Eur. J. Biochem. **271**, 1382–1390 (2004) © FEBS 2004 doi:10.1111/j.1432-1033.2004.04050.x

Membrane binding of SRP pathway components in the halophilic archaea *Haloferax volcanii*

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Across evolution, the signal recognition particle pathway targets extra-cytoplasmic proteins to membranous translocation sites. Whereas the pathway has been extensively studied in Eukarya and Bacteria, little is known of this system in Archaea. In the following, membrane association of FtsY, the prokaryal signal recognition particle receptor, and SRP54, a central component of the signal recognition particle, was addressed in the halophilic archaea *Haloferax volcanii*. Purified *H. volcanii* FtsY, the FtsY C-terminal GTP-binding domain (NG domain) or SRP54, were combined separately or in different combinations with *H. volcanii* inverted membrane vesicles and examined by gradient floatation to differentiate between soluble and membrane-bound protein. Such studies revealed that both FtsY and the FtsY NG domain bound to *H. volcanii* vesicles in a manner unaffected by proteolytic pretreatment of the membranes, implying that in Archaea, FtsY association is mediated through the membrane lipids. Indeed, membrane association of FtsY was also detected in intact *H. volcanii* cells. The contribution of the NG domain to FtsY binding in halophilic archaea may be considerable, given the low number of basic charges found at the start of the N-terminal acidic domain of haloarchaeal FtsY proteins (the region of the protein thought to mediate FtsY–membrane association in Bacteria). Moreover, FtsY, but not the NG domain, was shown to mediate membrane association of *H. volcanii* SRP54, a protein that did not otherwise interact with the membrane.

Keywords: Archaea; FtsY; *Haloferax volcanii*; protein targeting; signal recognition particle.

It is becoming increasingly clear that similarities exist not only in the membrane-associated complexes responsible for translocating proteins across membranes in Eukarya, Bacteria and Archaea [1,2], but also in the method by which extra-cytoplasmic proteins are targeted to these sites [3]. In higher Eukarya, the signal recognition particle (SRP), a ribonucleoprotein complex consisting of six polypeptides (SRP54, SRP19 and the SRP68/72 and SRP9/14 dimers) and a 7S RNA, binds to ribosomes in the process of translating proteins destined to cross the endoplasmic reticulum membrane [4–6]. Bacteria rely on a much simpler version of SRP, consisting of Ffh (an SRP54 homologue) and a 4.5S RNA, for the insertion of membrane proteins [7–9], although evidence implicating SRP in bacterial protein secretion has also been presented [10–14]. Archaeal SRP, comprised of 7S RNA, SRP19 and SRP54 subunits, is more reminiscent of its eukaryal counterpart, yet also possesses Archaea-specific traits in terms of the makeup of its subunits and mode of assembly [15]. For example, despite overall similar secondary structures, archaeal SRP RNA lacks helix 7 found in the eukaryal molecule, but includes the additional helix 1 not found in its eukaryal counterpart [16]. Archaeal SRP19 proteins also lack much of the polypeptide located between the so-called domain II and domain III regions of the eukaryal SRP19 protein [17]. In further contrast to the situation in Eukarya, where SRP19 binding to SRP RNA is a necessary prerequisite for SRP54 binding, a substantial amount of SRP54 can bind to SRP RNA in the absence of SRP19 in Archaea [18–20]. Morever, the precise role of archaeal SRP in protein translocation remains an open question.

During the SRP-mediated protein targeting cycle in both Eukarya and Bacteria, SRP interacts with the SRP receptor (SR). The peripheral SRα subunit in Eukarya, anchored to the endoplasmic reticulum membrane via the integral SRβ subunit [21,22], interacts with SRP in a GTP-dependent fashion [23,24]. In *Escherichia coli*, the SRα homologue FtsY exists as both a soluble and a membrane-associated protein [11]. While the precise roles and temporal positions of SRP and FtsY in the bacterial SRP cycle remain topics of on-going investigation [9], membrane binding of FtsY has been shown to be essential for the function of this targeting component [25]. Given the apparent absence of a bacterial homologue of the SRβ subunit, the nature of the FtsY–membrane association in Bacteria remains, however, unclear. FtsY is also present in Archaea, the other prokaryal domain. As in Bacteria, searches of completed archaeal genomes have failed to detect an archaeal SRβ homologue. Hence, little

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Abbreviations: A domain, acidic domain; IMV, inverted membrane vesicle; NG domain, C-terminal GTP-binding domain; SR, SRP receptor; SRP, signal recognition particle.

Note: A website is available at http://www.bgu.ac.il/life/Faculty/Eichler/index.htm

(Received 3 December 2003, revised 23 February 2004, accepted 24 February 2004)

is known presently of the nature of the FtsY–membrane interaction in Archaea. Moreover, the steps leading to delivery of SRP to the archaeal membrane have yet to be described.

Towards an understanding of the membrane interaction of SRP pathway components in Archaea, the membrane binding of SRP54 and FtsY in the halophilic archaea *Haloferax volcanii* was investigated. Such studies offer insight not only into the membrane-associating behavior of these proteins, but also address how interaction between SRP54 and FtsY and the membrane would occur inside halophilic archaea, where salt concentrations may reach as high as 5 M [26,27]. Furthermore, by examining the interplay between SRP54, FtsY and the membrane, these studies provide insight into the events that take place during the SRP-mediated protein targeting in Archaea.

Experimental procedures

Materials

H. volcanii DS2 was obtained from the American Type Culture Collection and grown aerobically at 40 °C in medium defined previously [28]. Ampicillin, DNase I, kanamycin and novobiocin came from Sigma. Proteinase K came from Boehringer (Mannheim, Germany). Yeast extract came from Pronadisa (Madrid, Spain), while tryptone came from United States Biochemicals (Cleveland, OH, USA). Molecular mass markers and goat anti-rabbit horseradish peroxidase-conjugated Igs were from Bio-Rad (Hercules, CA). FastStart DNA *Taq* polymerase was purchased from Roche. Nickel-nitrilotriacetic acid resin came from Qiagen. Restriction enzymes came from MBI Fermentas (Vilnius, Lithuania). An enhanced chemiluminescence kit came from Amersham-Pharmacia. *H. volcanii* SRP54 was prepared as described previously [20].

