Membrane Binding of Ribosomes Occurs at SecYE-based Sites in the Archaea Haloferax volcanii

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Whereas ribosomes bind to membranes at eukaryal Sec61αβγ and bacterial SecYEG sites, ribosomal membrane binding has yet to be studied in Archaea. Accordingly, functional ribosomes and inverted membrane vesicles were prepared from the halophilic archaea Haloferax volcanii. The ability of the ribosomes to bind to the membranes was determined using a flotation approach. Proteolytic pretreatment of the vesicles, as well as quantitative analyses, revealed the existence of a proteinaceous ribosome receptor, with the affinity of binding being comparable to that found in Eukarya and Bacteria. Inverted membrane vesicles prepared from cells expressing chimeras of SecE or SecY fused to a cytoplasmically oriented cellulose-binding domain displayed reduced ribosome binding due to steric hindrance. Pretreatment with cellulose drastically reduced ribosome binding to chimera-containing but not wild-type vesicles. Thus, as in Eukarya and Bacteria, ribosome binding in Archaea occurs at Sec-based sites. However, unlike the situation in the other domains of Life, ribosome binding in haloarchaea requires molar concentrations of salt. Structural information on ribosome–Sec complexes may provide insight into this high salt-dependent binding.

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Introduction

In Bacteria, proteins destined to live beyond the confines of the cytoplasm must first traverse the plasma membrane surrounding the cell. Similarly, eukaryal proteins designated to enter the secretory pathway must cross the topological homologue of the bacterial plasma membrane, i.e. the membrane of the endoplasmic reticulum (ER). While bacterial and eukaryal protein translocation have been addressed extensively,1–4 little is known of how proteins cross the membranes of Archaea, the third domain of Life. At first analysis, the archaeal protein export system appears to correspond to a hybrid of the bacterial and eukaryal translocation systems.5,6 However, closer examination reveals the existence of archaea-specific aspects of the process.7–9 Still, numerous aspects of archaeal protein translocation have yet to be described. In particular, much remains to be learned concerning the relationship between archaeal protein synthesis and protein translocation.

Translocation across the mammalian ER membrane occurs co-translationally. Once a ribosome synthesizing a signal peptide-bearing polypeptide chain has assembled the first ~70 amino acid residues, the nascent chain and, in turn, the ribosome, are recognized by the signal recognition particle (SRP).10 SRP binding subsequently slows or prevents the continuation of further protein synthesis.11 The ternary complex of ribosome–nascent polypeptide chain–SRP then interacts with the ER membrane via the affinities of SRP for the membrane-associated SRP receptor.12,13 but also via the interaction between the ribosome and Sec61αβγ, the core of the translocation complex (the “translocon”).14,15 Once delivered to the translocon, the arrest of protein synthesis mediated by SRP is removed and synthesis of the nascent chain into the translocon, and hence, across the ER membrane, proceeds. It should be noted though that both co- and post-translational protein translocation
have been shown to occur in yeast.\textsuperscript{16,17} Post-translational translocation is the main manner in which secretory proteins cross the bacterial plasma membrane.\textsuperscript{18} Recently, however, SRP-mediated co-translational targeting of bacterial membrane proteins has been demonstrated.\textsuperscript{19} Moreover, the ability of bacterial ribosomes to bind to the bacterial homologue of the Sec61-based translocon, the SecYEG complex, has been shown.\textsuperscript{20,21} Indeed, ribosome binding to Sec complexes in both domains of Life occurs with comparable dissociation constants.

In Archaea, evidence for both post and co-translational protein translocation has been obtained. Such studies have focused largely on halophilic archaea, given the relatively large number of molecular biology and biochemical tools available for working with this archaeal group. In studies involving \textit{Haloferax volcanii} cells transformed to express chimeric proteins bearing the signal peptide of a native exported protein fused to different reporters, it was shown that protein synthesis and secretion need not be coupled events.\textsuperscript{22} In contrast, kinetic radiolabelling approaches revealed the co-translational insertion of the N-terminal portion of the \textit{Halobacterium salinarum} membrane protein bacterioopsin.\textsuperscript{23} Moreover, earlier studies reporting co-sedimentation of SRP (7 S) RNA and bacterioopsin mRNA with membrane-bound polysomes, together with puromycin-induced release of the SRP RNA from the polysomes,\textsuperscript{24} as well as more recent studies addressing SRP in \textit{H. volcanii}\textsuperscript{25,26} and other archaeal species,\textsuperscript{27–31} lend further support for the existence of a co-translational mode of protein translocation in Archaea. Direct demonstration of an interaction between ribosomes and the archaeal translocation complex, a central step in a co-translational mode of translocation, remains, however, to be presented.

