Membrane Targeting of Ribosomes and Their Release Require Distinct and Separable Functions of FtsY*

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Liat Bahari‡, Richard Parlitz§, Asa Eitan§, Goran Stjepanovic§, Elena S. Bochkareva§, Irmgard Sinning§, and Eitan Bibi†1

From the ‡Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel and the §Biochemiezentrum der Universität Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany

The mechanism underlying the interaction of the *Escherichia coli* signal recognition particle (SRP) receptor FtsY with the cytoplasmic membrane is not fully understood. We investigated this issue by utilizing active (NG+1) and inactive (NG) mutants of FtsY. In solution, the mutants comparably bind and hydrolyze nucleotides and associate with SRP. In contrast, a major difference was observed in the cellular distribution of NG and NG+1. Unlike NG+1, which distributes almost as the wild-type receptor, the inactive NG mutant accumulates on the membrane, together with ribosomes and SRP. The results suggest that NG function is compromised only at a later stage of the targeting pathway and that despite their identical behavior in solution, the membrane-bound NG-SRP complex is less active than NG+1-SRP. This notion is strongly supported by the observation that lipids stimulate the GTPase activity of NG+1-SRP, whereas no stimulation is observed with NG-SRP. In conclusion, we propose that the SRP receptor has two distinct and separable roles in (i) mediating membrane targeting and docking of ribosomes and (ii) promoting their productive release from the docking site.

Membrane-bound ribosomes are responsible for the biosynthesis of many integral membrane proteins that insert into the membrane in a co-translational manner (1, 2). Targeting of these ribosomes to the cytoplasmic membrane in *Escherichia coli* requires the signal recognition particle (SRP)2 receptor, FtsY (3). In addition, under FtsY depletion conditions, the expression of polytopic membrane proteins such as LacY (4), SecY (3), and MdfA2 is repressed. Besides its interaction with the inner membrane, FtsY functionally interacts with the SRP and the SRP protein Ffh in a nucleotide-dependent manner (reviewed in Ref. 5). Moreover, additional studies demonstrated that FtsY forms a complex with membrane-bound ribosomes (6) and the SecYEG translocon (7, 8). These observations thus underscore the central role of the SRP receptor in ribosome targeting and biogenesis of membrane proteins (1, 9). However, despite extensive genetic, biochemical, and structural studies, important aspects of the function of FtsY are not yet fully understood.

At steady state, FtsY is distributed between the cytoplasm and the membrane (10), but the cytosolic form does not seem to be essential for membrane protein biogenesis or cell survival (11). In contrast, various studies suggest that FtsY functions as a membrane-bound receptor (11, 12), in agreement with observations that it interacts with membrane-bound ribosomes (6) and the translocon (7, 8). Interestingly, however, FtsY has no known membrane anchor partner homologous to the mammalian β-subunit of the SRP receptor.

FtsY contains three domains. The C-terminal N- and G-domains (together 302 residues long) constitute a universally conserved SRP-GTPase (13) that interacts with the homologous NG domain of the SRP protein Ffh. In its N terminus, FtsY contains an acidic A-domain (195 residues long) that was proposed to mediate membrane targeting and association of the receptor. Nevertheless, A-domain-truncated FtsY versions exhibit strong affinity for membranes (14, 15), possibly through the N-domain (16). This interaction is dominated by electrostatic forces (15) and is abolished by protease treatment of the membranes. The current view of FtsY association with the inner membrane combines interactions with lipids and protein(s). However, the mechanisms underlying these interactions and their functional importance remain largely unknown.

Recently we have shown by deletion analysis that FtsY retains its essential functions in vivo in the absence of the 195-residue-long A-domain (termed NG+1) (17). Interestingly, removal of a single N-terminal amino acid (Phe196) from NG+1 (termed NG) inactivates the receptor by an unknown mechanism. By investigating the functional differences between NG+1 and NG in vivo, and their interactions with SRP, nucleotides, SecYEG translocon, and the cytoplasmic membrane, we now show that although NG functions properly during membrane targeting and docking of ribosomes, it is defective at a late stage of the pathway, which consequently leads to dissociation of the ribosomes from their putative docking site. The results strongly suggest that this later step requires stimulation of FtsY-GTPase activity by lipids and that the response of the inactive NG mutant to lipids is impaired.

