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Early targeting events during membrane protein biogenesis in *Escherichia coli*☆

Eitan Bibi *

Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT

All living cells have co-translational pathways for targeting membrane proteins. Co-translation pathways for secretory proteins also exist but mostly in eukaryotes. Unlike secretory proteins, the biosynthetic pathway of most membrane proteins is conserved through evolution and these proteins are usually synthesized by membrane-bound ribosomes. Translation on the membrane requires that both the ribosomes and the mRNAs be properly localized. Theoretically, this can be achieved by several means. (i) The current view is that the targeting of cytosolic mRNA-ribosome-nascent chain complexes (RNCs) to the membrane is initiated by information in the emerging hydrophobic nascent polypeptides. (ii) The alternative model suggests that ribosomes may be targeted to the membrane also constitutively, whereas the appropriate mRNAs may be carried on small ribosomal subunits or targeted by other cellular factors to the membrane-bound ribosomes. Importantly, the available experimental data do not rule out the possibility that cells may also utilize both pathways in parallel. In any case, it is well documented that a major player in the targeting pathway is the signal recognition particle (SRP) system composed of the SRP and its receptor (SR). Although the functional core of the SRP system is evolutionarily conserved, its composition and biological practice come with different flavors in various organisms. This review is dedicated mainly to the *Escherichia* (*E.*) *coli* SRP, where the biochemical and structural properties of components of the SRP system have been relatively characterized, yielding essential information about various aspects of the pathway. In addition, several cellular interactions of the SRP and its receptor have been described in *E. coli*, providing insights into their spatial function. Collectively, these *in vitro* studies have led to the current view of the targeting pathway [see (i) above]. Interestingly, however, *in vivo* studies of the role of the SRP and its receptor, with emphasis on the temporal progress of the pathway, elicited an alternative hypothesis [see (ii) above]. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

1. Introduction

Targeting of proteins to and across biological membranes of various organelles and cellular compartments represents one of the basic phenomena in cell biology [1,2]. A signal recognition event is crucial for proper localization of proteins to the endoplasmic reticulum (ER) membrane [3–5]. This takes place immediately after a nascent signal peptide protrudes from the ribosome (ribosome-nascent chain complex, RNC). The translation of ER-destined proteins starts in the cytosol and the RNC interacts with a signal recognition particle (SRP) [6–8]. Next, the RNC–SRP complex is targeted to the membrane where it associates with SR, a heterodimeric SRP-receptor complex (reviewed in [9]). From there, through GTP hydrolysis by the SRP–SR complex, the RNC assembles on the protein-conducting channel (translocon). Identification of the major components of the mammalian SRP system (SRP54 and the peripheral SR subunit SR-α) also revealed that the core of the system is evolutionarily conserved [10–12].

Despite the rather striking similarities in the SRP core elements, various organisms adopted different variations, additions, and utilities of the SRP system [11,13–15]. The archeal SRP system is closely related to the eukaryotic system although less complex and much less characterized [15] and this system will not be discussed in detail here. In contrast, some of the characterized bacterial SRP features are briefly summarized below because they are better understood and since the exceptions might sometime lead to important general concepts. In Gram-negative bacteria such as *Escherichia coli*, the SRP system contains two proteins, Ffh (SRP protein, homologous to the eukaryotic SRP54 subunit) and FtsY (SR, homologous to the α subunit of the eukaryotic receptor), and a small stable 4.5S RNA (SRP RNA) (Fig. 1A and E), all of which are essential for growth [16–19]. They function primarily in the biosynthetic pathway of many membrane proteins (reviewed in [20]). The *Bacillus* (*B.*) *subtilis* SRP system also contains, in addition to the core elements a histone-like protein (HBsu) [21] and a third SRP-related GTPase that is crucial for proper polar flagella assembly (FlhF), which was also found in other bacteria [22–25]. The *B. subtilis* SRP system has features that are required for either vegetative growth and/or sporulation [26,27]. However, the need for the *B. subtilis* SRP

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* Tel.: +972 8 9346435; fax: +972 8 9346334.
E-mail address: e.bibi@weizmann.ac.il.

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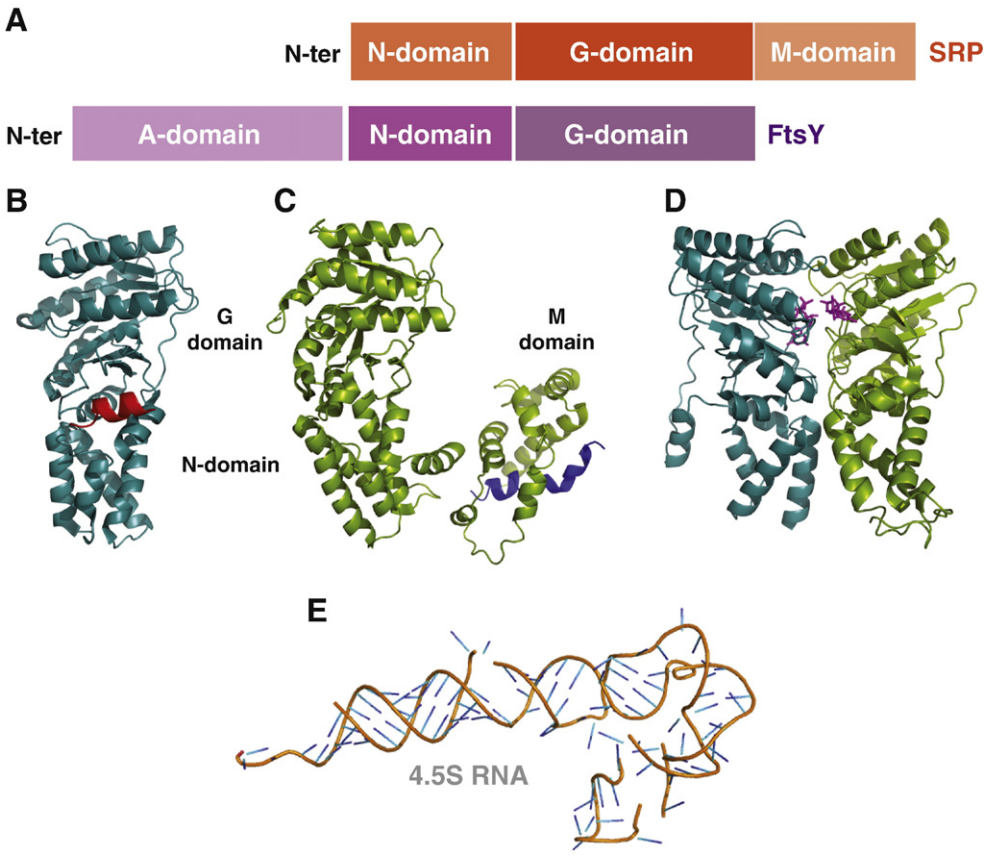