Thus, to address the relation between protein translation and translocation in Archaea, the interaction between archaeal ribosomes and inverted membrane vesicles, both prepared from \textit{H. volcanii}, is considered here. As such, results showing that membrane binding of \textit{H. volcanii} ribosomes occurs at proteinaceous sites containing SecY and SecE are presented, as is the first quantitative examination of ribosome membrane binding in Archaea. These findings suggest the evolutionarily conserved nature of ribosome–Sec complex binding in protein translocation. Moreover, this study reveals the ability of ribosomes to bind to SecYE sites in the presence of molar concentrations of salt, as found in the cytoplasm of halophilic archaea.\textsuperscript{32,33}

**Results**

**Purification of functional ribosomes from \textit{H. volcanii}**

As a first step towards describing the putative interaction between ribosomes and the plasma membrane in Archaea, ribosomes were purified from the cytosol of \textit{H. volcanii} cells relying on protocols previously used for purification of ribosomes from other haloarchaea such as \textit{H. salinarum},\textsuperscript{34} \textit{Haloarcula marismortui},\textsuperscript{35} \textit{Halobacterium cutirubrum},\textsuperscript{36} and \textit{Haloferax mediterranei}.\textsuperscript{37} In our hands, 20 \textit{A}\textsubscript{260} (500 pmol) units of 70 S ribosomes were isolated from 1 g of wet \textit{H. volcanii} cells. Portions of the purified \textit{H. volcanii} 70 S ribosomes were then dissociated and separated into their constituent 50 S large and 30 S small subunits by centrifugation in sucrose density gradients (Figure 1A). The migration pattern obtained was similar to that realized in the purification of the large and small subunits of other archaeal ribosomes.\textsuperscript{37,38} With purified 70 S ribosomes as well as 50 S and 30 S subunits available, the functional behaviour

**Figure 1.** Purification of active \textit{H. volcanii} ribosomes. A, \textit{H. volcanii} ribosomes were purified as described in Materials and Methods. The purified 70 S ribosomes were dissociated and separated by sucrose gradient centrifugation. Fractions were collected and \textit{A}\textsubscript{260} measured to detect the presence of 30 S and 50 S subunits. The 30 S marker represents the position of the small subunit, while the 50 S marker represents the position of the large subunit. B, Poly(U)-directed polyphenylalanine synthesis was performed as described,\textsuperscript{34} employing intact 70 S ribosomes (24 pmol), combined individual 50 S (18 pmol) and 30 S (36 pmol) subunits, 50 S or 30 S subunits alone or in the case of control samples, no ribosomal material. The measured \textsuperscript{14}C radioactivity (1 x 10\textsuperscript{5} cpm/pmol in the stock solution), reflecting polyphenylalanine polymerization, as well as the amount of polyphenylalanine polymerized, are presented for each case.
of the preparations was tested in a cell-free poly-peptide-synthesizing assay designed to examine the poly(U)-directed assembly of [14C]phenylalanine chains. As presented in Figure 1B, neither the 50 S nor the 30 S preparations synthesized peptides incorporating the radiolabelled amino acid at levels much higher than realized in control assays in which neither ribosomes nor ribosome-derived materials was included. In contrast, levels of [14C]phenylalanine biogenesis 40–50-fold higher than obtained in control experiments were obtained when either the 70 S intact ribosomes were tested or when the separate 50 S and 30 S subunits were combined and added to the reaction mixture. Hence, it could be concluded that the purified H. volcanii ribosomes were translationally active.

H. volcanii ribosomes bind to H. volcanii IMVs in a salt-dependent manner

With both active H. volcanii ribosomes and biologically functional H. volcanii IMVs available, the ability of the ribosomes to interact with the plasma membrane was addressed next. In preliminary studies, ribosomal markers were identified that, in subsequent experiments, could serve to distinguish the ribosomes from the IMVs when the two would be combined. Figure 2A reveals that polyclonal antibodies raised against the L11 and L12 proteins of H. marismortui ribosomes effectively recognized the homologous proteins in the H. volcanii ribosomes. In contrast, no such protein bands were recognized in an H. volcanii IMV preparation, confirming that the IMVs did not contain bound ribosomes or any cross-reactive proteins. Similarly, 23 S, 16 S and 5 S RNA species could be extracted only from the ribosomes and not from the membrane vesicles (Figure 2B). Finally, the ability to separate the 70 S purified ribosomes into separate pools of large 50 S subunits and small 30 S subunits was confirmed by demonstrating the presence of 23 S and 5 S ribosomal RNA only in the 50 S subunit pool and of 16 S ribosomal RNA solely in the 30 S subunit pool (Figure 2C).