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1 To whom correspondence should be addressed. Tel.: 972-8-9343464; Fax: 972-8-9344118; E-mail: e.bibi@weizmann.ac.il.

2 The abbreviations used are: SRP, signal recognition particle; Ni-NTA, nickel-nicotinamide adenine dinucleotide; PIP, inositol 1,4,5-trisphosphate; TgPTP, inositol 1,4,5-trisphosphate; FtsY, nickel-nicotinamide adenine dinucleotide 5-phosphate-dependent dimeric enzyme; DDM, n-dodecyl-β-D-maltosylpyranoside.

3 E. S. Bochkareva, I. Yosef, J. Adler, A. Seluanov, and E. Bibi, manuscript in preparation, unpublished observations.

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EXPERIMENTAL PROCEDURES

Materials—Antibodies to FtsY, Ffh, and ribosomes were described previously (6, 18). Antibodies to SecY and SecE were kindly provided by Prof. Hajime Tokuda.

Bacterial Strains—E. coli HB101 was used for propagation and preparation of various plasmid constructs. E. coli FJP10 (18) and E. coli IY28,4 which contain a chromosomal ftsY gene under the arabinose promoter, were used for FtsY complementation experiments. E. coli BL21 was used for the overexpression of SRP and FtsY mutants. E. coli UT5600 was used for studying membrane association of FtsY mutants, ribosomes, and SRP.

Interaction of NG Mutants with Guanosine Nucleotides—Dissociation constants were determined by fluorescence titration experiments as described previously (19). GTP hydrolysis experiments were carried out as described previously (15). Briefly, 0.5 nmol of protein were incubated in the presence of 0.5 nmol of SRP and GTP at a concentration of 1 mM (dotted with approximately 400 kBq of [γ-32P]GTP). The reactions (total volume of 250 μl) were monitored for 30 min, and the total amount of GTP hydrolyzed was determined.

Stimulation by liposomes was determined as described above (in the presence of SRP), after preincubation with preformed liposomes (70% phosphatidylethanolamine and 30% phosphatidylglycerol). Large unilamellar vesicles were prepared as described previously (14). Protein concentrations were determined by calculation of the absorption coefficients at 280 nm according to Gill and von Hippel (20) and measured in 5.4 M guanidinium HCl, 20 mM phosphate, pH 6.5.

Cell Fractionation, Gel Electrophoresis, and Immunoblotting—Harvested cultures were washed in buffer containing 50 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (for SDS-PAGE experiments) or 25 mM HEPES, pH 7.5, 10 mM MgOAc, 50 mM KAc, 5% sucrose, 250 mM NH4Cl, 0.1 mM EDTA (for membrane purification experiments), and resuspended in the same buffer. The cell suspensions were sonicated, and cell debris was removed by centrifugation (5 min at 13,000 rpm). The membranes were collected by ultracentrifugation (45 min at 150,000 g), and the supernatants were incubated with pre-equilibrated Ni-NTA. The dissociation of NG-His6 or NG-SRP complexes was studied as follows: cytolsolic fractions of E. coli overexpressing SRP (10 μg of total protein) were incubated (30 min at room temperature) with purified His6-tagged NG mutants (10 μg) with or without 2 mM GMP-PNP. After incubation the NG-SRP complexes were isolated using Ni-NTA and then separated by SDS-PAGE.