Fig. 1. Structural features of the SRP system. A. Schematic representation of the domain structures of Ffh and FtsY. N and G (NG-domain) of Ffh and FtsY are homologous. B. Crystal structure of a functional FtsY NG-domain from *E. coli* (NG + 1, PDB 2QY9). The lipid responsive domain at the N-terminus of NG + 1 is shown in red [58,59]. C. Crystal structure of Ffh from the hyperthermophilic archeon *Sulfolobus solfataricus* in complex with a yeast signal peptide (in blue) (PDB 3KL4) [56]. D. Crystal structure of the heterodimeric complex of the NG domains of FtsY (left) and Ffh (right) from *Thermus aquaticus*, with two molecules of the non-hydrolysable GTP analogue GMPPCP in the interface (shown in purple) (PDB 1RJ9) [44]. E. 3D representation of the SRP RNA molecule 4.5S from *E. coli* (adopted from http://rnp.uthct.edu/rnp/SRPDB/SRPDB.html).

components for growth is still debatable [28]. Interestingly, it was proposed that *B. subtilis* SRP is also required for biogenesis of secretory proteins, which usually have very hydrophobic signal peptides in this bacterium [28,29]. Notably, unlike *E. coli* and the debatable *B. subtilis*, several Gram-positive *Streptococci* were shown to tolerate disruption of all components in the SRP system under normal growth conditions [30,31]. Recent studies have shown that *Streptococcus* (*S.*) *mutans* may have developed a parallel co-translational targeting route via YidC2 and its demonstrated capacity to attract ribosomes to the membrane through a positively charged C-terminal domain [32]. The question of how YidC2 attracts only RNCs translating membrane proteins remains to be studied. As in *Streptococci*, FtsY can be deleted from *Streptomyces* (*S.*) *coelicolor*, and although the mutation affects sporulation and antibiotic production, the strain remains viable [33]. An emerging, specific role of SRP in bacterial pathogenicity (e.g. [31,34]) is also an interesting phenomenon, although very little is known about the differences between the housekeeping function of the SRP system and its involvement in virulence. Additional SRP-system flavors were revealed in other bacterial strains but more relevant to our later discussion is the *integral membrane FtsY* version in the phylogenetically distant Gram-positive bacteria of the order Actinomycetales [35–37].

The overall emerging picture of the SRP pathway in *E. coli* and other bacteria is still far from being fully understood: (i) Several *in vivo* studies do not readily conform to biochemical, *in vitro* data, and *vice-versa*. (ii) Very little is known about regulatory aspects at the transcription or translation level of SRP components and SRP substrates. (iii) Although proposed in several cases, it is unclear whether, in addition to the translocon, the SRP system targets proteins to other membrane-embedded insertion sites and what they are. (iv) As in all living cells, the prevailing view is that many *E. coli* membrane proteins are translated by membrane-bound ribosomes. However, the mode of ribosome targeting and association with the membrane *in vivo* has not yet been fully elucidated. Relevant to this, the question of whether ribosomes and mRNAs are targeted to the membrane independently or as assembled active RNCs has not yet found an *in vivo* solution. Moreover, the crucial interactions of SRP components with the membrane and with membrane proteins at various stages of the pathway still lack a detailed mechanistic understanding. The aim of this review is to encourage the awareness and consequently the investigation of gaps in our understanding of the SRP pathway in *E. coli* by emphasizing unanswered questions and experimental results that seek interpretations.

2. Structural, biochemical, and enzymological aspects of the *E. coli* SRP system

This section does not intend to offer a comprehensive account of the structural aspects of the SRP system [38–42]. The reviewed work, concepts, and recent developments in SRP structural research will be utilized here, as guides in our efforts to better understand biochemical and enzymological properties of the SRP system.

2.1. Components and complexes of the SRP system: Structural insight

The *E. coli* SRP system contains two proteins, Ffh and FtsY, both of which belong to the signal recognition-associated GTPase family of the ISMIBI class of GTPases and ATPases [43]. Ffh and FtsY contain a strikingly similar domain called NG (Fig. 1A, B, and C), and associate with each other through this domain with two nucleotides buried in the interface in an anti-parallel fashion (Fig. 1D) [44–46]. In both

proteins the N-domain forms a 4-helix bundle, which serves as a structural and functional platform for the GTPase G-domain.

In addition to their NG domains, Ffh and FtsY also harbor non-homologous additions (Fig. 1A). The NG of Ffh is connected via an α-helical linker to an evolutionarily conserved C-terminal methionine-rich M-domain, and the NG domain of *E. coli* FtsY has an N-terminal extension, which is termed the A-domain, which is extremely variable in length among bacteria. The Ffh M-domain interacts both with the SRP RNA and with hydrophobic nascent chains that emerge from ribosomes, as shown first in the eukaryotic system [47,48] and then in bacteria [49,50]. The structure of the M-domain, alone or in complexes, which was analyzed in several reports and variants [51–55] reveals that its helix-turn-helix motif interacts with a distorted RNA minor groove of the 4.5S RNA. On its opposite side, the M-domain exposes a conformationally flexible hydrophobic cleft that is appropriately situated for promiscuous interactions with hydrophobic nascent chains that emerge from the ribosome (Fig. 1C) [56]. It remains unclear, however, whether part of the 4.5S RNA also interacts with the nascent chain. The characterized features of the M-domain, including the fact that it is connected to the Ffh NG domain via a flexible linker that enables large conformational rearrangements upon functional interactions, make this domain a critical player in the selection of appropriate substrates and regulation of the targeting pathway.