Plasmid construction

The sequence of the *H. volcanii* FtsY-encoding gene was obtained from the partially completed *H. volcanii* genome sequence (http://zdna2.umbi.umd.edu/~haloweb/hvo.html) and amplified by PCR from *H. volcanii* genomic DNA, prepared as described previously [29]. The complete gene was cloned using primers designed to introduce *Nde*I (TATATATCATATGTTCGACGGACTGA) and *Hin*dIII (TTAAGCTTCTCGTCTTCACCGAG) sites at the 5′ and 3′ ends of the gene, respectively. The resulting gene was ligated into the pET-24b(+) vector (Novogen, Nottingham, UK) between the *Nde*I and *Hind*III sites to yield the plasmid pET-HVFtsY. The 801 bp C-terminal FtsY GTP-binding domain (NG domain) was PCR amplified from *H. volcanii* genomic DNA using primers designed to introduce *Nde*I (TATATA TCATATGGCGCTCCTCCAG) and *Xho*I (ATACTCG AGCTCGTCTTCACCGAG) sites at the 5′ and 3′ ends of the gene, respectively. The resulting gene was ligated into the pET-24b(+) vector (Novogen, Nottingham, UK) between the *Nde*I and *Xho*I sites to yield the plasmid pET-HVFtsYNG.

Expression and purification of *H. volcanii* FtsY and the FtsY NG domain

E. coli BL21 cells transformed with either plasmid pET-HVFtsY or plasmid pET-HVFtsYNG were grown in LB broth in the presence of 50 μg·mL^{-1} kanamycin to $D_{600} = 0.5$ and induced with 0.4 mM isopropyl thio-β-D-galactoside for 3 h. Cells were then harvested and disrupted by sonication (three times for 30 s with 30 s intervals between each pulse, 35% output, Misonix XL2020 ultrasonicator, Misonix Inc., Farmingdale, NY, USA). Soluble proteins were separated from membrane proteins by ultracentrifugation (Sorvall Discovery M120 ultracentrifuge, S120AT2 rotor, 190 000 ***g***, 10 min, 4 °C) and applied to nickel-nitrilotriacetic acid resin, equilibrated previously with 20 mM imidazole, 150 mM NaCl, 50 mM Tris/HCl, pH 7.9. Following a 1 h incubation at 4 °C, unbound proteins were removed by washing with the equilibration buffer. Specifically bound proteins were then eluted by addition of 500 mM imidazole, 150 mM NaCl, 50 mM Tris/HCl, pH 7.9. The purified proteins were concentrated in a Vivaspin concentrating unit (10 000 molecular mass cutoff; Satorius, Goettingen, Germany) and resuspended to a final concentration of 2–4 mg·mL^{-1} in buffer A (2 M NaCl, 50 mM Tris/HCl, pH 7.2).

Floatation assay

To assess the binding of *H. volcanii* FtsY, the FtsY NG domain or SRP54, to *H. volcanii* membranes, floatation was performed as described previously [30], with slight modifications. A 20 μL aliquot of FtsY, the FtsY NG domain or SRP54 was incubated with *H. volcanii* inverted membrane vesicles (IMVs) [31] on ice for 20 min. In some instances, SRP54 was preincubated with either FtsY or the FtsY NG domain. The mixture was then applied to the base of ultracentrifuge tubes for the S120AT2 rotor of the Sorvall Discovery M120 ultracentrifuge. In some cases, the membrane preparations were pretreated with proteinase K (1 mg·mL^{-1}, 4 h, 40 °C) and collected by centrifugation (S120AT2 rotor, 190 000 ***g***, 10 min, 4 °C) through a cushion of 0.4 M sucrose in buffer A to remove the protease. In either case, the samples were then mixed with 58% sucrose in buffer A to a final volume of 440 μL. In control experiments, the membranes were omitted. The various mixtures were overlaid with 680 μL of 52% sucrose in buffer A, 270 μL of 7.5% sucrose in the same buffer and centrifuged (357 000 ***g***, 90 min, 4 °C). Six fractions (200 μL) were collected from the top of the gradient and a 50 μL aliquot was precipitated with 15% trichloroacetic acid and analyzed by SDS/PAGE and Coomassie staining or immunoblotting.

Subcellular fractionation and immunoblotting

Subcellular fractionation was achieved by sonication (2 s on, 1 s off for 30 s, 35% output, Misonix XL2020 ultrasonicator) followed by centrifugation (8000 ***g***, 20 min) to clear unbroken cells and ultracentrifugation (S120AT2 rotor, 190 000 ***g***, 10 min, 4 °C). Immunoblotting was performed using antibodies raised against the

H. volcanii FtsY NG domain, against *H. volcanii* dihydrofolate reductase (obtained from M. Mevarech, Tel Aviv University, Israel) or against the *H. volcanii* S-layer glycoprotein [32]. Antibody binding was detected using goat anti-rabbit HRP-conjugated Igs and enhanced chemiluminescence.

Other methods

Menadione-dependent NADH dehydrogenase activity of the IMVs was assayed as described previously [31]. Protein concentration was determined using Bradford reagent (Bio-Rad), with BSA as standard. Densitometry was performed using IPLAB GEL software (Signal Analytics, Vienna, VI, USA).

