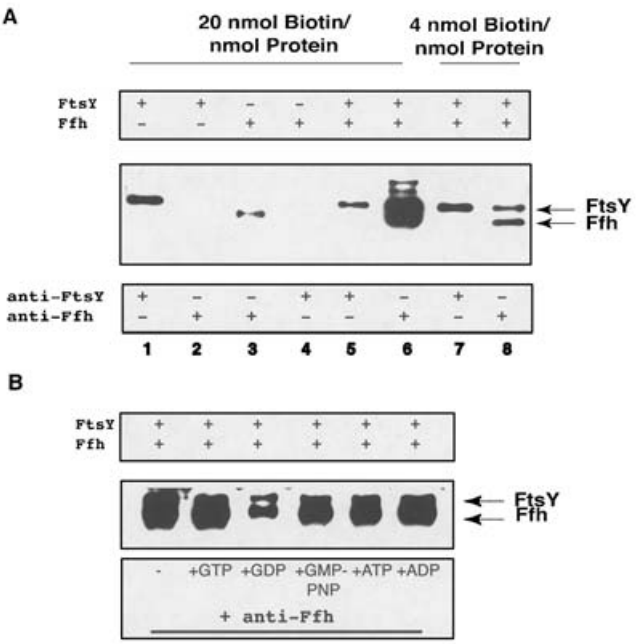

Figure 7 Protein–protein interaction between heterologously expressed *A. ambivalens* FtsY and Ffh

Ffh and FtsY were labelled either alone or together in equimolar amounts in the presence of p[NH]ppG/$MgCl_2$. The samples were further subjected to immunoprecipitation with α-Ffh and α-FtsY as indicated in (**A**). Immunoprecipitation of combined Ffh and FtsY with α-Ffh under various nucleotide conditions (**B**).

SRP receptor is composed of a single subunit in cytoplasmic supernatants of *A. ambivalens* cells lacking accessory components with high-affinity binding. In addition, the monomeric nature of *A. ambivalens* FtsY was further confirmed in gel-filtration experiments with the recombinant histidine-tagged protein from which a molecular mass of 56 kDa was determined (results not shown).

Since immunoprecipitation assays with anti-Ffh- and anti-FtsY antibodies did not co-precipitate the Ffh–FtsY complex in cytosolic supernatants (Figures 3 and 6), both components did not interact strongly under *in vivo* conditions.

In vitro interaction between heterologously expressed *A. ambivalens* Ffh and FtsY

Ffh and FtsY build a heterodimeric complex during protein targeting that dissociates after release of the nascent chain to the translocon and after GTP hydrolysis [23,24]. In a previous model, complex formation was deduced from the known protein structures of *A. ambivalens* Ffh-NG (-NG= N- and G-domains) and *E. coli* FtsY-NG [25,26]. In this model, both Ffh and FtsY interact in a head-to-head conformation in which switch I and II regions of the G-domains are involved. The Ffh–FtsY interaction displays a transient state in protein targeting which depends on other cellular factors such as nucleotide binding, binding of the nascent polypeptide chain and binding to phospholipids of the membrane. Despite these factors, Ffh and FtsY interact with low affinity. This low-affinity binding is demanded by the concerted-switch model in which empty-site and nucleotide-bound forms of both SRP GTPases are present in the heterodimeric complex transiently formed during the SRP cycle [27,28].

In order to address this question experimentally, the heterologously expressed *A. ambivalens* Ffh and FtsY were labelled and incubated for a prolonged period in order to achieve a state approaching binding equilibrium. Following immunoprecipitation using anti-Ffh- and anti-FtsY antisera, complex formation was displayed. When both proteins were incubated in this manner, anti-Ffh antibodies co-precipitated both proteins (Figure 7A, lane 6). However, using the same approach with anti-FtsY antibodies, only the FtsY component was precipitated without Ffh (Figure 7A, lane 5). These results qualitatively confirm that archaeal Ffh and FtsY constitute a specific heterodimeric complex *in vitro*. Complex formation is probably eliminated in the presence of anti-FtsY, owing to the binding of antibodies to critical interaction sites in the FtsY protein. However, the assay with anti-FtsY also served as an internal control to exclude non-specific interference between the two protein components. Furthermore the absence of cross-reactions between anti-Ffh and FtsY (lane 2) and anti-FtsY and Ffh (lane 4) underlines the high specificity of both antisera, which exclusively react with the protein component to which the antiserum has been raised (lane 1, lane 3). The signal intensity of the biotin-labelled complex was further increased when protein biotinylation increased from a 4 molar excess of biotin over protein to 20 molar excess (lane 8 and lane 6), indicating that enhanced biotinylation in this range did not interfere with complex formation.