In contrast to the conserved C-terminal extension in Ffh, the N-terminal A-domain of FtsY differs in size and properties among prokaryotes. Very little is known about the structure of the *E. coli* FtsY A-domain, which is thought to contain a large natively unstructured polypeptide (Parlitz, R. and Sinning, I. personal communication). There are, however, two stretches of amphipathic sequences in the A-domain of *E. coli* FtsY: (i) The most N-terminal 14 residues are proposed to form a helix, which was shown to contribute to the stability of the FtsY–membrane interaction [57]. (ii) Another structurally resolved, positively charged amphipathic helix is located between the A and N domains of FtsY [58]. This helix is required for stimulating the GTPase activity of the SRP-FtsY complex in response to acidic lipids [59,60]. A possible explanation for this activity is that the helix is packed against the surface of the NG domain and thus it transduces conformational information upon interaction with lipids. Although in many other bacteria the A-domain is minimal, structural analysis revealed that a similar amphipathic helix precedes the N-domain in other FtsY homologues [61]. Taken together, these structure-driven studies offer a functional clue of the role of lipids in the SRP targeting pathway (see later), as shown for other proteins such as ATPase MinD of the same SIMIBI class [62,63].

The third critical building block in the core SRP system is the SRP RNA, termed 4.5S RNA in *E. coli* [14]. Although previous studies have shown SRP-independent interactions of 4.5S RNA, we will focus here on its role as part of the SRP system. Structurally, 4.5S RNA is a 114-nucleotide-long molecule, which comprises the most evolutionarily conserved part of SRP RNAs, termed helix 8 (Fig. 1E). This domain has a short stem-loop structure with two internal loops that contain the most conserved nucleotides in SRP RNAs. The high-resolution structures of parts of 4.5S RNA were resolved using X-ray crystallography [64] or NMR [65]. Very informative, however, is the comparison between the structures of the RNA alone and those in the context of Ffh or its M-domain [51,55,66]. The RNA has a stabilizing effect on the M-domain [50,67] and it also stabilizes and stimulates the GTPase activity of the SRP–SR complex [68–72] (see later). These results strongly suggest structural rearrangements when 4.5S interacts with Ffh. The structures of the functional regions of the SRP–RNA with Ffh or the M-domain showed that indeed, both the RNA and the M-domain are affected conformationally in the complex. Moreover, if possible to deduce from another system, the interaction of the RNA with a full archeal SRP54 molecule leads to a movement of the M-domain relative to the NG-domain and promotes interaction between M- and N-domains, raising the possibility of intermolecular communication upon signal peptide binding [55]. Consequently, the current view is that the SRP–RNA is a functional determinant, rather than only a structural support element in the SRP.

A critical open question that has vaguely been discussed is whether free Ffh (without 4.5S RNA) exists in *E. coli* cells and whether it also functions as a free entity. Based on Ffh/4.5S RNA stoichiometry, the stability of Ffh in the presence of the RNA [69], and the fact that no 4.5S RNA-independent function for Ffh has been identified so far, it is likely that under normal conditions, Ffh is always in complex with 4.5S RNA. In this regard, recent studies using cells overexpressing Ffh or its M-domain alone have raised the possibility that free Ffh may play a role in the pathway through its capacity to interact with RNCs also in the absence of 4.5S RNA [67]. If this turns out to be true, then the described comparison between the structure (and enzymatic activity, see later) of Ffh alone and in its complex with the RNA [51,55,66] is of great importance.

2.2. Components and complexes of the SRP system: Biochemical insight

A central regulatory theme in the targeting pathway is driven by an intricate cycle of GTP binding and hydrolysis by the SRP protein, its receptor, and by the complex that they form with each other. Briefly, both Ffh and FtsY bind GTP before complex formation and GTP hydrolysis is required later in the pathway for dissociation of the complex, during the transfer of RNCs to the translocon. Functionally, Ffh and FtsY belong to a group of GTPases that are activated by nucleotide-dependent dimerization (GADs) and act in a pseudo *trans*-homodimerization manner [73]. This distinction is important because Ffh and FtsY are not regulated by classical external guanine nucleotide-exchange factors (GEFs) or GTPase-activating proteins (GAPs), and they fulfill these functions autonomously [44,74]. This does not imply that Ffh and FtsY are not regulated by other means during the targeting pathway, such as by interaction with the SRP RNA and other non-SRP system players. The following biochemical properties of the SRP and SR GTPases explain their enzymological capabilities. (i) Structurally, their nucleotide binding sites are loose and open [75,76] and therefore, their independent affinities to nucleotides are very low compared with classical GTPases [71,77,78]. The structural data also explain the relatively fast nucleotide exchange rates in SRP and SR. (ii) The two GTPases seem to be indifferent conformationally to nucleotide binding [75,76,79,80]. (iii) Separately, the SRP and SR proteins exhibit low GTPase activities [71], and this property may also be explained by structural data. (iv) In contrast, when SRP and SR form a stable heterodimeric complex together with two nucleotides (Fig. 1D) [44] they stimulate each other's GTPase activity dramatically and consequently dissociate [71]. Translated into biological terms, when RNCs are ready to be released from their docking site to a vacant translocon, this transfer probably occurs quickly because the GDP-bound SRP–SR complex is unstable. As discussed recently, the two GTPases undergo a series of conformational changes during the cycle, which are affected by external factors, and exhibit a prerequisite for proper regulation and function [42].