Results

Heterologous expression and purification of *H. volcanii FtsY* and the FtsY NG domain

The sequence of the *H. volcanii ftsY* gene was obtained from a partially completed version of the *H. volcanii* genome sequence as described above, and amplified by PCR from *H. volcanii* genomic DNA. *E. coli* BL21 cells were transformed with plasmid pET-HVFtsY, encoding for a C-terminally polyhistidine-tagged version of the protein. Given that the presence of a His_6-tag at the C-terminus of the *E. coli* FtsY NG domain did not interfere with determination of the 3D structure of this FtsY region [33], it was assumed that the presence of a polyhistidine tag at the same position in the *H. volcanii* protein would not hamper proper protein expression or folding. Induction of the transformed cells led to the enhanced expression of a 75 kDa protein, which, following nickel-nitrilotriacetic acid-based purification from the soluble fraction of the cells, was confirmed by N-terminal amino acid sequencing as *H. volcanii* FtsY (Fig. 1A). Although the *H. volcanii ftsY* gene sequence predicts a 48.2 kDa species, the slower migration of FtsY proteins in SDS/PAGE was not unexpected, having been previously reported in the case of *E. coli* FtsY [11] and attributed to the amino acid composition of the acidic domain (A domain) of the protein [25,34]. Moreover, the aberrant behavior of halophilic proteins in SDS/PAGE is well known, resulting from the negatively charged character and subsequently diminished SDS binding capacity of such proteins [35,36].

In addition, *E. coli* BL21 cells were also transformed with plasmid pET-HVFtsYNG, encoding for a polyhistidine-tagged version of the C terminal NG domain of the protein. The FtsY NG domain has been previously expressed and studied as a separate structural unit [33,37]. Induction of the transformed bacterial cells led to the appearance of a prominent 30 kDa protein band, in agreement with the predicted molecular mass of this domain (Fig. 1B). Incubation of the cytosolic fraction of the induced cells with nickel-nitrilotriacetic acid resin and subsequent elution with imidazole led to purification of the C-terminally tagged 30 kDa species. The identity of the eluted protein as the *H. volcanii* FtsY NG domain was confirmed by N-terminal amino acid sequencing.

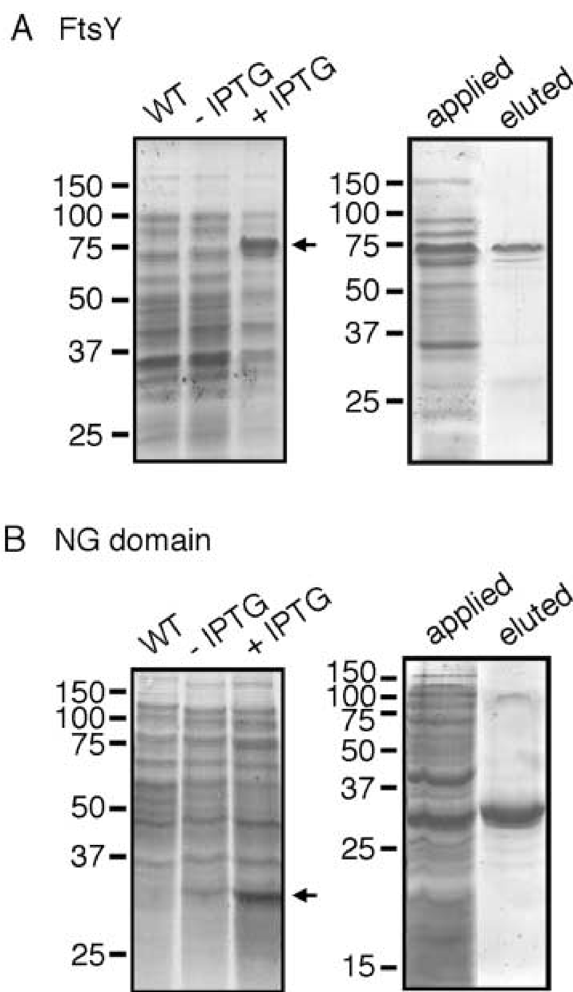

Fig. 1. Purification of *H. volcanii* FtsY (A) and the FtsY NG domain (B). *E. coli* BL21 cells were transformed with either plasmid pET-HVFtsY or plasmid pET-HVFtsYNG, encoding for His_6-tagged versions of *H. volcanii* FtsY or the FtsY NG domain, respectively. The transformed cells were induced with 0.5 mM isopropyl thio-β-D-galactoside (IPTG) for 3 h prior to harvesting and examination of cellular protein contents by SDS/PAGE and Coomassie staining. The soluble fraction of the induced cells was applied to a nickel-nitrilotriacetic acid column and eluted with 0.5 M imidazole. In (A) purification of FtsY and (B) purification of the FtsY NG domain, wild type cells (WT), uninduced transformed cells (–IPTG), induced transformed cells (+IPTG), the supernatant applied to nickel-nitrilotriacetic acid resin (applied) and the purified protein (eluted), are shown. In all panels, molecular mass markers are shown on the left. In the left panel of both (A) and (B), the position of FtsY and the FtsY NG domain, respectively, are depicted by an arrow on the right.

Characterization of *H. volcanii* FtsY interaction with the membrane

With purified *H. volcanii* FtsY in hand and *H. volcanii* IMVs available [31], the membrane binding ability of the protein was assessed, relying on a floatation assay adapted for halophilic conditions [30]. Aliquots of FtsY were incubated with IMVs and the resulting mixture was applied to the base of an ultracentrifugation tube and overlaid with

a step gradient of sucrose prepared in 2 M NaCl, as described above. Following centrifugation, six fractions were collected from the top of the gradient down, and each fraction was analyzed for the presence of FtsY. In gradients containing FtsY alone, the protein was localized to the bottom fractions of the gradient (Fig. 2A, top panel). When *H. volcanii* FtsY was preincubated with IMVs prior to centrifugation, a substantial amount of the protein migrated to the upper gradient fractions (Fig. 2A, middle panel). Indeed, densitometric quantitation of the membrane binding of FtsY revealed that almost half of the FtsY protein present floated to the upper half of the gradients following preincubation with *H. volcanii* IMVs (Fig. 2B). When centrifuged alone, a major fraction of the IMVs also migrated to the upper gradient fractions. Analysis of the SDS/PAGE profile of these fractions, however, failed to reveal the presence of any intensely stained protein bands at the position of FtsY (Fig. 2A, lower panel). Moreover, immunoblotting of the IMVs with anti-FtsY serum (see below) failed to detect the presence of significant levels of FtsY associated with the IMVs (Fig. 6C). Finally, the presence of 5 mM GTP, GDP or GTPγS had no discernable effect on the interaction of FtsY with the membrane preparations (not shown).