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RESULTS

Nucleotide Binding and Hydrolysis by NG and NG+1—To examine their enzymological properties, we purified and analyzed NG and NG+1 for their nucleotide binding activities and GTP hydrolysis in the presence of SRP. The binding affinities of NG+1 and NG for nucleotides were determined by measuring the steady state dissociation constant of each mutant for GDP (Fig. 1, A and B) and GTP (Fig. 1, C and D), using tryptophan fluorescence (19). No significant differences in the dissociation constants of the mutants were detected, although with both GDP and GTP, the change in fluorescence intensity of the single tryptophan 343 is somewhat smaller in NG+1, compared with NG. In the presence of SRP, GTP hydrolysis is almost identical for both NG and NG+1 (Fig. 1E). Therefore, nucleo-

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4 I. Yosef and E. Bibi, unpublished observations.
tide binding and hydrolysis by NG and NG+1 in vitro do not provide a satisfactory explanation for the observed differences in their functionality in vivo.

Nucleotide-mediated Complex Formation (and Dissociation) of NG or NG+1 with SRP—Previous biochemical studies of FtsY have established the importance of its functional association with SRP in a guanosine nucleotide-dependent manner and its ability to hydrolyze GTP (21, 22). Recent x-ray structures show a tight complex between the NG domains of FtsY and SRP54 (Ffh) from Thermus aquaticus and Sulfolobus solfataricus with bound nucleotides (23, 24).5 To search for possible functional differences between the truncated FtsY mutants, we characterized the interaction of NG and NG+1 with SRP. Both NG·His₆ and NG+1·His₆ were purified and tested for their ability to bind SRP in extracts prepared from cells overexpressing SRP (both Ffh and 4.5 S RNA). In these cells, the amount of SRP is ~50-fold higher than that in wild-type cells, and more than 70% of the SRP is found in the soluble fractions (data not shown). As shown in Fig. 2A, NG and NG+1 bind SRP to a similar extent in a GMP-PNP-dependent manner. In both cases much less complex is formed in the absence of GMP-PNP, presumably because the SRP-containing extracts also contain small amounts of nucleotides. To analyze the GMP-PNP-dependent interaction quantitatively, we assayed equilibrium binding by using a fixed amount of SRP-containing ultracentrifuged cell extract (devoid of membranes and ribosomes) and increasing concentrations of purified NG or NG+1 (Fig. 2B). The results indicate specific binding that reaches saturation at about the same concentration for NG or NG+1, with an estimated $K_d$ for both mutants in the range of ~0.22–0.25 μM. This $K_d$ value is in good agreement with the value obtained for full-length FtsY (25).

As shown in Fig. 2A, efficient association of NG or NG+1 with SRP requires the slow hydrolyzable GTP analog. Previously, it was demonstrated that GTP hydrolysis drives the dissociation of SRP from its receptor (21, 26). Also shown was that the SRP-FtsY complex dissociation occurs even in the absence of membranes and upon exchange of the stabilizing nonhydrolyzable guanosine nucleotide with GDP (27). Here we tested whether NG and NG+1 behave similarly regarding their release from SRP in a GTP-dependent manner. Following the incubation of the purified NG·His₆ or NG+1·His₆ with SRP extracts in the presence of GMP-PNP, the complexes were immobilized by Ni-NTA beads. Next, the beads were washed and incubated with either GMP-PNP (Fig. 2C, lanes 1) or GTP (Fig. 2C, lanes 2). Finally, the beads were collected, and their elution samples were analyzed by immunoblotting with antibodies against Ffh. As shown, SRP is released from either NG or NG+1, in a GTP-dependent manner. In summary, these results indicate that the formation and dissociation of the SRP-SRP receptor complex in solution is preserved both with the active (NG+1) and the inactive (NG) mutants of FtsY.

Interaction of NG and NG+1 with the Translocon—Previous studies suggested two sites of interaction between FtsY and the cytoplasmic membrane in E. coli lipids (14, 15, 28) and the

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5 G. Bange and I. Sinning, unpublished data.
translocon (7, 8). To test possible differences between NG and NG/H11001 in their association with the translocon, we performed co-precipitation experiments with solubilized membranes, utilizing equal amounts of overexpressed His$_6$-tagged NG and NG/H11001 and Ni-NTA resin. As shown in Fig. 3, both NG and NG/H11001 co-precipitate SecY. Surprisingly, however, in addition to SecY, Ffh was also precipitated by NG or NG/H11001, despite our prediction and previous studies (8). Notable, NG co-precipitated substantially more Ffh than NG/H11001, suggesting that Ffh might get stuck on the NG membranes together with NG and/or the translocon (see below).