How does the third component of the *E. coli* SRP system, the 4.5S RNA, affect the interaction between Ffh and FtsY? The first biochemical clue was offered by Peluso et al. [81] who showed that 4.5S RNA facilitates both the assembly and disassembly of the Ffh–FtsY complex. It was also suggested that the RNA modestly regulates the GTPase activity of the Ffh–FtsY complex [71]. This proposal was further elucidated and put in a physiological context. Convincingly, it was shown that the 4.5S RNA indeed enhances the GTPase activity of the complex just above a critical threshold required for cell viability [72]. Moreover, it was shown genetically in this study that the 4.5S RNA tetraloop is responsible for this effect. Further investigation of the role of the tetraloop has confirmed its importance for complex formation [82]. In this regard, it is interesting that deleting the first helix of the N-domain

of both Ffh and FtsY mimics the stimulatory effect of 4.5S RNA on complex formation, suggesting that in the RNA-unbound form, the N-domain has a suppressing regulatory function [70] (see also [83]). However, many of these studies were influenced dramatically by the inclusion of detergents in the assays [84]. Moreover, in the absence of detergent, it has recently been shown that 4.5S RNA does not stimulate complex formation between Ffh and FtsY, unless the SRP is associated with a hydrophobic nascent chain [68] (see later). It was proposed that the 4.5S RNA mediates its stimulatory effect through transient interaction between its tetraloop and the G-domain of FtsY [85]. Finally, in the context of the SRP alone, structural and biochemical studies showed that the 4.5S RNA affects cross talk between the NG- and M-domains of Ffh with possible implications regarding the role of the SRP-nascent chain association and its interaction with FtsY [66,86].

When integrating all these structural and biochemical insight into a biological context, a question arises: Are there cellular situations or steps during the targeting pathway where Ffh and FtsY or the SRP and FtsY form complexes free from other cellular components? The answer is probably no, and it was even proposed that the GTPases of the SRP system evolved to ensure that the SRP and SR engage external ligands before interacting with each other [87]. Therefore, characterizing the biochemical properties under additional conditions is crucial for better understanding how these complexes function in the cellular context. Several studies in this direction are described below.

2.3. Structural and biochemical aspects of other cellular interactions of components of the SRP system

The structural and biochemical studies described above shed critical light on the properties of the SRP components and their dynamic interactions with each other. An important question would be what could one learn from these studies regarding the *order of events* during the biosynthetic pathway of membrane proteins? To answer this question, obtaining structural and biochemical insight into the interactions of SRP components with other cellular factors is crucial. In *E. coli*, the main postulated extra-SRP system interactions are with the ribosomes, the emerging hydrophobic nascent chain, membrane lipids, and membrane proteins. Structurally, only several of these interactions have been resolved at high resolution.

2.3.1. Interaction of SRP with the ribosome

Obviously, the ribosome itself is a critical player in co-translational processes [88]. In fact, even non-translating ribosomes have been shown to interact with the SRP, although with a lower affinity than RNCs [89]. The ribosome senses and responds conformationally to nascent chains of various secondary structures and chemical properties even before they exit the large subunit tunnel, and the consequent response may affect the elongation rate [90,91] and the association of the RNC with cellular factors [92–94]. Biochemical work has shown that a main determinant of the ribosome–SRP interaction is L23 and that this interaction occurs independently of a nascent chain in the exit tunnel of the ribosome [95,96]. Additional cross-linking studies revealed an interaction site between 4.5S RNA and the ribosomal 23S RNA in a location that is not very close to the exit tunnel [97]. Other interaction sites between the *E. coli* ribosome and SRP were identified through cryo-electron microscopy of stalled ribosomes and by successfully fitting high-resolution molecular models of the interacting components [98,99]. In these studies the reconstructed particles were assembled *in vitro* from isolated components. All together, the results show that in addition to the previously identified L23 (adjacent to the N-domain of Ffh), the SRP is in relatively close contact with L29 (NG-domain of Ffh), L22, L24, and helices 24, 50, and 59 of 23S RNA (M-domain of Ffh) and L18 (or L32 [100]) (4.5S RNA). In cases where the interacting large subunit proteins have loops that extend all the way to the tunnel (e.g. L22) or affect the tunnel indirectly, binding of SRP might influence the movement of nascent chains through the tunnel. It is interesting that the M- and NG-domains of Ffh interact on opposite sites of the tunnel exit and therefore might have a domain-specific influence on the ribosome (discussed in [67]). In its complex with the ribosome, the SRP NG domain undergoes a conformational rearrangement that meets the requirement for co-association with the receptor (see also [101]). Of great importance is the finding that the same ribosomal components at the tunnel exit are involved in *several* molecular interactions that also include the SRP and the translocon (reviewed in [100]), suggesting temporally ordered interactions between the ribosome (or RNC) with the various factors or competitive regulatory means [102]. In this regard, the cryo-electron microscopy studies suggest that when the SRP-receptor is present in the complex (possibly via its interaction with both the ribosome and the SRP), a ribosomal region required for docking on the translocon becomes exposed [103]. This scenario supports previous conclusions from biochemical studies of the eukaryotic system [104,105].

2.3.2. Interaction of SRP with the hydrophobic nascent chain

A major biological role of the SRP is to scan for hydrophobic nascent chains that emerge from the ribosome. The recent crystal structure of a model complex between the SRP protein and a signal sequence [56] has confirmed suggestions made based on structures devoid of signal sequences [54,55] that the M-domain has a hydrophobic grove appropriate for promiscuous interactions. The crystal structure revealed a putative induced-fit mechanism (for the signal peptide) that leads to extensive interactions between the polypeptide and hydrophobic residues in the M-domain groove [56]. How is the information that a hydrophobic nascent chain is present at the M-domain transferred to the GTPase domain? Cross-linking experiments have suggested close but transient contacts between the hydrophobic core of the signal peptide and the NG domain [106]. However, since these results have not yet been confirmed by other methods, the possibility of the NG domain being directly involved in signal sequence recognition is still under debate. Another study showed that the SRP-FtsY interaction, which results in the stimulation of GTP hydrolysis, can be inhibited by synthetic signal peptides [107]. These results do not conform to later studies of the system (see below). It has been predicted that the binding of 4.5S RNA to Ffh may contribute to the association of hydrophobic nascent polypeptides [51], indirectly suggesting a role for the RNA in the intra-molecular information transfer. Such an alternative information-transfer scheme was recently proposed based on reverse genetics and biochemical studies [68,108]. According to this scheme, binding of a hydrophobic nascent chain at the M-domain of Ffh induces a conformational change that activates the 4.5S RNA, which in turn, stimulates SRP–SR complex formation by dislocating the intra-molecular negative regulators of Ffh and FtsY (the N-terminal helices of their NG domains). Recent studies have suggested that this occurs through a transient link between the RNA tetraloop and the G-domain of FtsY [85]. According to the emerging scenario, it is unlikely that *free* Ffh would enter the targeting pathway in a productive manner. Therefore, if Ffh has an RNA-independent function (as discussed above), it must be different from that of SRP.