The NG domain contributes to *H. volcanii* FtsY membrane association

In *E. coli*, membrane association of FtsY has been proposed to be mediated by clusters of lysine and arginine residues situated at the start of the N-terminal A domain [25,34,38–40]. Analysis of various archaeal FtsY sequences confirmed the presence of clusters of positively charged residues within the first 46 residues of the A domain (Fig. 3), a length of FtsY shown to be important for membrane localization of the protein in *E. coli* [38]. For example, *Archaeoglobus fulgidus* FtsY contains 14 positively charged residues within the first 46 positions, while *Pyrococcus furiosus* FtsY contains 12 arginine and lysine residues in this portion of the protein. In contrast, examination of the A domain sequence of FtsY in *Halobacterium* sp. NRC-1, the only halophilic archaeal species for which complete sequence data has been published [41], reveals that only three positively charged residues are found within the N-terminal 46 residues. Despite its two additional lysine and additional arginine residues, *H. volcanii* FtsY can also be placed within the group of archaeal FtsY proteins containing the fewest number of basic residues within the A domain N-terminal region. A similar number of arginine and lysine residues are found in the initial 46 residues of *Haloarcula marismortui* FtsY (http://zdna2.umbi.umd.edu/~haloweb/hma.html). This raises the question of whether the A domain alone is responsible for FtsY membrane binding in haloarchaea. Indeed, given the molar salt concentrations present in the haloarchaeal cytoplasm [26,27], it is conceivable that haloarchaeal FtsY proteins rely on an additional mode of membrane association, apart from that thought to be mediated by the A domain of the archaeal protein.

Accordingly, experiments addressing the membrane-binding behavior of the purified *H. volcanii* FtsY NG domain were performed, relying on the floatation assay described above. The results of such studies paralleled those

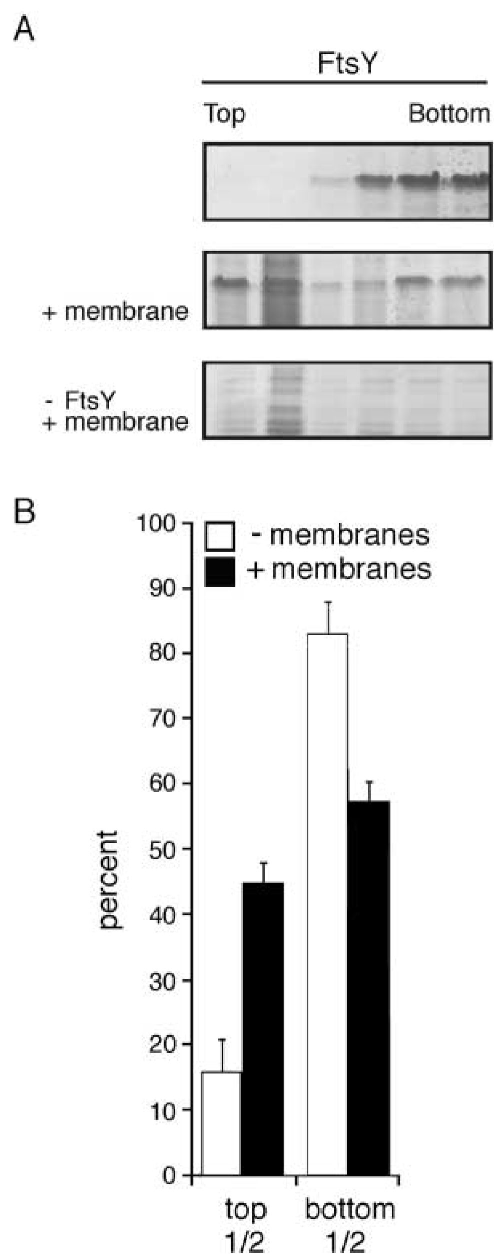

Fig. 2. Membrane binding of *H. volcanii* FtsY. (A) Purified FtsY (80 μg) was incubated in the absence (upper panel) or presence (middle panel) of *H. volcanii* IMVs (50 μg). The reactions were then applied to the base of ultracentrifuge tubes and overlaid with a sucrose density step gradient, as described in Experimental procedures. Six fractions of 200 μL were collected from the top of each gradient, and examined by SDS/PAGE and Coomassie staining. In the lower panel, membranes were centrifuged alone. (B) Densitometric quantitation of FtsY floatation. The results of four experiments such as that described in (A) were scanned and densitometrically quantitated. Values shown represent the average values obtained ± SEM in the top and bottom halves of the gradients.

obtained using full length FtsY; in both instances, substantially more protein migrated to the upper portions of the gradient in the presence of membranes (compare Fig. 2A with Fig. 4A). In the case of the FtsY NG domain,