Having defined markers of the H. volcanii ribosomes, the ability of the ribosomes to bind to H. volcanii IMVs was tested in a flotation protocol based on centrifugation of preincubated ribosome–IMV mixtures in sucrose density gradients. Following centrifugation, six fractions were collected from the top of the gradients downwards

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**Figure 2.** Immunoblotting of and RNA extraction from H. volcanii ribosomes and IMVs. A, Aliquots of H. volcanii ribosomes and IMVs (3 A260 units and 20 μg of protein, respectively) were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies raised against H. marismortui ribosome large subunit L11 and L12 proteins. B, RNA was extracted from aliquots of H. volcanii ribosomes and IMVs and separated on denaturing 1.5% (w/v) agarose gels. Lanes 1 and 4, 1.7 A260 units (40 pmol) of RNA; lanes 2 and 5, 3.4 A260 units (80 pmol) of RNA; lanes 3 and 6, 5.1 A260 units (120 pmol) of RNA. The positions of 23 S, 16 S and 5 S RNA are shown. C, Separation of H. volcanii ribosomal large (50 S) and small (30 S) subunits. The RNA content of each subunit was extracted to confirm the efficiency of separation. In the left lane (50 S), six A260 units (200 pmol) of RNA were applied to the gel, while in the right lane (30 S), 1.5 A260 units (60 pmol) of RNA were applied.
and the position of the ribosomes was revealed by determining the distribution of \textit{H. volcanii} ribosomal protein or RNA markers. When intact ribosomes were subjected to flotation on their own, subsequent immunoblotting of the gradient fractions with anti-L11 antibodies revealed the failure of the ribosomes to float, instead remaining at the base of the ultracentrifuge tubes (Figure 3A). Identical behaviour was revealed using anti-L12 antibodies as well as by analysing the distribution of ribosomal 23 S RNA. In contrast, following prior incubation with \textit{H. volcanii} IMVs, the distribution of all three ribosomal markers revealed that the ribosomes had floated to the top of the gradients, concentrating in the uppermost two gradient fractions, due to interaction with the floated vesicles found in the same fractions.

To determine whether membrane binding of the ribosomes was mediated through the large 50 S subunit or via the small 30 S subunit, the ability of each separated subunit to interact with \textit{H. volcanii} IMVs was tested. Flotation of the large subunit revealed behaviour identical with that obtained using the intact ribosomes (Figure 3B). In contrast, no binding of the small subunit was obtained, as revealed upon extraction of 16 S RNA from the gradient fractions (Figure 3C). Similarly, no ribosome flotation was achieved when right-side-out vesicles were employed (Figure 3D), reflecting the necessity of inverted vesicle topology for ribosomes binding.

Considering the high salt content of the halophilic archaeal cytoplasm,\textsuperscript{32,33} experiments designed to study the salt-dependence of the ribosome binding to \textit{H. volcanii} membranes were undertaken. In these studies, \textit{H. volcanii} ribosomes were suspended in buffer containing 0–3.4 M KCl, incubated with \textit{H. volcanii} IMVs and subjected to flotation on a gradient prepared with the same concentration of KCl as was found with the ribosomes, in a given experiment. As shown in Figure 4A, when flotation was performed from 0 M to

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**Figure 3.** \textit{H. volcanii} ribosomes bind to \textit{H. volcanii} IMVs via the 50 S particle. A, \textit{H. volcanii} ribosomes were subjected to flotation in the absence or presence of IMVs, as described in Materials and Methods. The position of proteins L11, L12 and 23 S RNA in each case is shown. B, The experiment described in A was repeated, using separated 50 S subunits. C, The experiment described in A was repeated, using separated 30 S subunits. Here, the position of 16 S RNA following flotation in the absence or presence of IMVs is shown. D, Ribosomes were subjected to flotation in the presence of IMVs or right-side-out vesicles (RSOs). In each case, the position of protein L11 is shown.
1.5 M KCl, no ribosome flotation was observed. In the presence of 2.0 M or 2.5 M KCl, a major portion of the ribosomes was able to bind to the IMVs and float to the upper fractions of the gradient. From the 3.0 M KCl level, the total pool of ribosomes was found to be bound to the IMVs. To discount the possibility that the failure of the ribosomes to float in low-salt solutions is not due to a loss of ribosome-binding ability. H. volcanii ribosomes bind to protein sites in the membrane

**Figure 4**. H. volcanii ribosomes bind to H. volcanii IMVs in a salt-dependent manner. A, H. volcanii ribosomes in buffer containing 0–3.4 M KCl were preincubated with IMVs and subjected to flotation on gradients containing the same concentration of KCl. Gradient fractions were subjected to immunoblotting using antibodies against L11. B, The position of H. volcanii IMVs in gradients containing 0–3.4 M KCl was determined by immunoblotting gradient fractions with antibodies to the H. volcanii S-layer glycoprotein. The upper panel shows a gradient (prepared in 1.0 M KCl) representative of gradients prepared in 0–1.5 M KCl, while the lower panel shows a gradient (prepared in 3.0 M KCl) representative of gradients prepared in 2.0–3.4 M KCl.

**Figure 5**. Proteolytically-digested H. volcanii IMVs no longer bind ribosomes. A, H. volcanii ribosomes were subjected to flotation using IMVs pre-treated (four hours, 40 °C), in the presence or in the absence of proteinase K (1 mg/ml). The positions of proteins L11 and L12 are depicted. B, The experiment described in A was repeated using separated 50 S subunits. C, The experiment described in A was repeated and the level of H. volcanii S-layer glycoprotein was determined. In some cases, the IMVs were first treated with 1% (v/v) Triton X-100 (Tx-100) prior to proteolytic digestion.

failure of the IMVs to float but rather due to a lack of ribosome-binding ability.

