Escherichia coli Signal Recognition Particle Receptor FtsY Contains an Essential and Autonomous Membrane-binding Amphipathic Helix

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Escherichia coli membrane protein biogenesis is mediated by a signal recognition particle and its membrane-associated receptor (FtsY). Although crucial for its function, it is still not clear how FtsY interacts with the membrane. Analysis of the structure/function differences between severely truncated active (NG+1) and inactive (NG) mutants of FtsY enabled us to identify an essential membrane-interacting determinant. Comparison of the three-dimensional structures of the mutants, combined with site-directed mutagenesis, modeling, and liposome-binding assays, revealed that FtsY contains a conserved autonomous lipid-binding amphipathic α-helix at the N-terminal end of the N domain. Deletion experiments showed that this helix is essential for FtsY function in vivo, thus offering, for the first time, clear evidence for the functionally important, physiologically relevant interaction of FtsY with lipids.

Escherichia coli FtsY is a membrane-associated homologue of the eukaryotic SRP receptor α-subunit. Together with the universally conserved SRP particle, FtsY is responsible for the cotranslational targeting of many integral membrane proteins that are consequently inserted into the membrane via the SecYEG translocon (1, 2). For proper function, FtsY interacts with the SRP protein Ffh in a nucleotide-dependent manner (reviewed in Ref. 3). The structure of the complex between the two NG domains of Ffh and FtsY has recently been resolved by x-ray crystallography (4, 5). Targeting of ribosomes to the cytoplasmic membrane in E. coli is dependent on the expression of FtsY (6). In addition, under FtsY-depletion conditions, the expression of polytopic membrane proteins such as LacY (7), SecY (6), and MdfA§ is repressed. Other studies demonstrated that the SRP receptor forms a complex with membrane-bound ribosomes at the endoplasmic reticulum (8) and in E. coli, as well as in the absence of SRP or the translocon (9). In addition, recent studies identified interactions between the SRP receptor and the translocon in the endoplasmic reticulum (10) and in E. coli (11, 12). These observations thus underscore the central role of FtsY in ribosome targeting and biogenesis of membrane proteins (13). However, despite extensive genetic, biochemical, and structural studies, important aspects of the function of FtsY are not yet fully understood. FtsY contains three distinct domains: the C-terminal N- and G-domains (together 302 residues long), which constitute a universally conserved SRP-GTPase (14), and an N-terminal A-domain (195 residues long) that was shown to be dispensable under various growth conditions (15).

At steady state, FtsY is distributed between the cytoplasm and the membrane (16), and it has no known membrane anchor partner homologous to the mammalian β-subunit of the SRP receptor (SR-B), which coordinates the transfer of ribosomes translating SRP substrates to the translocon (17). Nevertheless, various studies have suggested that FtsY functions as a membrane-bound receptor (18, 19). Interestingly, A-domain-truncated FtsY versions exhibit strong affinity for membrane lipids (20, 21), possibly through the N-domain (22), an interaction that seems to be dominated by electrostatic forces (21). However, the precise lipid-interacting domain in FtsY has not been defined, and the mechanism and functional role of lipid binding to the receptor remain elusive.

Interestingly, an A-domain-truncated version of FtsY (termed NG+1) that is functional in vivo was identified by deletion analysis (15). Removal of the N-terminal amino acid Phe2 (hereafter termed Phe196§) according to the sequence of wild-type FtsY from NG+1 inactivated the receptor (termed NG). In the present study, we describe comparative structure/function analyses of the two mutants, which shed light on the mechanism underlying FtsY interaction with membrane lipids and revealed an essential and autonomous lipid-binding domain of the receptor.

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The atomic coordinates and structure factors (code 2QY9) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S2.

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4 The abbreviations used are: SRP, signal recognition particle; Trx, thioredoxin; LUV, large unilamellar vesicle; MTS, membrane-targeting sequence.
5 G. Bange and I. Sinning, unpublished data.

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EXPERIMENTAL PROCEDURES

Materials—Antibodies to FtsY were described previously (6). Bacterial Strains—E. coli HB101 was used for propagation and preparation of various plasmid constructs. E. coli FJP10 (23), harboring ftsY under control of the araB promoter, was used for FtsY-depletion experiments. E. coli BL21 was used for the overexpression of SRP and FtsY mutants.

Crystalization and Structure Determination of NG+1—Crystals of NG+1 were obtained by the vapor-diffusion method as described previously (24). Structure was solved by molecular replacement with the NG structure (Protein Data Bank code 1FTS) as a search model (14) and using CCP4 (25). The structure was manually built using the program O (26) and refined with CNS (27). Details of the refinement statistics are given in supplemental Table S2.