2.3.3. Interaction of FtsY with membrane ribosomes

Whereas the association of SRP with the ribosome seems obvious, identifying FtsY in complex with *E. coli* membrane ribosomes [109], an interaction that exist also in the mammalian system [110], came as a surprise. Even more surprising was the observation that FtsY associates with membrane ribosomes even in the absence of the SRP or the translocon [109]. Unfortunately, the properties and structure of the FtsY-membrane ribosome complex have not yet been fully characterized in *E. coli*. In this regard, it is interesting to note the

exceptionally interesting results of Mandon et al. [110], who studied the mammalian system *in vitro* by utilizing biosensor experiments, and characterized more extensively the SR-ribosome complex. Their studies revealed high-affinity, saturable binding of ribosomes or large ribosomal subunits to the receptor. Remarkably, they showed that the receptor has a 100-fold higher affinity for the ribosome than for SRP and that an N-terminal 319-residue segment of the SR is necessary and sufficient for binding to the ribosome. The authors proposed that: "the ribosome–SR interaction accelerates targeting of the ribosome nascent chain complex to the RER, whereas the SRP–SR interaction is crucial for maintaining the fidelity of the targeting reaction". More recently, the high-resolution cryo-electron microscopy structure of a "docking complex" consisting of an SRP-bound 80S ribosome and the SRP receptor was described [103]. This structure showed that the SRP receptor causes rearrangement in SRP, which exposes the ribosomal binding site for the translocon. However, structural studies of a complex devoid of the SRP have not yet been reported. Taken together, these results might have a major implication, which suggests that ribosomes have an SRP- and translocon-independent membrane docking site, as described in detail later in this review.

2.3.4. Interaction of FtsY with membrane lipids

Previous studies have established that the bacterial SRP-receptor (FtsY) functions as a membrane-bound protein [35,111,112]. The question of how FtsY associates with the membrane prior to its association with the translocon has not yet been fully resolved. It has been shown convincingly that the receptor interacts with membrane lipids [113–115], and that acidic lipids stimulate its GTPase activity [59,114]. Trypsin digestion experiments highlighted the central role of the N-domain in lipid contacts [116]. All these issues have been investigated further *in vivo*, and by structural, and additional biochemical approaches. The *E. coli* FtsY has a unique ~195 amino acid-long N-terminal domain (termed A-domain), which was found dispensable for the receptor function *in vivo* [117]. Nevertheless, the A-domain has in its N-terminus a stretch of 14 amino acids that interacts with acidic lipids and thus strengthens the association of FtsY with the membrane [57]. As described earlier in this review, structural analysis of several FtsY molecules, which do not contain such a large A-domain, revealed a short, positively charged amphipathic helix at the N-terminus of their N-domain (see Fig. 4 in [118]), as was also observed with the functional, truncated *E. coli* FtsY mutant NG + 1 [58]. Apparently, this short extension of the NG-domain of FtsY was found responsible for the lipid-stimulatory effect on FtsY-SRP GTPase activity [59], strongly suggesting that lipids serve as functional regulators of the receptor activity. The question of which lipids interact with the lipid-responsive domain was addressed in the past by *in vitro* experiments and these studies demonstrated the involvement of acidic lipids [57,114]. This notion has finally received *in vivo* support through genetic studies, which clearly showed that over-production of acidic lipids restores the function of NG [60], an otherwise inactive truncated construct of *E. coli* FtsY [58,59,117]. Why is it necessary to regulate FtsY by acidic lipids? One possible scenario is based on the observation that acidic lipids near the translocon play important roles in its function (e.g. [119]). If acidic lipids indeed cluster in the vicinity of the translocon, this might provide a regulatory means during late stages of the ribosomal targeting pathway. After docking, membrane-bound ribosomes translating membrane proteins must be transferred to the translocon for proper co-translational insertion of the nascent chains into the membrane. Prior to interaction of the RNCs/FtsY/SRP complex with the translocon, it must remain intact to prevent premature release of SRP from the nascent chain. Upon association with a free translocon, acidic lipids would stimulate the FtsY–SRP GTPase activity to promote the fast release of the RNC and its assembly on the translocon.

2.3.5. Interaction of FtsY with the translocon

Studies of the defect in cold-sensitive *E. coli* Sec40 mutant, which carries a single mutation in SecY (A363T), showed that excess FtsY restores membrane protein integration *in vitro* and partially also the growth at a non-permissive temperature [120]. This indirect indication for FtsY–SecY interaction was further confirmed by cross-linking, co-immunoprecipitation, and co-purification experiments. All together, these and additional studies in *E. coli* [121] and yeast [122–124] strongly suggest a functionally important role for the translocon in the SRP–SRP-receptor function late during the targeting process. The exact interaction sites between the translocon and the SRP-receptor remain to be identified.

3. The *E. coli* SRP-mediated targeting pathway

In addition to the structural and biochemical insights described above, *in vitro* and *in vivo* targeting studies also critically contributed to our understanding of the SRP system. Specifically, these studies revealed the need for various components of the SRP system to support co-translational targeting to the bacterial membrane and the SecYEG translocon. However, in several cases, conclusions that were derived from biochemical and *in vitro* studies could not be fully reconciled with the results obtained *in vivo* and *vice-versa*. This part of the review offers a critical view of the *in vitro* and *in vivo* studies. As a general perspective, I propose that sometimes, what one sees *in vitro* might not really happen *in vivo*. On the other hand, results obtained *in vivo* might represent indirect effects and thus must be reconstituted in the test tube in order to prove their relevance. *In vitro* reconstitution will only be feasible if all the native functional determinants and their biosynthetic modes have been identified and characterized.