**H. volcanii** ribosomes bind to protein sites in the membrane

Characterization of the H. volcanii membrane
ribosome-binding site was next undertaken. In the first of these experiments, the IMVs were digested with proteinase K prior to incubation with *H. volcanii* ribosomes. Whereas the ribosomes migrated to the top of the gradients when non-proteolyzed control membranes (also incubated at 40 °C for four hours, as were the protease-exposed IMVs) were employed, immunoblotting with anti-L11 and anti-L12 antibodies revealed that pre-treatment with proteinase K eliminated essentially all ribosome binding (Figure 5A). Identical results were obtained when the effects of proteolytic pre-treatment of the membranes on large subunit membrane binding were considered (Figure 5B). To confirm that the loss of ribosome-binding capacity was not the result of unrestricted, protease-mediated destruction of the membranes, control experiments were performed to confirm that IMV integrity had been maintained. In these studies, the behaviour of the S-layer glycoprotein, the sole protein component of the surface layer surrounding *H. volcanii* cells,40 was tested immunologically using antibodies raised against the first 13 amino acid residues of the protein.41 In the inverted membranes, the S-layer glycoprotein is oriented towards the vesicle lumen and thus protected from externally-added protease.39 Indeed, proteolytic treatment had no effect on the level or distribution of the internally oriented S-layer glycoprotein present in fractions collected following flotation (Figure 5C). Hence, the treatment with proteinase K that led to a loss in *H. volcanii* ribosome binding did not disrupt IMV integrity. To confirm that the conditions of proteolysis were, however, adequate for digestion of the S-layer glycoprotein, given protease accessibility, control experiments were performed in which membrane integrity was destroyed by including 1% Triton X-100 with the protease. Under such conditions, the S-layer glycoprotein was degraded completely. Finally, to ensure that no residual protease had compromised ribosome integrity, thereby preventing effective binding, ribosomes that had failed to bind to the proteolytically treated IMVs were subjected to a second round of flotation, this time using control IMVs. As expected, effective flotation was achieved (not shown).

Quantitative analysis revealed the saturable nature of the binding of *H. volcanii* ribosomes to the IMVs (Figure 6). Analysis of the binding revealed that ribosome binding occurred with an apparent binding affinity (K_d) of 14.6 nM and a binding-site density (B_max) of 28.5 nM. This value is comparable to those obtained in studies addressing ribosomal binding to eukaryal and bacterial membrane vesicles and purified Sec complexes.15,20,21,42

**H. volcanii** ribosomes bind at SecYE sites

Having confirmed the existence of proteinaceous ribosome-binding sites in the IMVs, experiments designed to identify the *H. volcanii* ribosomal membrane receptor were undertaken. Given that the Sec61αβγ and SecYEG protein translocation complexes have been shown to serve as ribosome receptors in ER and bacterial plasma membranes, respectively,14,15,20,21 it was hypothesized that a measuring the radioactivity found in the bottom 400 μl aliquots from the beginning of binding saturation. This radioactivity, originating from dissociated 30 S subunits and/or ribosome fragments, was subtracted from the difference between the total radioactivity applied to the gradient and the measured radioactivity bound, yielding the radioactivity associated with unbound, intact ribosomes (i.e. free ribosomes). In the graph, the data shown represent the average of three separate experiments. The standard deviation of each point was less than 5%. The inset presents the results of the binding experiment according to Scatchard analysis. Curve-fitting was achieved using MacCurveFit.
membrane-embedded Sec complex serves a similar function in Archaea. Thus, to determine whether *H. volcanii* ribosomes bind to membranes at Sec-based sites, IMVs were prepared from *H. volcanii* cells transformed to express chimeras of either SecE or SecY fused to the 17 kDa cellulose-binding domain (CBD) of the *Clostridium thermocellum* cellulose. Earlier studies have shown these chimeras to be expressed stably and localized exclusively to the membranes of the transformed haloarchaeal cells. In such IMVs, the presence of the CBD moiety, fused to the cytoplasmically facing N terminus of *H. volcanii* SecE and SecY, would be expected to present a steric hindrance to any ribosomal binding at sites incorporating the CBD-SecE or CBD-SecY chimeras.