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**Cellular Distribution of NG and NG/H11001**—Because mutant NG does not support growth in the FtsY complementation assay (17) (see also Fig. 7), despite the fact that its apparent interactions with nucleotides, SRP, and the translocon were not impaired, another function of FtsY must be compromised in this mutant. Previously, it was shown that FtsY distributes between the cytosolic and membrane fractions of disrupted cells (10). Although at steady state most of the FtsY molecules are cytosolic, an appreciable amount of a membrane-bound form cannot be released from the membrane even by elevated ionic strength, alkaline pH, or chaotropic agents (10), suggesting that a strong interaction with the membrane takes place in vivo. To address the possibility that the inactive mutant NG might be defective in its ability to interact with the membrane in vivo, we examined the cellular distribution of both mutants. Wild-type *E. coli* cells transformed with plasmids encoding IPTG-inducible versions of either NG/H11001 or NG were induced at the mid-log phase. As shown in Fig. 4A, the growth of NG cells was arrested, possibly because of a dominant negative

**FIGURE 3. Co-purification of NG/NG+1 with the translocon.** NG-His$_6$ or NG/H11001-His$_6$ were purified by nickel-NTA from 1% DDM-solubilized membranes. Elution samples containing equivalent amounts of NG and NG/H11001 were analyzed by Western blotting using antibodies against FtsY, Ffh, and the translocon subunit, SecY. As a control, the procedure was repeated with untagged NG+1.
Effect of overexpressed NG.6 The cells were harvested after 5.5 h, and then membranes were purified by floatation in a stepwise density gradient and analyzed by immunoblotting with anti-FtsY antibodies. Surprisingly, an unanticipated behavior for NG was observed (Fig. 4B). Instead of an impaired membrane association as predicted, the results revealed that the inactive NG mutant accumulates on the membrane in contrast to NG+1, of which only a small fraction co-purifies with membranes (see also Ref. 17).

Previously, a linkage was identified between the amount of membrane-bound FtsY and ribosomes under various experimental conditions (3, 6), suggesting that FtsY plays a direct role in ribosome targeting and docking. Therefore, we tested whether NG retains these essential functions of the receptor. This was done by subjecting membranes purified from cells expressing NG+1 or NG to Western blotting with antibodies against proteins of the large and small ribosomal subunits. Figure 4C shows that remarkably, ribosomes also accumulated on NG membranes, compared with NG+1 membranes, suggesting that the function of NG during the early stages of the pathway (ribosome targeting and docking) is not defective, whereas its function at a later step is compromised.

Does the accumulation of NG on the membrane reflect non-relevant membrane aggregation of this toxic protein in arrested cells? Although this is unlikely because ribosomes also accumulate on membranes of NG-expressing cells, we examined further the cellular distribution of NG, ribosomes, and also Ffh, at various times after induction of expression of NG or NG+1 (Fig. 5). The results show that in NG cells, both NG and ribosomes accumulate, and their membrane-bound amount increases with time. Interestingly, under the same conditions, the results show that although the amount of Ffh in NG+1 membranes decreases during growth, it remains relatively constant in NG membranes (Fig. 5, bottom panel; see also Fig. 3).

These results suggest that whereas SRP releases membrane-associated ribosome-NG+1 complexes efficiently, it is unable to release NG-associated ribosomes. These observations raise the possibility that NG behaves differently in solution (Figs. 1 and 2) than on the membrane, regarding its functional interaction with SRP. A possible explanation could be that the GTPase-mediated dissociation of the NG-SRP complex, compared with that of NG+1-SRP, is less efficient on the membrane than in solution.