3.1. Selected in vivo and in vitro studies of the E. coli SRP system

3.1.1. Identification of SRP substrates

Based on a variety of experimental approaches it is currently accepted that the *E. coli* SRP interacts with very hydrophobic nascent polypeptides that emerge from the ribosome. Indeed, several *in vitro* studies have helped to define which proteins are SRP substrates (reviewed in [20]). However, whereas several studies showed that the *E. coli* SRP interacts *in vitro* with nascent pre-secretory proteins (signal peptides) (e.g. [125,126]), this does not really happen *in vivo*, where the *E. coli* SRP functionally interacts only with transmembrane helices of membrane proteins or with secretory proteins that harbor extremely hydrophobic signal peptides [127–129]. These studies represent a classical example where genuine results that were obtained *in vitro* do not represent the physiological situation. In this specific case, the differences between the *in vitro* and *in vivo* results may have several explanations, such as competition among various nascent chains for SRP binding *in vivo*, or the fact that additional players are important for the interaction, or the mode of interaction of SRP with nascent chains [128,130,131].

3.1.2. Interaction of SRP with RNCs

In vivo studies have shown that the SRP-mediated targeting and insertion of membrane proteins occurs in a co-translational fashion [127,132,133]. *In vitro* studies [134] suggested the same, and from these experiments one can learn about the order of events during the targeting pathway: The hydrophobic nascent chain first interacts with the ribosomal proteins L4, L22 inside the tunnel, and subsequently with protein L23 at the tunnel exit. Next, the nascent polypeptide interacts with the SRP and only later with SecY. The question when does the SRP functionally interacts with nascent hydrophobic polypeptides *in vivo*, whether it happens in the cytoplasm or on the membrane, remains to be investigated.

What happens if SRP binds to cytosolic ribosomes translating membrane proteins? The *E. coli* SRP lacks an Alu domain in its RNA

subunit, which is responsible for elongation-arrest in eukaryotes (e.g. [135,136]), and is therefore unlikely to impose elongation-arrest in the bacterium. However, *in vitro* and *in vivo* experiments have yielded conflicting results [137,138] and the issue of translation arrest in *E. coli* remains unresolved. Recently, the effect of SRP on the translation of many membrane and cytosolic proteins in *E. coli* has been analyzed. The results showed that overexpressed SRP, Ffh or its M-domain alone are all able to *selectively* inhibit the translation of membrane proteins [67]. Since, as mentioned, the *E. coli* SRP does not have a eukaryotic like elongation-arrest domain, and since the M-domain alone was able to retard translation, the mechanism of such a regulated membrane protein translation must be different from that proposed for the eukaryotic system. However, despite the observed selectivity towards membrane proteins, one cannot rule out the possibility that the effect is indirect, because defined *in vitro* studies have so far failed to detect any influence of SRP on membrane protein translation (e.g. [138]). If the described *in vivo* effect is direct and physiologically relevant, then one possible explanation for the conflicting results would be that additional unknown factors should be included in the *in vitro* assay. In addition, it would be interesting to study the effect of the M-domain alone on membrane protein translation *in vitro*.

3.1.3. FtsY biogenesis

As described earlier in this review, FtsY functions as a membrane-bound protein [35,111,112]. The questions of when and how FtsY functionally associates with the membrane have not yet been fully answered and thus represent a critical gap in our understanding of the targeting pathway. Importantly, several observations in the bacterial [35,112,139] and mammalian [140] systems raised the possibility that FtsY and its eukaryotic homolog are targeted to the membrane co-translationally in an SRP-independent manner. If this turns out to be true and essential for membrane protein biogenesis, it would cast some doubt on the physiological relevance of several *in vitro* observations. In contrast, unfortunately, it has never been tested *in vitro* whether the FtsY co-translational targeting is critical for membrane protein biogenesis in *E. coli*. Again, resolving this issue is of central importance to our understanding of the targeting pathway and the order of events during membrane protein biogenesis (see later).

3.1.4. SRP and SRP–RNCs interaction with FtsY

Although complex formation between SRP or RNC–SRP and FtsY can occur in the cytosol *in vitro* [44,131,141,142], it was shown that soluble FtsY is not sufficient to induce dissociation of SRP from the RNC; a process that requires membrane-bound FtsY [130,143]. Importantly, in addition, these studies showed that pre-assembled FtsY–SRP–RNC complexes could not efficiently target FtsY-depleted membrane, possibly due to steric hindrance in FtsY, whereas SRP–RNCs targeting does occur when purified FtsY was added to FtsY-depleted membranes. Since FtsY binds membranes (as discussed earlier), it is quite predictable that SRP–RNCs would interact with FtsY-reconstituted membranes. Is this reconstituted SRP–RNC targeting functionally productive or entirely futile? This question depends on whether or not FtsY needs to be targeted to membranes co-translationally for efficient biological activity. In any case, this and other studies disfavor the possibility that soluble FtsY plays a critical role in the targeting pathway [35,111,112].

Are hydrophobic nascent polypeptides necessary for targeting SRP–RNCs to membrane bound FtsY *in vivo* and *in vitro*? The accepted view is that only RNCs exposing such nascent polypeptides can productively interact with SRP and target the membrane bound receptor (e.g. [143]). A recent study, however, demonstrated convincingly by an elegant set of *in vitro* experiments that exposure of hydrophobic nascent chains is not at all required for targeting of RNCs by the SRP [93]. This observation recapitulates the notion that what one observes *in vitro* might or might not necessarily happen *in vivo*. The conclusion of the authors is that all of the translating ribosomes, regardless of whether they translate a cytosolic or a membrane protein, can enter the SRP cycle. However, another possible conclusion would be that the *in vitro* experimental conditions in this study might not fully represent the intracellular *in vivo* conditions, especially regarding additional factors that are likely to interact with the ribosome. An obvious example is the observation that although SRP binds to RNCs exposing signal peptides of secretory proteins *in vitro*, this binding did not occur when the chaperonin trigger factor was included in the reaction [130].

3.2. Targeting of ribosomes: Alternative concepts

3.2.1. SRP-mediated targeting of RNCs to membrane-bound FtsY

The prevailing view of membrane protein targeting in *E. coli* has been described in several review articles (e.g. [20]) and in text books. Briefly, the presented scenario proceeds according to the following order of events: 1. SRP binds to a hydrophobic nascent polypeptide as it emerges from a cytosolic ribosome. It is still debatable whether this interaction might arrest further translation or not in the bacterium. 2. The entire SRP–RNC complex is then targeted to the membrane-bound FtsY, but the model does not describe how FtsY reaches the membrane. 3. The RNC is transferred to, and assembles on the SecYEG translocon. 4. GTP hydrolysis by the SRP–SR complex triggers its dissociation.