Construction of ftsY Mutants—Plasmids pET-14b (Novagen) and pTfTsY (18) were digested with XhoI and NcoI, respectively, treated with Klenow, and cleaved again with HindIII. The large fragment released from pTfTsY was ligated to produce plasmid pET-14b(6His-fTsY). The resultant plasmid was cut with Ncol and HindIII, and the small fragment was ligated with the large fragment released from pGEX-2T (Amersham Biosciences) cut with the same enzymes to create plasmid pGEX(6His-fTsY). Mutant NG was generated by PCR with pGEX(6His-fTsY) as a template, using the 5′ primer: 5′-ttattcagttccgctctggccagctg and a homologous 3′ primer. The PCR fragment was cloned into pGEX(6His-fTsY) by Ncol andSacII digestion, to create pGEX(NG). Mutant NG+1 was created by two stages of PCR using pGEX(FtsY-NG) as a template with two internal primers: 5′-agttacatattgctgcagctccgagacgg and 5′-tcagccgctcagacagtagctgacgccgtttccc, and two external primers: 5′-tctgctctggagTCGACgattctcctcggctgcagctg and 5′-tctgctctggagTCGACgattctcctcggctgcagctg. The final PCR product was cloned into pGEX(6His-fTsY) by Ncol and SacII digestion, to create pGEX(NG+1). The same vector was prepared from pGEX(FtsY-NG) by digestion with Ncol and Aval, Klenow treatment, and self-ligation of the large fragment. The indicated mutations were created by site-directed PCR mutagenesis using pGEX(NG) or pGEX(NG+1) as templates. pGEX(NG+1)(R198C/D479C) was prepared by digesting pGEX(NG+1)(R198C) with BspEI and Bsu36I and ligating the 0.5-kb fragment with the 4.8-kb fragment of pGEX(NG+1)(D479C) digested with the same enzymes. Plasmids used for protein expression and purification were prepared as follows: the coding region of Laciα was amplified by PCR on pGEX-2T with the 5′ primer 5′-attaataacagttagctcagccatcataagggttcg and the 3′ primer 5′-attataacagttagctcagccatcataagggttcg and the 3′ primer 5′-attataacagttagctcagccatcataagggttcg, and both the PCR products and pT7-5(NG) and pT7-5(NG+1) were digested with MluI and BspEI and ligated to create pT7-5(NG-6H) and pT7-5(NG+1-6H). pT7-5(NG+1-6H) was used for expression and purification of NG+1 for crystallization. To construct tagged mutants we used a 5′ primer, 5′-ataataaagttcctgctccatcataagggttcg and a 3′ primer, 5′-ttggtgtctacaacacccgagctg for PCR amplification using as templates pGEX(NG+1)(R198C), pGEX(NG+1)(F196A), and pGEX(NG+1)(F196D). Each of the PCR products and pT7-5(NG+1-6H) were digested with EcoRI and BspEI and ligated to produce pT7-5(NG+1-R198C-6H), pT7-5(NG+1-F196A-6H), and pT7-5(NG+1-F196D-6H). All the mutations were verified by sequencing through the PCR-generated segments, or the Klenow-treated ligation junctions.

For mutating the amphipathic helix in NG+1 (NG+1/quadro), pTfTsY was digested with XhoI and NcoI, respectively, treated with Klenow, and cleaved again with HindIII. The large fragment released from pTfTsY was ligated to produce plasmid pET-14b(6His-fTsY). The resultant plasmid was cut with Ncol and HindIII, and the small fragment was ligated with the large fragment released from pGEX-2T (Amersham Biosciences) cut with the same enzymes to create plasmid pGEX(6His-fTsY). Mutant NG was generated by PCR with pGEX(6His-fTsY) as a template, using the 5′ primer: 5′-ttattcagttccgctctggccagctg and a homologous 3′ primer. The PCR fragment was cloned into pGEX(6His-fTsY) by Ncol and SacII digestion, to create pGEX(NG). Mutant NG+1 was created by two stages of PCR using pGEX(FtsY-NG) as a template with two internal primers: 5′-agttacatattgctgcagctccgagacgg and 5′-tcagccgctcagacagtagctgacgccgtttccc, and two external primers: 5′-tctgctctggagTCGACgattctcctcggctgcagctg and 5′-tctgctctggagTCGACgattctcctcggctgcagctg. The final PCR product was cloned into pGEX(6His-fTsY) by Ncol and SacII digestion, to create pGEX(NG+1). A plain vector was prepared from pET-14b (Novagen) that encoded the four mutations: sense, 5′-ctgctctggagTCGACgattctcctcggctgcagctg and antisense, 5′-ctgctctggagTCGACgattctcctcggctgcagctg. The PCR product was then treated with DpnI endonuclease for the digestion of methylated, non-mutated parental DNA templates, purified from 0.8% agarose gel, and ligated. Next, the ligation mixture was transformed into E. coli HB101, and purified plasmids were verified by DNA sequencing. A similar procedure was utilized for deleting the helix from full-length FtsY (Phe196-Lys207).