In a series of control experiments, IMVs were prepared from CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells and characterized. The inverted nature of the IMVs was determined by addressing the orientation of menadione-dependent NADH dehydrogenase, a membrane protein associated with the inner face of the *H. volcanii* membrane accessible only upon membrane disruption, such as upon treatment with Triton X-100. In IMVs, however, the enzymatic activity is readily detectable from the medium, and as such can serve as a reporter of the topology of *H. volcanii* membrane vesicles. In IMVs prepared from wild-type *H. volcanii* cells, 89% of the normally inward-facing enzyme activity was accessible (Table 1), in agreement with earlier studies. In the case of CBD-SecE- and CBD-SecY-containing IMV preparations, 79% and 90% of the menadione-dependent NADH dehydrogenase activity now faced the exterior, respectively. However, since the normally sequestered enzyme would be accessible to added substrate in membrane fragments as well as IMVs, the sealed nature of the vesicles was tested. To do so, protease accessibility of the normally outward-facing S-layer glycoprotein was addressed. Whereas the S-layer glycoprotein is digested readily in intact *H. volcanii* cells, the glycoprotein is not accessible to added protease in IMVs. In agreement with these earlier studies, 88% of the starting level of S-layer glycoprotein in IMVs prepared from wild-type *H. volcanii* cells survived treatment with proteinase K (Table 1). However, upon detergent-mediated disruption of membrane integrity, only 18% of the S-layer glycoprotein survived the proteolytic treatment. When similar studies were performed on CBD-SecE- and CBD-SecY-incorporating vesicles, 78% and 81% of the starting amount of S-layer glycoprotein was protected from the added protease, respectively.

The ability of *H. volcanii* IMVs prepared from wild-type, CBD-SecE- and CBD-SecY-expressing cells was next determined, employing the flotation assay described above, following control experiments designed to confirm the ability of all three vesicle types to float equally well (not shown). Whereas ribosomes floated readily with wild-type IMVs, the presence of either CBD-SecE or CBD-SecY decreased significantly the amount of ribosomes bound to the membrane, as reflected in immunoblots using anti-L11 antibodies (Figure 7A). Similar results were obtained in immunoblots using anti-L12 antibodies (not shown). Quantification of ribosomal binding to the wild-type, CBD-SecE and CBD-SecY IMVs by densitometric analysis of the immunoblots obtained using anti L11-antibodies revealed that the presence of the CBD moiety reduced binding by 73% when fused to SecE and by 84% when fused to SecY, calculated by determining the proportion of ribosomes applied to the gradient and then found in the top two gradient fractions following flotation (Figure 7B).

The decreased ability of ribosomes to bind to IMVs prepared from CBD-SecY- or CBD-SecE-expressing cells could be due to either reduced levels of endogenous SecY and SecE in the transformed cells or to the presence of mixed oligomers of chimeric and endogenous SecYE proteins. Since several observations, such as the similar patterns of cell growth and comparable secretion of the S-layer glycoprotein (the major extracellular protein of *H. volcanii*) in wild-type, CBD-SecY- and CBD-SecE-expressing cells (not shown), argue against a decreased level of endogenous SecYE in the transformed cells, it is likely that CBD-SecY and CBD-SecE exist in complexes together with

### Table 1. Characterization of IMVs from wild-type, CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells

<table>
<thead>
<tr>
<th>Assay</th>
<th>Wild-type</th>
<th>CBD-SecE</th>
<th>CBD-SecY</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Menadione-dependent NADH dehydrogenase activity</strong>a</td>
<td>IMVs</td>
<td>89.1 (2.8)</td>
<td>79.2 (2.8)</td>
</tr>
<tr>
<td></td>
<td>RSOsb</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>S-layer glycoprotein protection</strong>c</td>
<td>IMVs</td>
<td>87.9</td>
<td>78.4</td>
</tr>
<tr>
<td></td>
<td>RSOb</td>
<td>19.5</td>
<td>11.7</td>
</tr>
</tbody>
</table>

a Percentages of the total activity, obtained following preincubation with 0.01% Triton X-100, are given. The values reported represent the average of two separate experiments. In all cases, values differed by less than 1%. The values in parentheses correspond to the specific activity of the average of two experiments, given as ΔA550/minute per 1 mg of protein.

b Right-side-out vesicles (RSOs) were obtained from the gradients used to obtain IMVs.

c Percentages of the starting amount of S-layer glycoprotein remaining following four hours exposure to proteinase K are given. The values reported represent the average of two separate experiments. In all cases, values differed by less than 1%.
endogenously expressed SecYE proteins. The stability of CBD-SecY in the transformed haloarchaeal cells supports this hypothesis, since it has been shown that uncomplexed bacterial SecY is degraded rapidly. Not all Sec-based ribosome-binding sites, however, include a chimeric component, as 20–30% of the ribosome binding obtained with wild-type IMVs was detected in CBD-SecY- and CBD-SecE-containing vesicles (Figure 7B).

Finally, the ability of cellulose to interact with the CBD moiety fused to either SecE or SecY in a salt-independent manner was exploited. Whereas cellulose pretreatment had no effect on ribosomal binding to wild-type IMVs, incubation of the CBD-SecE- or CBD-SecY-containing H. volcanii IMV preparations with cellulose prior to ribosome binding and flotation served to completely block the access of the ribosomes to binding sites in the membrane (Figure 7C). The cellulose treatment, however, had no effect on IMV flotation (not shown). Collectively, these experiments offer strong evidence that SecE and SecY contribute to the ribosome-binding site in H. volcanii membranes.