This model, which I term here **S**RP-**m**ediated **r**ibosome **t**argeting (S-MRT), is supported by numerous *in vitro* experiments. However, one central step in the model (step 2) is difficult to reconcile with *in vivo* studies. Whereas according to the model both FtsY and SRP are critical for ribosome targeting, depletion studies showed that only FtsY seems crucial for this process [144]. Moreover, immunoprecipitation assays showed that FtsY is present in a complex with membrane-bound ribosomes in the absence of SRP [109], suggesting an SRP-independent membrane docking of ribosomes (see also [110]). Furthermore, other studies indirectly indicate mechanistic differences between the roles of SRP and FtsY in membrane protein expression. Whereas FtsY-depletion leads to dramatic inhibition of membrane protein synthesis [145,146], SRP-depletion has a minor effect on expression, although functional assembly of membrane proteins is disrupted in the Ffh-depleted cells [146,147]. As stated earlier in this review, the main problem with the *in vivo* studies is that their outcomes might represent unpredictable indirect effects, despite the use of proper control experiments. Nevertheless, these studies have elicited an alternative view of the targeting pathway, which deserves further investigation. A preliminary account of this hypothetical view, which was published in the past [148], has since received additional, though still mostly indirect experimental support.

3.2.2. FtsY-mediated membrane-targeting of ribosomes

The alternative model, which is termed here **S**RP **R**eceptor-**m**ediated **r**ibosome **t**argeting (SR-MRT, see Fig. 2) follows a different order of events compared to S-MRT: 1. FtsY is targeted to the membrane co-translationally and both the ribosome and FtsY dock on an unknown membrane site. 2. mRNA encoding a membrane protein is targeted to the membrane-bound FtsY-containing ribosome complex. 3. SRP interacts with a hydrophobic nascent polypeptide as it emerges from the membrane-associated ribosome. 4. The membrane associated FtsY–SRP–RNC complex interacts with a vacant SecYEG translocon (or other insertion sites, see: [149]) and the SRP–FtsY GTPase is then stimulated by acidic lipids ([59,60], leading to rapid transfer of the RNC to the SecYEG translocon. 5. GTP hydrolysis by the SRP–SR complex also triggers its dissociation.

Obviously, the S-MRT and the SR-MRT models are identical in the steps that follow the recruitment of SRP (step 3 in the SR-MRT model). Therefore, the downstream events in the SR-MRT model are compatible with many of the *in vitro*, biochemical, and structural studies described earlier of inter- and intra-SRP system interactions

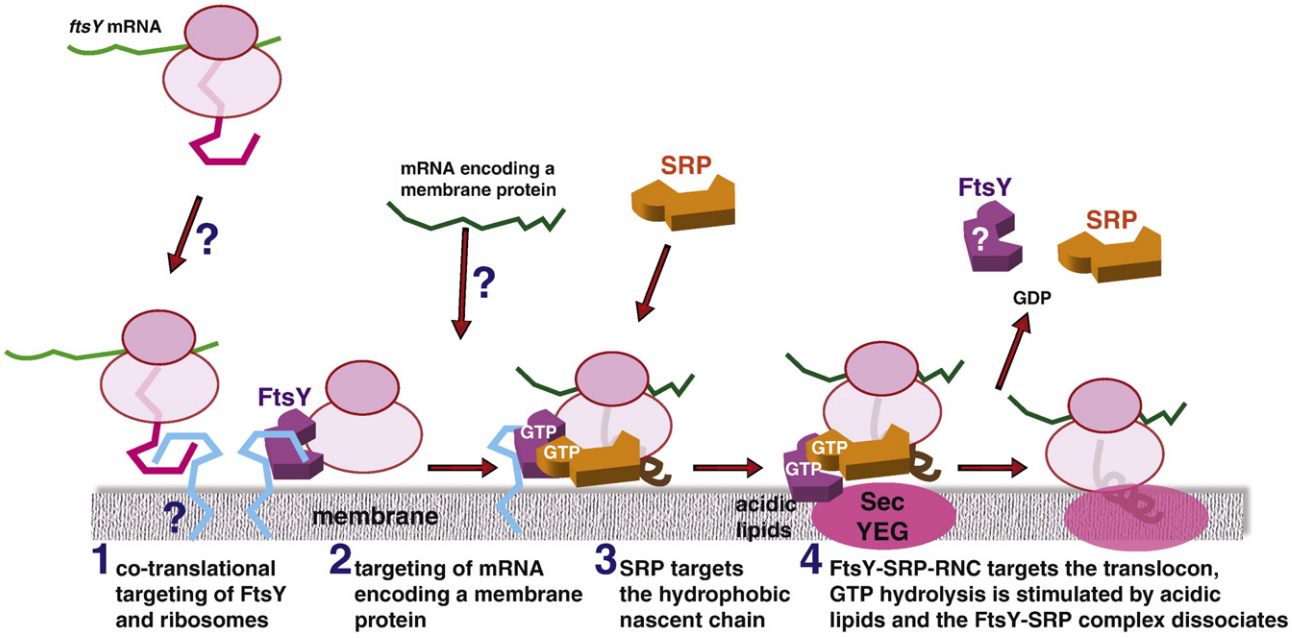

Fig. 2. SR-MRT model for targeting ribosomes and mRNAs to the *E. coli* membrane and SRP-dependent targeting of membrane proteins to the SecYEG translocon. 1. FtsY is targeted to the membrane co-translationally through the information embedded in its N-domain, and assembles on membrane lipids and/or on an yet unknown integral membrane protein (light blue). After FtsY targeting and termination of translation, the ribosome or its large ribosomal subunit remains membrane-bound. 2. mRNA encoding an integral membrane protein is targeted to the membrane bound ribosomes and forms a new translation initiation complex. 3. The emerging nascent hydrophobic polypeptide is recognized by SRP that binds to it, to the ribosome and to the SRP receptor. 4. The RNC–SRP–FtsY complex interacts with a vacant SecYEG translocon (or other membrane protein insertion sites) through FtsY. The SRP–FtsY GTPase activity is stimulated by the interaction of FtsY with acidic lipids. This activation step leads to the release of the RNC from SRP–FtsY, its assembly on the SecYEG translocon, and the concomitant dissociation of the SRP–FtsY complex. The question mark on FtsY means that the fate of FtsY at the end of the cycle is currently unknown.

and the pathway related enzymatic activities. The main differences between the models are in the initial steps, namely, according to the SR-MRT model, ribosomes are targeted to the membrane during translation of FtsY, and this implies that mRNAs encoding membrane proteins should be targeted to the membrane independently of translation. These two issues are discussed below.