Effect of Overexpressed SRP on the Cellular Distribution and Biological Function of NG—The experiments described above utilized wild-type E. coli cells overexpressing NG. The amount of the natively expressed SRP (Ffh and 4.5 S RNA) in these cells is significantly lower than that of overexpressed NG. Assuming that the efficiency of dissociation of the membrane-bound NG-SRP complex is lower than that of NG+1-SRP, we reasoned that overexpressing SRP might affect the equilibrium toward more dissociation in vivo. To test this possibility and the apparent effect of SRP on the cellular distribution of NG and ribosomes, we grew cells simultaneously overexpressing both NG and SRP. As shown in Fig. 6A, overexpression of SRP clearly improves the growth of NG-expressing cells in a reproducible manner. The membranes purified from these cells were analyzed by Western blotting (Fig. 6B), and the results show that overexpression of SRP indeed reduces the amount of membrane-associated NG and ribosomes.

This observation led to the suggestion that high SRP concentrations might also restore the function of NG in vivo, and this was examined by FtsY complementation assays. To this end, FtsY depletion cells were transformed with plasmid(s) encoding NG alone or NG together with SRP (Ffh and 4.5 S RNA). The transformants were grown in LB broth (Fig. 7A) or plated on LB agar plates (Fig. 7B) with or without arabinose (the inducer of chromosomal FtsY) and IPTG (for induction of NG). The expression of FtsY, NG, and Ffh in these cells is shown in Fig. 7C. Results of this assay indicate that although NG alone is unable to complement FtsY depletion, co-expression of SRP had a marked positive effect on the growth, both in liquid and solid media.

Effect of Lipids on the GTPase Activity of NG and NG+1—What is the reason for the proposed inefficient dissociation of the membrane-bound NG-SRP complex? To address this critical issue, we tested the effect of lipids on the enzymological properties of various mutants, because FtsY-SRP dissociation requires GTP hydrolysis. It has been previously shown that phospholipids stimulate the GTPase activity of full-length FtsY, but not NG (15). Here we repeated these experiments utilizing SRP together with either NG, NG+1, or FtsY, in the presence of liposomes containing E. coli-mimicked phospholipid mixture (70% phosphatidylethanolamine and 30% phosphatidylglycerol). The results show that the GTPase activity of NG-SRP is not stimulated by phospholipids, whereas NG+1- and FtsY-SRP are stimulated (Fig. 8), suggesting that indeed, the interaction of NG with SRP on the membrane differs from that

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6 E. Malul and E. Bibi, unpublished observations.
observed in solution (Fig. 1E), possibly because of its impaired capacity to respond to lipids.

**DISCUSSION**

Previous studies of the overall domain structure of FtsY and the functional importance of its subdomains have suggested that both the A- and N-domains play a role in membrane association of the receptor (14–16). This interaction is dominated by electrostatic forces and requires acidic phospholipids (15). Later, our studies revealed that the A-domain (residues 1–195) is dispensable in vivo, raising the possibility that essential contact with the membrane is mainly dictated by the N-domain. This notion is supported by the observation that shortening the N-domain of the functional mutant (NG/H1001) by one N-terminal residue (NG) abolished the in vivo activity of the receptor (17).

The proposal that FtsY is peripherally associated with membranes was first raised by Luirink et al., (10), who noted that at steady state, FtsY is distributed between the cytoplasm and the membrane. However, FtsY has no known membrane anchor partner homologous to the mammalian β-subunit of the SRP receptor. Nevertheless, further studies supported the original suggestion that FtsY functions as a membrane-bound receptor (11, 12). The exact mechanism by which FtsY associates with the membrane is yet not fully understood. Previous studies showed that membrane attachment of FtsY, like that of SecA (29, 30), involves lipids (15) and proteins (8). Strong support for
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functional interaction of FtsY with lipids, preferentially acidic ones, was obtained by in vitro studies, which demonstrated marked stimulation of the FtsY GTPase activity by lipids (15). On the other hand, it has been shown that a postulated FtsY membrane assembly domain binds to E. coli inner membrane inverted vesicles via an interaction with a trypsin-sensitive component (28) and that FtsY is associated with the translocon (7, 8). Based on these and additional data, a model was proposed that FtsY binding to the membrane occurs initially through phospholipid binding, followed by targeting to translocation sites via an interaction with an unknown membrane protein. However, our present studies suggest that interaction with lipids is required only at a late stage in the pathway and not for membrane targeting and docking of the receptor.