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A.amb   IICFEKLKKAFSSFTEKLRQKGEEKSEEPQQTLSNSTTQLVTQEAQ
A.ful   MFKALKEKLSGLRKKIEEEEKEEIEEIRKEAEEKREAKVEEKKVGL
A.per   MSRRLFGRLREALNRASEHIARGIVDTLAYKELKPEDLEPVLDDIL
M.ace   MFNKFKEKLGSFKKALSKTIDEKAVEVEPVVVEQMPQSEESLEEEI
M.bar   MFNKFKEKLGNFKKSLSRTIDDKAVDIEPVVEPVPEAKEGFEEDIE
M.jan   MFGKLKEKLLETASKITEKIYSKGEAEEVKEEKEEKSKISFTSLFK
M.kan   MPSSLFGKMKSVLSKVKEKVAKRVEERAEEAEEAKVKEVETMPEDL
M.the   MFESLKKKFSETVGKITEKVSSGQEPRSEEDKSEDRGPEPIPEGKP
P.aby   MFGKLKEKLRSFIKKVEENVEKEEKEAEKKGILEKILMVEIKEKDV
P.aer   MFERLKRTFSKFVESVVSVVKEETLSEKDVDALTSDLYIDLVESDV
P.fur   MFGKLREKLKSFVKRVEEEVEKEEEEVEKKGLLDRILTVEIKEKDV
P.hor   MVVEMLGKLKEKLQSFIRRVEENVEKEEDEAKKKGLLDKILMIEIK
S.aci   MICFDRLKKAFSNFLDKISGEENKKEPETRQTDQLESKKEETIQQQ
S.sol   MICFDRLKKAFSNFLDKISGEENKKEPETRQTDQLESKKEETIQQQ
T.aci   MFEKLKKKFAEIFHRKKIDPDEVADEIPLKLVEADVSLEAAEDLAS
T.vol   MFEKFKKKLVEIFSRKKIDPDEAAEEIPLKLIEADVSVETAEYLGN
T.zil   MLGKLREKLKRFTKQVEEKVEEEEKTVISGEEKKEEKAGLLDRLLQ

H.mar   CNSKSRKTSKAAMFDGLKDKLSGFTSDVEEDVDDDALEAEDEPDAD
H.NRC   MFDGLKDKLSNFREDAEEVAEENAEELADQPDDEAADTEAAAADSA
H.vol   MFDGLKKKLNRFRNDVEETAEEKAEAAADEAESDADAEAESAPADT
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Fig. 3. Haloarchaeal FtsY A domains contain fewer arginine and lysine residues than A domains of other archaeal FtsY proteins. The amino acid composition of the first 46 positions of archaeal FtsY proteins are shown. Arginine and lysine residues are highlighted in bold. The strains examined (and their accession numbers or source) were: A.amb, *Acidianus ambivalens* (CAA65233); A.ful, *Archaeoglobus fulgidus* (NP_070886); A.per, *Aeropyrum pernix* (NP_147702); M.ace, *Methanosarcina acetivorans* str. C2A (NP_618977); M.bar, *Methanosarcina barkeri* (ZP_00078816); M.jan, *Methanococcus jannaschii* (NP_247264); M.kan, *Methanopyrus kandleri* AV19 (NP_614896); M.the, *Methanothermobacter thermoautotrophicus* (NP_276720); P.aby, *Pyroccocus abyssi* (NP_126193); P.aer, *Pyrobaculum aerophilum* (NP_560489); P.fur, *Pyrococcus furiosus* (NP_579495); P.hor, *Pyrococcus horikoshii* (NP_143516); S.aci, *Sulfolobus acidocaldarius* (S53703); S.sol, *Sulfolobus solfataricus* (CAA41429); T.aci, *Thermoplasma acidophilum* (NP_394537); T.vol, *Thermoplasma volcanium* (NP_111051); T.zil, *Thermoplasma zilligii* (AAB58327); H.mar, *Haloarcula marismortui* (http://zdna2.umbi.umd.edu/~haloweb/hma.html); H.NRC, *Halobacterium* sp. NRC-1 (NP_281058) and H.vol, *Haloferax volcanii* (http://zdna2.umbi.umd.edu/~haloweb/hvo.html).

densitometric analysis revealed that two-fold more protein floated to the upper half of the gradient following preincubation with *H. volcanii* membranes, as compared to when the protein was subjected to floatation alone (Fig. 4B). Finally, as observed with the membrane-mediated floatation of FtsY, the presence of 5 mM GTP, GDP or GTPγS did not affect the association of the FtsY NG domain with the membrane (not shown).

Proteolytic treatment does not prevent membrane binding of FtsY or the FtsY NG domain

Despite the failure of searches of completed archaeal genome sequences to detect an archaeal version of the eukaryal integral SRβ subunit, studies designed to probe for the presence of a proteinaceous FtsY receptor were undertaken. In these experiments, *H. volcanii* IMVs were preincubated with proteinase K to remove any polypeptides associated with the external surface of the membrane. Following subsequent removal of the protease via passage of the mixture through a 0.4 M sucrose cushion, the membranes were resuspended and incubated with either *H. volcanii* FtsY or the FtsY NG domain, and once again subjected to floatation. As reflected in Fig. 5A, the

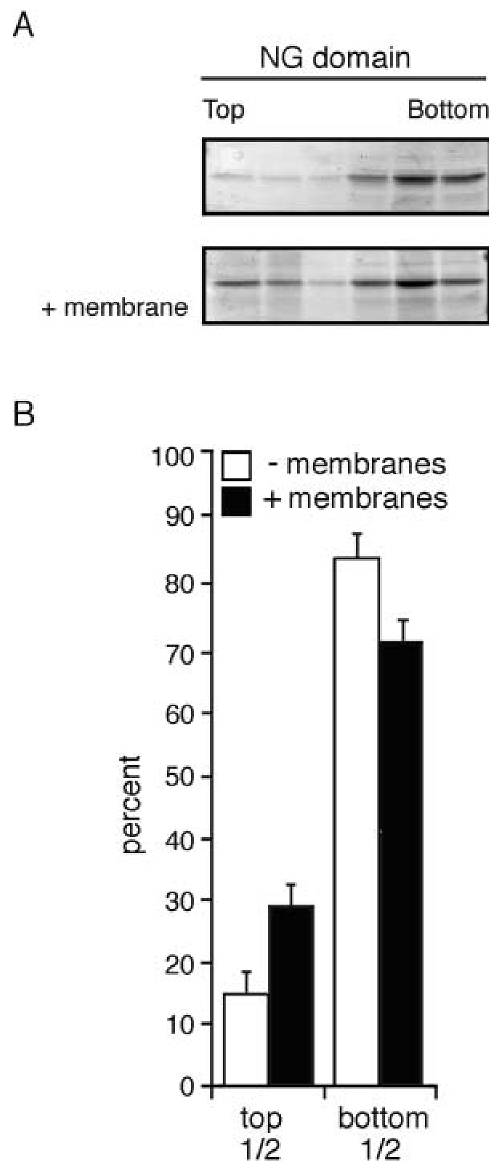

Fig. 4. Membrane binding of the *H. volcanii* FtsY NG domain. (A) Purified FtsY NG domain (40 μg) was incubated in the absence (upper panel) or presence (lower panel) of *H. volcanii* IMVs (50 μg). The reactions were then applied to the base of ultracentrifuge tubes and overlaid with a sucrose density step gradient, as described in Experimental procedures. Six fractions of 200 μL were collected from the top of each gradient, and examined by SDS/PAGE and Coomassie staining. (B) Densitometric quantitation of FtsY NG domain floatation. The results of five experiments such as that described in (A) were scanned and densitometrically quantitated. Values shown represent the average values obtained ± SEM in the top and bottom halves of the gradients.