Discussion

At the onset of co-translational protein translocation in Eukarya, ribosomes in the process of synthesizing signal peptide-bearing nascent polypeptides are recognized by SRP and delivered to the ER membrane through the affinities of SRP for its receptor and of the ribosome for the Sec61 complex. Similarly, bacterial ribosomes have been shown to specifically bind to SecYEG complexes, although the physiological significance of this finding remains the subject of investigation. In Archaea, the nature of the ribosomal receptor had, until now, not been defined. In part, this shortcoming could be attributed to several obstacles. First, the molecular composition, subunit stoichiometry and oligomeric status of the archaeal translocation complex are unknown, although genomic and single-gene analyses have revealed the existence of archaeal versions of eukaryal Sec61ag/bacterial YE, eukaryal Sec61b and bacterial SecDF. Secondly, the well-characterized, genetically modified strains used to describe the bacterial ribosome-binding site do not exist in Archaea. Thirdly, studies exploiting antibodies raised against translocon components to delineate the ribosome–translocon interaction are not feasible with halophilic archaea such as H. volcanii, given that the molar concentrations of salt required for the study of biochemical and cellular processes in this species would interfere with antibody binding. Finally, until recently, no reliable protocol for large-scale preparation of archaeal IMVs was available. Now, relying on ribosomes and IMVs prepared from H. volcanii cells, membrane interaction of archaeal ribosomes, mediated through the large subunit, has been shown. Moreover, whereas earlier studies had proposed that membrane binding of archaeal polysomes to the membrane occurs via ribosome-linked nascent polypeptide chains, the present report reveals, for the first time, binding of archaeal ribosomes to proteinaceous sites in the archaeal membrane.
Employing IMVs prepared from cells transformed to express chimeric CBD-SecE or CBD-SecY, binding of the archaeal ribosomes at SecYE-based sites was, presumably, shown. While it is conceivable that the presence of the CBD moiety blocked access, and hence binding, of the ribosomes to other membrane proteins, consideration of the proposed sizes of the Sec complex and the CBD moiety argue against this scenario. Moreover, the fact that the measured binding affinity of the haloarchaeal ribosomes was similar to that obtained for eukaryal and bacterial ribosome–Sec complex interactions further supports the idea that the Sec complex serves as the site of ribosome binding in H. volcanii. As such, the ability of ribosomes to bind to the Sec complex is universally conserved, occurring in Eukarya and Bacteria, and in Archaea.

The binding of ribosomes to translocation complexes appears to be a dynamic process, given that the conditions needed for release of ribosomes bound to the ER membrane at Sec61 sites change during the course of nascent polypeptide translocation, as addressed using in vitro translation/translocation systems. To disrupt the association between translating ribosomes and the Sec61 complex into which the nascent chain is fed, both puromycin and high salt are required. However, for those ribosomes bound at Sec61 sites during the early phases of the translocation event, or for Sec61-bound non-translating ribosomes, high-salt washes alone are sufficient for release from the membrane. Similarly, binding of non-translating bacterial ribosomes to SecYEG was not possible at concentrations of salt above 500 mM. As such, it is striking that a salt-dependent interaction between non-translating functional H. volcanii ribosomes and membranous SecYE-based sites could be demonstrated here. Indeed, should such ribosome binding to the haloarchaeal membrane occur in vivo, then these interactions would take place in concentrations of salt as high as 5 M, as reportedly found in haloarchaeal cytoplasm.

The basis for the ability of H. volcanii ribosomes to remain membrane-bound at elevated concentrations of salt is not known. Although H. volcanii ribosomes bind to IMVs with a dissociation constant similar to that detected in eukaryal and bacterial ribosome-binding studies, the nature of the contact site between the ribosome and the Sec complex could, conceivably, explain the observed high salt-requiring binding. Whereas biochemical approaches had suggested the existence of a tight seal between the two, a series of more recent structural studies of ribosome–Sec61 complexes suggests such interaction to be mediated by only four points of contact. It is thought that both protein and RNA components of the large subunit contribute to the ribosomal portion of these connections, although 28 S and 23 S ribosomal RNA bound to eukaryal and bacterial Sec complexes, respectively, with affinities similar to intact ribosomes. Examination of the regions of the large subunit proposed to comprise the points of connection reveals that the overall arrangement of these sites is conserved in Eukarya, Bacteria and Archaea.

### Table 2. Alignment of Saccharomyces cerevisiae and H. marismortui ribosome protein sequences proposed to mediate connection with the Sec complex

<table>
<thead>
<tr>
<th>Contact site</th>
<th>Protein</th>
<th>Sequence*</th>
</tr>
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<tbody>
<tr>
<td>C1</td>
<td>S. cerevisiae (L19)</td>
<td>PNETSEIAQANSRNNAIRKLKNGT</td>
</tr>
<tr>
<td></td>
<td>H. marismortui (L19)</td>
<td>PERQGIDAIATREDVRELVDEGA</td>
</tr>
<tr>
<td>C2</td>
<td>S. cerevisiae (L25)</td>
<td>SETAMKKEVDEGNI</td>
</tr>
<tr>
<td></td>
<td>H. marismortui (L23)</td>
<td>TEKAMNDDMFQNK</td>
</tr>
<tr>
<td>C3</td>
<td>S. cerevisiae (L24)</td>
<td>TKEKYNCGAVP</td>
</tr>
<tr>
<td></td>
<td>H. marismortui (L26)</td>
<td>TLEKTDGEVVP</td>
</tr>
<tr>
<td>C4</td>
<td>S. cerevisiae (L35)</td>
<td>AGVK</td>
</tr>
<tr>
<td></td>
<td>H. marismortui (L29)</td>
<td>TVLH</td>
</tr>
<tr>
<td></td>
<td>S. cerevisiae (L35)</td>
<td>KKELAELKVQKL5RPSLP-KIKTVKR</td>
</tr>
<tr>
<td></td>
<td>H. marismortui (L29)</td>
<td>KTELINARAVQAAGGAPENPRKELR</td>
</tr>
</tbody>
</table>