Growth Experiments and FtsY Complementation Studies—Cultures were grown at 37 °C in LB medium, supplemented with ampicillin (100 μg/ml), kanamycin (10 μg/ml), chloramphenicol (10 μg/ml), and spectinomycin (33 μg/ml) when necessary. For growth experiments in liquid media, cells were grown overnight at 37 °C, and then diluted to 0.01 A600. In experiments with E. coli FJP10, cells were diluted to A600 of 0.025. FtsY complementation experiments were executed by plating transformed E. coli FJP10 (23), harboring various constructs, on LB agar plates with or without arabinose, the inducer of the chromosomal ftsY.
the 3'-end, and an NcoI site was introduced at the 5'-end. The resulting PCR product was digested with Ncol and BamHI and ligated into vector pETM13 (kindly provided by Gunter Stier) digested with the same enzymes. NG +1-Trx was constructed by using the PCR template described above and the primers 5'-ggaaagccatggtgccgctgctaaaaacagattaattacccctgcttg and 5'-gggtgggccaggttagcgtcgagg, which introduce a 5'-end PciI restriction site and a 3'-end BamHI site and a 6-histidine tag. The PCR product was digested with PciI and BamHI and then ligated into the pET24d vector (Novagen) cut by Ncol and BamHI. NG-Trx was constructed by using the PCR template described above and the primers 5'-ggaaggccatgcgcgcctgaaacgcagcctgttaaaaaccaaagaaaatcctcggtagcgataaaattattcacctg and 5'-ccggtggaagaaatcgctatcgagggtctgggtgggccaggttagcgtcgagg, which introduce a 5' Ncol cleavage site, a 3' BamHI cleavage site and a 3'-end PciI cleavage site and a 5'-end BamHI cleavage site. The PCR product was digested with Ncol and BamHI and ligated into the pET24d vector (Novagen) cut by Ncol and BamHI. The final constructs were confirmed by DNA sequencing. Proteins were overexpressed in E. coli BL21(DE3), nucleic acids were precipitated after cell lysis with prolammine sulfate, and the proteins were purified by nickel affinity chromatography and gel filtration as described for NG FtsY (24).

Expression and Purification of the A-domain (FtsY1–188)—A Factor Xa cleavage site (Q185I, Q187G, and E188R) was introduced into the plasmid containing FtsY6His (Luirink et al., 16), using the QuickChange kit (Stratagene) and the following primer pair: forward primer, 5'-cctggtgcagcctgtaaattattcacctg and reverse primer, 5'-ctggtgcagcctgtaaattattcacctg, which introduce a 5'-end NcoI site and a 3'-end BamHI site. The PCR product was digested with NcoI and BamHI and then ligated into vector pETM13 (kindly provided by Gunter Stier) cut by Ncol and BamHI. The final constructs were confirmed by DNA sequencing. Proteins were overexpressed in E. coli BL21(DE3), nucleic acids were precipitated after cell lysis with prolammine sulfate, and the proteins were purified by nickel affinity chromatography and gel filtration as described for NG FtsY (24).

Results—Preparation of liposomes and flotation ultracentrifugation were carried out essentially as described previously (21). Briefly, purified FtsY, NG, NG+1, Trx, NG-Trx, or NG +1-Trx (20 µg) were incubated for 20 min at 37 °C in the absence or presence of 125 nmol (~100 µg) of LUVs prepared from synthetic POPE (2-oleoyl-1-palmitoyl-sn-glycerol-3-phosphoethanolamine) and POPC (1-palmitoyl-2-oleoyl-sn-glycerol-3-(phospho-rac-(1-glycerol))-ammonium salts) phospholipids (Sigma) in assay buffer (20 mM Tris/HCl, pH 8, 1 mM CaCl2, 100 mM NaCl). Cleavage products were isolated by gel filtration on an S200 column (Amersham Biosciences) in 20 mM Tris/HCl, pH 8, 100 mM NaCl, 10 mM MgCl2, 100 mM NaCl. Cleavage products were confirmed by electrospray ionization-mass spectrometry and in-line static-light scattering (Wyatt Technologies).