proteinase K treatment had no effect on the ability of either FtsY or the FtsY NG domain to bind to the membrane, with floatation of the proteins to the upper gradient fractions occurring to similar degrees both prior to and following proteolysis. This visual assessment was confirmed by densitometric quantitation, which showed that 93% ± 1% (SD, $n = 2$) of the starting amount of

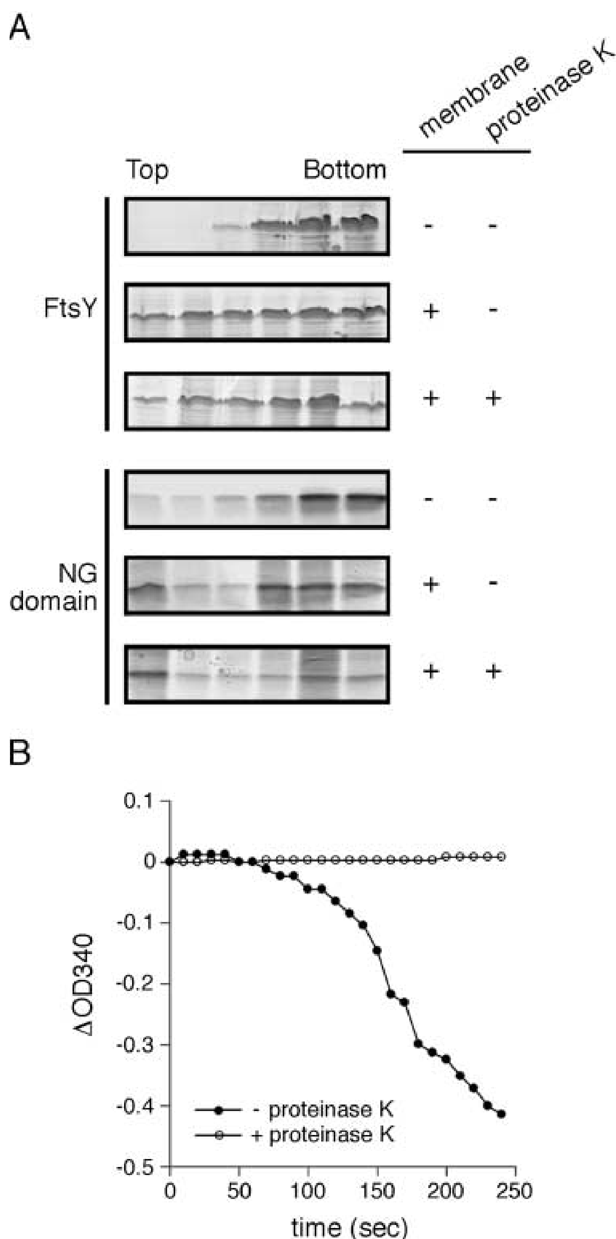

Fig. 5. Proteinase K treatment does not prevent FtsY or FtsY NG domain binding to membranes. (A) *H. volcanii* FtsY or the FtsY NG domain was incubated in the absence or presence of *H. volcanii* IMVs, either untreated or pretreated with proteinase K (1 mg·mL^{-1}, 4 h, 40 °C) and collected by centrifugation (S120AT2 rotor, 190 000 ***g***, 10 min, 4 °C) through a cushion of 0.4 M sucrose to remove the protease. The reactions were then subjected to floatation as described in Experimental procedures. (B) The level of menadione-dependent NADH dehydrogenase activity of IMVs either untreated (●) or pretreated (○) with proteinase K is shown.

IMV-bound FtsY detected in the upper half of the gradient remained following proteolysis. To confirm the effectiveness of the protease treatment under the conditions employed, the activity of menadione-dependent NADH dehydrogenase, a marker of the *H. volcanii* inner surface that is outwardly exposed in the inverted membrane preparation and hence accessible to added protease, was addressed [31]. As shown in Fig. 5B, pretreatment with proteinase K led to a complete loss in enzymatic activity, as reflected by the unchanged level of NADH, measured at 340 nm. Thus, it appears that in *H. volcanii*, FtsY and the FtsY NG domain interact with the lipid phase of the membrane.

H. volcanii FtsY is membrane-associated *in vivo*

To determine whether the membrane interaction detected using purified *H. volcanii* FtsY and IMVs was of physiological relevance, the distribution of FtsY in *H. volcanii* was addressed by subcellular fractionation and immunoblotting with antibodies raised against the *H. volcanii* FtsY NG domain. As shown in Fig. 6A, the antibodies effectively recognized both the heterologously expressed FtsY NG domain (lane 3) and the full length FtsY protein (lane 5). Moreover, the antiserum successfully labeled FtsY in *H. volcanii* cell extracts (lane 6). Immunoblotting of the soluble and membrane portions of the cells revealed FtsY to be present in both fractions (Fig. 6B). To confirm the effectiveness of the subcellular fractionation, each fraction was probed with antibodies raised against marker proteins of known cellular localization [32,42]: the cytoplasmic marker dihydrofolate reductase-1 was restricted to the soluble fraction, while the S-layer glycoprotein, a marker of the cell surface, was restricted to the membrane fraction of the cells.