* As reported by Beckmann et al. Acidic amino acid residues are in bold. Alignment was performed using ClustalW v.1.8 (at http://clustalw.genome.ad.jp).
the *H. volcanii* ribosomes to bind to SecYE-based complexes in the presence of high salt. In terms of the profile of the membrane contribution to the site of interaction with the ribosome, binding in Eukarya has been shown to involve the Sec61α cytoplasmic loops 6 and 8, although Sec61β has been implicated in this binding by some, but not others. Examination of the amino acid sequence of *H. volcanii* SecY reveals, however, that the homologous regions of the haloarchaeal SecY are 41% identical (71% similar) and 34% identical (57% similar) to canine Sec61α cytoplasmic loops 6 and 8, respectively.

It is possible that the high salt binding observed in *H. volcanii* could be mediated by archaeal translocation complex subunits other than the core SecYE components or the recently proposed archaeal homologue of Sec61β, given the overall similarity of these elements to their eukaryal homologues. In the ER, proteins other than the Sec61 complex, including p180 and p34, have been implicated in ribosomal membrane-binding, although the involvement of these proteins has been questioned. Translocon-associated protein (TRAP) and oligosaccharide transferase (OST) have been proposed to contribute to the eukaryal ribosome-binding site. BLAST searches of completed genome sequences predict the existence of archaeal proteins resembling p180 to various extents, although in most cases, the function of these proteins is unknown. No archaeal homologues of either p34 or TRAP was detected, while little is known of the components involved in archaeal protein glycosylation. The existence of currently unidentified, uniquely archaeal translocon components involved in ribosome binding remains a possibility.

Alternatively, the membrane-associating ability of non-translating ribosomes could be regulated physiologically, even in high-salt conditions. In Eukarya and Bacteria, such considerations have been shown to modulate ribosome association with membranes. In yeast, the presence of Sec62/63 complex prevented ribosome binding to the Sec61 complex. Most recently, it was reported that in *Escherichia coli*, SecA acts to dissociate translocon-bound translationally inactive ribosomes. However, given the apparent absence of an archaeal SecA homologue, it is difficult to imagine a similar mechanism at play in Archaea. It is tempting to speculate, however, that ribosomal membrane-binding in Archaea could be modulated by the translation status of the ribosome or by the characteristic sequence of the nascent polypeptide being translated. Accordingly, in *E. coli* cells depleted for Ffh, the SRP54 homologue, ribosomes remain membrane-associated in a puromycin-resistant and high salt-resistant manner, suggesting a role for SRP in modulating the nature of ribosomal binding to the membrane. In the eukaryal system, it has been shown that non-translating ribosomes bind the SRP receptor with similar affinity as the Sec61 complex. The universal nature of the SRP pathway raises the possibility that this system could also modulate ribosome–membrane interactions in Archaea.

The finding that *H. volcanii* ribosomes are capable of binding to the inner surface of the *H. volcanii* membrane at translocation sites offers insight into the archaeal protein translocation process. For example, the observation of ribosomal binding at Sec-based sites in Archaea lends support to earlier reports describing the co-translational insertion of the N-terminal portion of *H. salinarum* bacterioopsin. Still, deciphering the relationship between protein translation and translocation in Archaea would greatly benefit from the availability of an *in vitro* system capable of performing these activities. While the availability of *H. volcanii* IMVs and reconstituted *H. volcanii* SRP suggests that it will soon be possible to reconstruct the translocation event, efforts at developing a haloarchaeal *in vitro* protein translocation system have met with only limited success. However, the availability of an *in vitro* translation system from the thermoacidophilic archaea *Sulfolobus solfataricus* offers hope that a homologous haloarchaeal system will one day be developed.

### Materials and Methods

#### Materials

- Aluminium oxide, ATP, diethylpyrocarbonate, GTP, 2-mercaptoethanol, phospho(enol)pyruvate, and polyuridylic acid were obtained from Sigma (St. Louis, MO). Brewer’s yeast tRNA, pyruvate kinase, and RNase-free DNase came from Roche (Mannheim, Germany). Sepharacryl 200 superfine resin, molecular mass markers and horseradish peroxidase conjugated goat anti-rabbit antibodies were purchased from BioRad (Hercules, CA).
- An ECL enhanced chemiluminescence kit was obtained from Amersham (Buckingham, UK), as were [*14C]*phenylalanine (50 μCi/ml) and [*125I]*Na (100 mCi/ml). Radio-labelling with [*25I] was performed essentially as described.