Strikingly, complex formation does not strongly depend on the presence of GTP or the non-hydrolysable analogue p[NH]ppG (Figure 7B). Both proteins were complexed, both in the presence and in the absence of added nucleotide. Apparently, complex formation is slightly increased in the absence of any nucleotide (lane 1), but was reduced in the presence of GDP (lane 3). GDP acts as a strong inhibitor in the intrinsic GTPase assays of both proteins [5,11]. Therefore GTP binding to Ffh and FtsY is assumed not to be the major prerequisite for complex formation, but GDP binding interferes with complexation. In addition, overall GTP-hydrolysing activities recorded in the presence of both unlabelled *A. ambivalens* Ffh and FtsY were roughly the sum

of the two activities and diminished in the presence of SRP RNA transcribed *in vitro* (results not shown). Therefore complex formation in the absence of other cellular factors is assumed not to be an absolute prerequisite for GTP binding and hydrolysis in the archaeal SRP cycle. If enhanced GTPase activities are necessary in archaeal SRP-dependent targeting, as shown for mammalian and bacterial SRP systems [29,30], other still-unidentified GTPase activating factors must also play a role in archaea. The direct association of the two components is in accordance both with the structural model proposed in [25] and with the concerted switch model [27,28]. In the structural proposal, the complex can be modelled for the apoproteins in the absence of nucleotide. However, GTP binding presumably leads to the prevention of a structural clash between the interacting surfaces of both proteins after conformational changes involving an insertional sequence element specific to all SRP-GTPases [31].

DISCUSSION

On the basis of the immunoprecipitation assays, the *A. ambivalens* SRP is composed at least of Ffh and SRP RNA under *in vivo* conditions. Although biotinylation covalently modifies proteins at exposed lysine residues, the cellular ribonucleoprotein remains stable, indicating strong binding between both components inside the complex. Smaller, as-yet-unclassified, components may complete this unique archaeal structure. The SRP 19-homologous protein is regarded as a strong candidate, as it is present in almost all archaeal genomes except *Thermoplasma acidophilum*. As shown by *in vitro* reconstitution, the SRP 19-homologous protein binds to helix 6 of SRP RNA and thus indirectly strengthens the binding of Ffh to RNA probably by conformational changes in the RNA moiety [32]. In the present study, the cellular SRP 19-homologous component may correspond to the biotinylated band between 14.5 and 20.5 kDa. Detection of an even smaller protein in the range between 6.5 and 14.5 kDa suggests another accessory protein component in the *A. ambivalens* SRP. In view of its size, this band is comparable with the histone-like *B. subtilis* HBsu protein binding to the Alu domain of small cytoplasmic RNA [33]. Since polyclonal antisera may both precipitate cross-reacting proteins artificially and proteolytically derived Ffh degradation fragments, further attempts to identify these *Acidianus* components unambigiously are in progress. In striking contrast with the eukaryal particle SRP 68/72, homologous proteins were not found. The two components have been localized in the nucleolus of mammalian cells, where the SRP68/72 dimer might contribute to SRP assembly or activation [34,35]. As archaea, like bacteria, do not contain nucleoli, different mechanisms may be active in assembly which do not require SRP 68/72.

A. ambivalens Ffh and FtsY demonstrate an intrinsic affinity to bipolar tetraether phospholipids when qualitatively tested in flotation gradient analysis. The two components are therefore able to associate with vesicles without other factors. Although the biological importance and the structural fundamentals of these lipid–protein binding processes still need to be resolved, they may represent a necessary combination of protein targeting as a primarily cytosolic occurring reaction and protein translocation as a membrane-mediated process. For this reason, it is speculated that the intrinsic affinities of the two proteins to tetraether lipids pull the targeting complex to the archaeal plasma membrane. Owing to the absence of an integral receptor component such as SRβ, such linkage appears reasonable. Strong candidates for lipid association are anionic phospholipids interacting with positive K/R (lysine/arginine) charge clusters in the acidic FtsY protein (calculated pI 5.13) and the basic Ffh (calculated pI 9.89). The first evidence for this kind of interaction has been found for the *E. coli* SRP receptor [12], but now appears to be clear also for the archaeal Ffh and FtsY. It remains to be clarified whether bacterial apo Ffh also demonstrates phospholipid binding. In summary, these results, for the first time, indicate soluble regulatory targeting proteins of archaea containing intrinsic membrane binding sites in their apo forms.

The results presented here are the first indications of a receptor function of FtsY for Ffh, since a nucleotide-independent complex formation between the two proteins was demonstrated. *A. ambivalens* Ffh RNA and FtsY were detected uncomplexed in cytosolic supernatants, whereas *in vitro* binding was observed with Ffh and FtsY after prolonged incubation overnight, suggesting an intrinsic affinity between the two components. This low-affinity binding did not depend on the presence of nucleotides under *in vitro* conditions, assuming that nucleotide-free forms of the two archaeal proteins can associate. Binding of the ribosome–nascent-chain complex and other as-yet-unidentified factors would enhance the Ffh–FtsY interaction under cellular conditions in order to generate a transiently active targeting complex.

I thank Professor Dr Irmgard Sinning (Biocentre, University of Heidelberg, Heidelberg, Germany) for giving me the opportunity to perform the TUV preparations during an EMBL (European Molecular Biology Laboratory) fellowship in her laboratory, and Dr Kai te Kaat (European Molecular Biology Laboratory, Heidelberg, Germany) for an introduction to vesicle preparation. I also thank Professor Dr Günter Schäfer for his continuous support to this project. I thank Professor Dr Stefan Anemüller for his kind donation of *A. ambivalens* cells, and Miss Silke Schmidtke for her excellent technical assistance.

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Received 27 March 2003/6 May 2003; accepted 30 May 2003
Published as BJ Immediate Publication 30 May 2003, DOI 10.1042/BJ20030475