3.2.3. Concomitant targeting of FtsY and ribosomes during FtsY translation

It seems plausible, in light of the earlier discussion of FtsY biogenesis, that FtsY (and its mammalian homolog) may be targeted to the membrane co-translationally. With the *E. coli* receptor, this scenario [139] needs to be thoroughly re-examined *in vitro*, especially in light of our later findings that except for a short helical peptide that precedes the N-domain, the A-domain of FtsY is dispensable *in vivo* [117]. In other *in vivo* studies it was suggested that a deleted FtsY construct (termed NG), which does not catalytically respond to lipids is still able to target the membrane [58,59]. Moreover, our unpublished *in vivo* studies demonstrate that several GTPase defective FtsY mutants also accumulate on the membrane together with ribosomes (L. Bahari and E.B., unpublished). These results suggest that determinants in the N domain of FtsY are required for targeting. In addition to further identification of the necessary domains *in vivo*, these results should be evaluated *in vitro* as described for the mammalian receptor [140].

If a co-translational pathway is functionally involved in membrane protein biogenesis, how could we explain the results of *in vitro* studies where purified FtsY was shown to support targeting? There are several possible explanations. The simplest one is that FtsY can also sometimes assemble on the membrane properly in a post-translational manner, although this might or might not happen *in vivo*. However, if this is not the case, then reconciliation is more difficult. As discussed above, it has been shown that purified FtsY can interact with membranes or liposomes and thus targeting of SRP–RNCs to reconstituted, membrane-associated FtsY is then mediated via the SRP–FtsY complex formation. The remaining caveat is, however, whether such reconstitution indeed leads to proper, quantitative insertion of the translated membrane proteins. It should be taken into account that membrane proteins may insert spontaneously into lipid bilayers, sometimes properly (e.g. [150]), even though such pathways are catalyzed efficiently by membrane-insertion machineries (e.g. [151]). The spontaneous insertion of short SRP substrates, as those that have usually been utilized in many targeting assays, might be largely facilitated if their translation occurs on membrane-bound ribosomes. Intuitively, although not fully supported by experimental data, I favor the possibility that at least a portion of the reconstituted membrane-bound FtsY would be able to support proper targeting and assembly of SRP substrates on the translocon *in vitro*. Whether this also happens *in vivo* remains to be investigated.

The practical implication of co-translational targeting of the receptor to the membrane is that both the receptor and its translating ribosome reach the membrane simultaneously. According to the S-MRT model, after FtsY targeting and translation, the ribosome may dissociate from the membrane, leaving the newly targeted FtsY ready to accommodate a new, SRP-targeted ribosome, now translating an SRP substrate. Alternatively, according to the SR-MRT model, after termination of FtsY translation, the large ribosomal subunit may remain membrane bound, ready to assemble and translate a new mRNA. Provided that the new mRNA encodes a membrane protein, transfer of this ribosome to the translocon requires recognition of the hydrophobic nascent polypeptide by SRP. Conceptually, the SR-MRT model suggests a constitutive mechanism for continual supply of ribosomes to the membrane that is mediated by the SRP receptor. In addition, this scenario implies that mRNAs encoding SRP substrates are targeted, via a specific mechanism, to membrane bound ribosomes, as discussed next.

3.2.4. Targeting of mRNAs encoding SRP substrates

The phenomenon of specific mRNA localization has recently been studied extensively in eukaryotic systems also in the context of the biosynthetic pathway of SRP substrates [152]. Evidence exists that mRNAs of SRP substrates utilize specific targeting machineries [153], and recent studies have demonstrated translation- and SRP-

independent targeting of mRNAs to the ER membrane [154,155]. In *E. coli*, investigation of the possibility that mRNAs encoding membrane proteins are targeted to the plasma membrane in a translation-independent manner is currently in progress. The main questions in the field are: (i) What is the targeting signal(s)? And, (ii) what is the cellular machinery that mediates selective targeting? It has been proposed that the targeting signal in mRNAs encoding eukaryotic secretory proteins is embedded in the signal sequence-coding region [153]. For membrane proteins devoid of signal sequences, both in eukaryotes and prokaryotes the signal must be different. Because most membrane proteins across evolution are translated by membrane bound ribosomes, we decided to search for universally conserved specific features in this group of mRNAs [156]. The bioinformatics analysis revealed that codons of very hydrophobic amino acids, highly represented in integral membrane proteins, are composed of 50% uracils (U), which are largely overrepresented in regions encoding transmembrane helices. This conclusion and additional results suggest that the U-richness is an evolutionarily ancient feature of mRNAs encoding integral membrane proteins, which might serve as a physiologically relevant distinctive signature to this group of mRNAs [156]. Whether the repeated ~60-nucleotide long U-rich sequences of mRNAs encoding membrane proteins harbor the information for membrane targeting remains to be investigated. A strong support for this possibility has come from a recent study of the biogenesis of PMP1, a very short membrane protein from *S. cerevisiae* [154]. The results demonstrated that the targeting information is spread throughout a long 3′ UTR. Surprisingly, when we examined the uracil content in the sequence of the PMP1 3′ UTR, we observed that it contains repeating U-rich stretches that mimic those of a typical polytopic membrane protein (E.B., unpublished). In conclusion, U-richness represents the only known difference between mRNAs of membrane proteins and other mRNAs and therefore raises the attractive, though still speculative, hypothesis that U-richness is required for mRNA targeting to membranes across evolution. Further investigation of this possibility may close a central gap in our understanding of membrane protein biogenesis.

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