To gain functionally related mechanistic insight into the various interactions of FtsY, we investigated the functional differences between active NG+1 and inactive NG, and the results enabled us to identify distinct and separable functions of the receptor in (i) targeting of ribosomes to the membrane and (ii) release of the ribosomes. Whereas NG retains the receptor role in the ribosome-targeting step, it is unable to function during the release of ribosomes from their docking site, possibly because of its defective functional response to membrane lipids. These conclusions are supported mainly by the following observations: (i) The inactive mutant NG accumulates on the cytoplasmic membrane, together with ribosomes and SRP, indicating proper targeting function. (ii) However, the accumulation per se indicates that the release of these complexes from the membrane is defective. (iii) The GTPase activity of the lipid-associated SRP-NG complex is substantially lower than that measured for NG+1 or FtsY.

These results also indicate that deletion of one amino acid from the N terminus of NG+1 damages the lipid responsive domain of the receptor. As discussed previously (31), the N-terminal peptide of the FtsY N-domain protrudes into a conserved hydrophobic interface (32) between the N- and G-domains. In addition, an intimate linkage has been identified between this peptide and the C-terminal helix in FtsY of different organisms, also recently including NG+1 from E. coli. As such, in addition to sensing lipids, this peptide might be able to transmit lipid-mediated structural information to the active site of FtsY (probably via motif IV of the G-domain).

Although it has not yet been fully elucidated whether the receptor must associate with lipids for its physical attachment to the E. coli cytoplasmic membrane, our results strongly suggest that regulation of FtsY GTPase activity by lipids is essential for proper function only at a later step of the pathway. A possible scenario would be that the receptor associates with a membrane protein (the translocon or another receptor; see Refs 33 and 34), and in addition, it has a membrane-responsive domain that is not essential for membrane attachment of the receptor. This domain would then be responsible for transferring lipid-mediated regulatory information to the receptor active site. Why would that be important for the receptor’s function? We favor the possibility that there is a mechanism that delays the dissociation of the SRP-FtsY complex and transfer of the associated ribosomes to the translocon, until a free translocon is ready to accept the nascent chain. In vivo, one such scenario would be that prior to its association with the free translocon, the receptor is kept nonresponsive to lipids. This repressed form of the receptor can be activated and converted into a lipid-responsive form only upon interaction with the free translocon and release of the hydrophobic nascent chain from SRP. Although speculative, we propose that lipid receptor interaction in E. coli might fulfill a task conceptually analogous to that proposed for the mammalian β-subunit of the SRP receptor of the SRP receptor (35) in regulating the last step of targeting, by utilizing an entirely different mechanism. Currently, this hypothesis is being evaluated by in vivo and in vitro studies.

No external GTPase-activating proteins have so far been identified for FtsY or Ffh, although such regulation is probably needed to prevent premature SRP cycles during the biogenesis of membrane proteins. Recently, an interesting mechanism was proposed for constitutive Ffh activation by the 4.5 S RNA tetraloop (36). It was suggested that activation of Ffh by the RNA is suppressed by hydrophobic nascent chains but only when SRP associates with ribosomes translating SRP substrates. Similarly, our studies raise the interesting possibility that lipids provide a functionally relevant GTPase-activating mechanism for FtsY, which regulates a later step in the pathway. Interestingly, although certain 4.5 S RNA mutants (in the tetraloop) were found inactive in in vivo complementation studies (36), different results were obtained by Jagath et al. (37). Siu et al. (36) suggest that this apparent contradiction could be resolved because different expression levels of the mutants were utilized in the two studies and that activation of Ffh by these mutants was only decreased and could be compensated for by higher amounts of the RNA. A similar scenario was observed in our studies, where overexpression of SRP partially restored the ability of NG to complement FtsY depletion in vivo. These results suggest that the expression level of components of the SRP machinery might be tightly controlled in vivo to assure proper regulation of the SRP-related GTPases Ffh and FtsY during different stages of the targeting pathway.

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