#### Purification of *H. volcanii* ribosomes

*H. volcanii* ribosomes were prepared essentially as described. *H. volcanii* cells were grown at 40 °C to mid-exponential phase, to a concentration of 3 g of cells per litre. The cells were harvested, frozen in liquid nitrogen and stored at −80 °C. For ribosome extraction, the cells were ground four times (two minutes each time) at 4 °C in a pre-cooled mortar with twice their weight of alumina. The resulting paste was diluted with two volumes (2 ml per 1 g of wet cells) of buffer AR (3.4 M KCl, 100 mM MgCl₂, 6 mM 2-mercaptoethanol, 10 mM Tris–HCl, pH 7.6) and treated with 1 μg/ml of RNase-free DNase (30 minutes, 4 °C). The aluminium oxide and cell debris were removed by centrifugation (27,000g, 15 minutes). The supernatant was then subjected to a second centrifugation (65,000g, 40 minutes), collected and ultracentrifuged for five hours at 255,000g in a Beckman Ti60 rotor to pellet the 70 S ribosomes. The supernatant, referred to as S100,
was stored at −80°C in aliquots. The pellet was first rinsed and then resuspended with buffer AR. After a low-speed centrifugation, the 70 S ribosomes were frozen rapidly in liquid nitrogen and stored in aliquots at −80°C.

For the isolation of *H. volcanii* ribosomal 50 S and 30 S subunits, the 70 S ribosomes were dialyzed against dissociation buffer D10 (2.7 M KCl, 0.45 M NH₄Cl, 10 mM MgCl₂, 6 mM 2-mercaptoethanol, 20 mM Tris–HCl, pH 7.6). The dissociated ribosomes were then loaded onto a linear sucrose gradient containing 15–30% (w/v) sucrose prepared in buffer D10 and centrifuged in a Beckman SW-27 rotor (14 hours, 18,000 rpm, 4°C). Fractions (0.5 ml) were collected and tested for the presence of 30 S or 50 S particles by measuring absorbance at A₂₆₀. Fractions containing the 30 S or 50 S subunits were pooled, centrifuged (Ti60 Beckman rotor, 16 hours, 50,000 rpm, 4°C) and resuspended in a small volume of buffer D10. Sucrose was next removed by gel-filtration using Sephacryl 200 superfine resin (40 cm × 1 cm column) equilibrated with buffer D10. The sucrose-free subunits eluted from the column were concentrated by centrifugation (Beckman Ti60 rotor, 16 hours, 50,000 rpm, 4°C) and resuspended in buffer D30 (2.7 M KCl, 0.45 M NH₄Cl, 30 mM MgCl₂, 6 mM 2-mercaptoethanol, 20 mM Tris–HCl, pH 7.6). The separated subunits were then overlaid with 400 μl of buffer F4 (1.5 M sucrose in buffer AR) and transferred to the bottom of a Sorvall Discovery M120 ultracentrifuge S120AT2 rotor tube. The samples were then overlaid with 400 μl of buffer F3 (1.5 M sucrose in buffer AR) and 270 μl of buffer AR. After ultracentrifugation (100,000 rpm, two hours, 4°C), the upper (400 μl), middle (200 μl), and lower (400 μl), phases of each gradient were collected and the radioactivity was measured using a DPC γ-C12 counter.

### RNA extraction

RNA extraction was performed essentially as described, but with slight modifications. 100 μl samples were mixed with 20 μl of 10 mg/ml proteinase K, 20 μl of buffer SE (10% (w/v) SDS, 0.2 M EDTA–KOH, pH 8.0) and 60 μl of diethylpyrocarbonate-treated water, and incubated at 40°C for 30 minutes. Two phenol-extractions were performed to remove residual peptides, with back extractions of the phenol phases performed with buffer TE (1 mM EDTA, 10 mM Tris–HCl, pH 7.5). After extraction with chloroform/isopropanol (49:1, v/v), 0.2 vol. of 5.5 M NH₄Cl (pH 6) and 2.5 vol. of ethanol were added. Following incubation at −80°C for 30 minutes, the RNA in the samples was precipitated by centrifugation (13,000 rpm, three minutes) and washed with 100 μl of cold 70% (v/v) ethanol. The RNA pellet was dried and dissolved in diethylpyrocarbonate-treated water. After addition of loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 95% (v/v) formamide, 20 mM EDTA–KOH, pH 8.0), the samples were examined by electrophoresis on denaturing 1.5% agarose gels.

### Other methods

Protein concentration was determined with Bradford reagent (BioRad, Hercules CA), using bovine serum albumin as a standard. Antibody binding was detected using goat anti-rabbit horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence using an ECL kit. Densitometry was performed using IPLab Gel software (Signal Analytics, Vienna VI). Curve-fitting was achieved using MacCurveFit.

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