Experiments were next undertaken to describe the nature of the association of *H. volcanii* FtsY with the membrane. Accordingly, membranes prepared by sonication and isolated by ultracentrifugation were incubated with 6 M urea or 100 mM sodium carbonate and once again collected. Release of bound FtsY was then determined by immunoblotting of the pelleted membrane fraction with anti-FtsY serum. Despite the predicted absence of any membrane-spanning domains, the inability of either urea or sodium carbonate to solubilize membrane-bound *H. volcanii* FtsY suggests that the protein relies on a stronger mode of membrane association than normally employed by peripheral proteins (Fig. 6D).

FtsY, but not the NG domain, mediates membrane association of *H. volcanii* SRP54

To provide information on the sequence of events that occur during SRP-mediated protein targeting in Archaea, the *in vitro* membrane binding behavior of SRP54 in *H. volcanii* was next considered. In such studies, bacterially expressed, purified polyhistidine-tagged *H. volcanii* SRP54 [20] was subjected to the same floatation protocol as FtsY or the FtsY NG domain, described above. As observed with full-length FtsY (Fig. 2A) and the purified FtsY NG domain (Fig. 4A), *H. volcanii* SRP54 was concentrated in the lower fractions of gradients containing the protein alone (Fig. 7A). However, unlike the situation with either FtsY or the FtsY NG domain, preincubation of SRP54 with *H. volcanii* IMVs did not affect the migration of the protein. The failure of SRP54 to bind to the membrane is in agreement with earlier *in vivo* studies addressing the subcellular distribution of SRP54 in *H. volcanii* cells, where the protein was localized to the soluble fraction of the cell [20].

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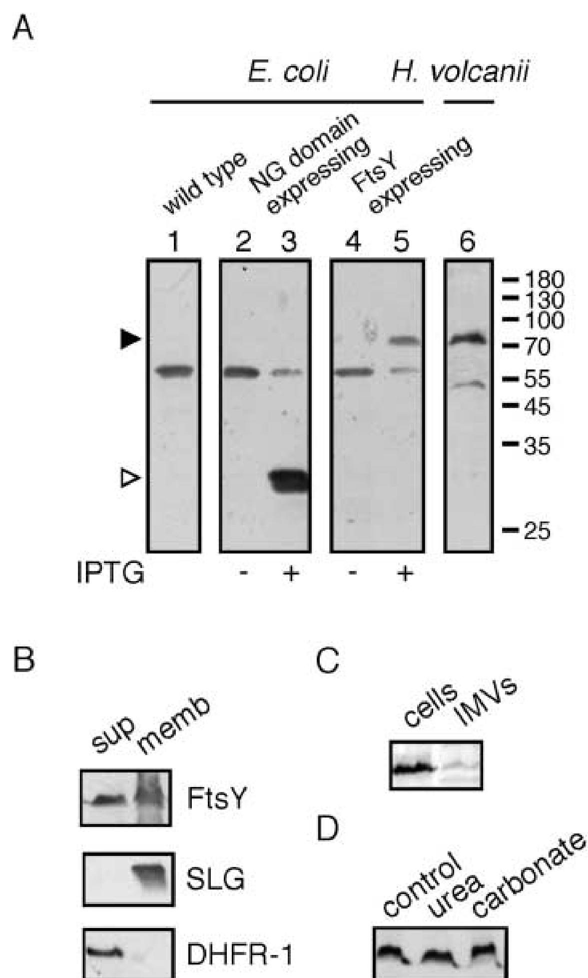

Fig. 6. *H. volcanii* FtsY is associated with the membrane *in vivo*. (A) Antibodies were raised against the *H. volcanii* FtsY NG domain and used for immunoblotting of wild type *E. coli* cells (lane 1), *E. coli* cells transformed to express the *H. volcanii* FtsY NG domain in the absence (lane 2) or presence (lane 3) of isopropyl thio-β-D-galactoside (IPTG), *E. coli* cells transformed to express *H. volcanii* FtsY in the absence (lane 4) or presence (lane 5) of IPTG, or *H. volcanii* cells only (lane 6). The positions of FtsY (▶) and the FtsY NG domain (▷) are shown on the left, while molecular mass markers are shown on the right. (B) *H. volcanii* cells were separated into soluble and membrane fractions and probed with antibodies against FtsY (upper panel), the *H. volcanii* S-layer glycoprotein (SLG; middle panel) or *H. volcanii* dihydrofolate reductase-1 (DHFR-1; lower panel). (C) *H. volcanii* cells and IMVs (20 µg each) were probed with anti-FtsY serum. (D) Isolated *H. volcanii* membranes, in some cases following incubation (1 h on ice) in 6 M urea or 200 mM sodium carbonate in buffer A, were subjected to ultracentrifugation, rinsed in buffer A, once again collected by ultracentrifugation and probed with anti-FtsY serum.

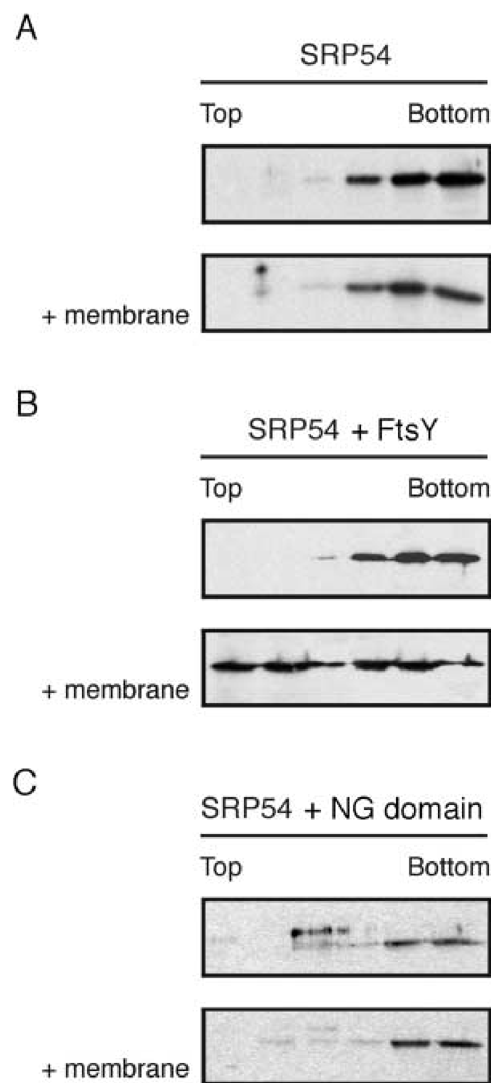

Fig. 7. *H. volcanii* FtsY mediates membrane association of *H. volcanii* SRP54. (A) Purified *H. volcanii* SRP54 (40 µg) was incubated in the absence (upper panel) or presence (lower panel) of *H. volcanii* IMVs and subjected to the same analysis as FtsY, Fig. 2 legend. The gradient fractions were then immunoblotted using polyclonal antibodies raised against *H. volcanii* SRP54 [20]. (B) Purified *H. volcanii* SRP54 (40 µg) was incubated with *H. volcanii* FtsY (80 µg) in the absence (upper panel) or presence (lower panel) of *H. volcanii* IMVs and subjected to analysis as Fig. 4 legend. (C) As in (B), except that the FtsY NG domain was employed in place of FtsY. Antibody binding was visualized by enhanced chemiluminescence.

Next, to determine whether FtsY could mediate membrane association of *H. volcanii* SRP54, SRP54 was premixed with FtsY, incubated with *H. volcanii* IMVs and subjected to floatation. Whereas in the absence of membranes both FtsY and SRP54 remained in the lower portions of the gradient, preincubation of the two proteins with *H. volcanii* IMVs led to cofloatation of both FtsY and SRP to the upper gradient fractions (Fig. 7B). Thus, in *H. volcanii*, SRP54 association with the membrane is mediated through FtsY. Given the membrane-binding behavior of the FtsY NG domain, experiments were carried out to determine whether this FtsY fragment was also capable of mediating SRP54 membrane association. In contrast to the full-length protein, preincubation of the FtsY NG domain with SRP54 did not result in SRP54 binding to the membrane (Fig. 7C).

Discussion

All examined organisms encode for the SRP54 subunit and for FtsY or its eukaryal homologue, SRα, underlying the importance of these elements in protein targeting. In the present report, the membrane binding behavior of SRP54 and FtsY in the halophilic archaea *H. volcanii* was addressed. The results reveal not only the *in vivo* and *in vitro* membrane binding capability of FtsY, but also the ability of the isolated *H. volcanii* FtsY NG domain to interact with *H. volcanii* membranes. The results also reveal that FtsY can serve as the link between the membrane and *H. volcanii* SRP54.

At present, the mode of FtsY binding to the archaeal membrane is not known. The ability of the purified *H. volcanii* FtsY NG domain to specifically interact with membranes, as revealed in the present report by floatation techniques, suggests that this region of the archaeal protein includes a membrane binding site. Indeed, given the relatively low number of positive charges at the start of the A domain of haloarchaeal FtsY proteins, it is not unreasonable to implicate an additional portion of the protein in membrane binding. In *E. coli*, it has also been reported that in addition to the cluster of positive charges found at the start of the N-terminal FtsY A domain, a second region contained within the NG domain of the protein participates in FtsY membrane binding [34,40], although this observation has been questioned [39]. As proteolytic pretreatment of the *H. volcanii* membranes did not prevent membrane binding of either *H. volcanii* FtsY or the FtsY NG domain, it is likely that these interactions are mediated through the lipid phase of the membrane. In *E. coli*, where FtsY–membrane binding has been best-studied, a lipid-mediated mode of FtsY–membrane binding has also been proposed [40], although evidence for the participation of protein–protein interactions in such binding has also been presented [39].

At some stage in the SRP-mediated protein targeting cycle, SRP interacts with its receptor, regardless of the nature of the receptor–membrane association. In Eukarya, the membrane-localized SR binds SRP following the formation of a ribosome–nascent polypeptide–SRP complex. In Bacteria, the order of events leading to the eventual interaction of SRP with FtsY remains an open question [9]. Similarly, the interplay between SRP and FtsY in Archaea has yet to be defined. In a recent paper addressing SRP pathway components in the hyperthermoacidophilic archaea *Acidianus ambivalens*, Moll [43] reported the formation of a soluble SRP54–FtsY complex, yet also described the ability of both SRP54 and FtsY to interact with liposomes prepared from tetraetheric archaeal membrane lipids. In contrast to the situation in *A. ambivalens*, the present report, relying on components prepared from *H. volcanii*, showed interaction of SRP54 with inverted membrane vesicles to be FtsY-dependent. This observation is in agreement with our earlier *in vivo* studies, which failed to detect any membrane-associated SRP54 in *H. volcanii* [20]. Of course, it should be noted that despite having been shown both *in vivo* [44] and *in vitro* [20] to be a component of *H. volcanii* SRP, it still remains to be proven that the membrane-associating behavior of the isolated SRP54 subunit accurately reflects the behavior of the intact ribonucleoprotein particle.

In Archaea, not only the mechanism, but indeed the role of the SRP targeting pathway remains unknown. While SRP has been proposed to be involved in the cotranslational insertion of at least one membrane protein, i.e. bacterioopsin [45,46], it has also been shown that protein secretion [47] and membrane insertion [31] in haloarchaea can occur post-translationally, and hence, presumably independent of the SRP system. In future, the role of the SRP pathway in archaeal protein export will be facilitated in studies employing *H. volcanii* IMVs [31], functional *H. volcanii* ribosomes [48], *H. volcanii* SRP [20,44] and *H. volcanii* FtsY combined in a reconstituted protein targeting and translocation system.

Acknowledgements

This work was supported by the Israel Science Foundation (grant #433/03).

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