Binding to LUVs—Preparation of liposomes and flotation ultracentrifugation were carried out essentially as described previously (21). Briefly, purified FtsY, NG, NG+1, Trx, NG-Trx, or NG +1-Trx (20 µg) were incubated for 20 min at 37 °C in the absence or presence of 125 nmol (~100 µg) of LUVs prepared from synthetic POPE (2-oleoyl-1-palmitoyl-sn-glycerol-3-phosphoethanolamine) and POPC (1-palmitoyl-2-oleoyl-sn-glycerol-3-(phospho-rac-(1-glycerol))-ammonium salts) phospholipids (Sigma) in assay buffer (20 mM Tris/HCl, pH 8, 100 mM NaCl, 10 mM MgCl2, 100 mM NaCl). Cleavage products were isolated by gel filtration on an S200 column (Amersham Biosciences) in 20 mM Tris/HCl, pH 8, 100 mM NaCl, 10 mM MgCl2, 100 mM NaCl. Cleavage products were confirmed by electrospray ionization-mass spectrometry and in-line static-light scattering (Wyatt Technologies).

The expression of selected mutants harboring hydrophobic and large (leucine, isoleucine, and tryptophan), small (alanine), or charged (lysine) residues at position 196 was examined by immunoblotting, and their activity was assayed by complementation of FtsY-depleted cells.

**TABLE 1**

Characterization of the various FtsY mutants

| Mutant     | Activity | Expression | N-terminal sequence |
|------------|----------|------------|---------------------|
| wt FtsY    | ++       | ++         | ARLK                |
| NG         | -        | ++         | MFACLK              |
| NG+1       | ++       | ++         | MFACLK              |
| NG+1(R198C)| --       | ++         | MIARLKV             |
| NG+1(D479C)| ++       | ++         | AARLKV              |
| NG+1(D479C)/D479C| -- | ++ | MIARLKV |
| NG+1(D479A) | ++ | ++ | MIARLKV |
| NG+1(D479R)| ++ | ++ | MIARLKV |
| NG+1(F196A)| -- | ++ | MIARLKV |
| NG+1(F196L)| ++ | ++ | MIARLKV |
| NG+1(F196L)| ++ | ++ | MIARLKV |
| NG+1(F196K)| -- | ++ | MIARLKV |
| NG+1(F196K)| -- | ++ | MIARLKV |
| NG+1(F196L/A197E)| -- | ++ | MIARLKV |

**RESULTS**

Site-directed Mutagenesis of the N Terminus of NG +1—As described previously (15), the functional NG +1 mutant of FtsY was created by insertion of a single phenylalanine residue at position 2 of the inactive mutant NG (Phe196). The dramatic functional difference between NG and NG +1 was now investigated by mutating this phenylalanine in an attempt to explore the importance of the chemical properties at the N terminus of NG +1. The expression of selected mutants harboring hydrophobic and large (leucine, isoleucine, and tryptophan), small (alanine), or charged (lysine) residues at position 196 was examined by immunoblotting, and their activity was assayed by complementation of FtsY-depleted cells (Fig. 1). The results (summarized in Table 1) indicated that a large hydrophobic residue is required at position 196. However, because additional truncation studies and characterization of other mutants indicated that the length of the N terminus might also be important (data
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The N- and C-terminal helices are in proximity (Fig. 2D), and this interaction might be involved in regulation of the GTPase, as suggested earlier (29–31). Taken together, comparison of the three-dimensional structures and the results presented above enabled us to draw the following conclusions: (i) the Arg\(^{198}\)--Asp\(^{479}\) interaction might be important for the functional assembly of the receptor, (ii) this interaction might also facilitate the formation of a regular \(\alpha\)-helix, and (iii) the \(\alpha\)-helical N terminus might be important for NG+1 function. These possibilities were explored further (see below). Importantly, the recently solved structure of the full-length FtsY\(^7\) revealed that the N-terminal \(\alpha\)-helix of NG+1 is part of an authentic structural element of the wild-type receptor, where the helix is even longer. Moreover, a comparison with recent structures of FtsY from *Mycoplasma mycoides* (29) and *Thermotoga maritima* (Joint Center for Structural Genomics, PDB entry code: 1vma) indicates that this helix is conserved in FtsY homologues.

**Site-directed Mutagenesis**—The interaction between Arg\(^{198}\) and Asp\(^{479}\) (Fig. 2C) was not observed in the NG structure (14) and could be of structural and functional relevance. Sequence alignment shows that Asp\(^{479}\) is universally conserved, whereas Arg\(^{198}\) is partially conserved among SRP receptors (data not shown). We performed non-conservative replacements at positions Asp\(^{479}\) and Arg\(^{198}\), independently and simultaneously, to analyze the importance of these residues. The mutants were expressed (Fig. 3A), and their in vivo functions were tested by complementing FtsY depletion.

Surprisingly, replacement of the fully conserved residue Asp\(^{479}\) for cysteine (D479C), alanine (D479A), or even arginine (D479R) did not have any appreciable effect on NG+1 function in vivo (Fig. 3B). In contrast, replacing residue Arg\(^{198}\) had a dramatic inhibitory effect. Mutation R198C strongly inhibited the function of NG+1, and mutant R198D could not even be constructed, possibly due to its toxicity even under non-induced, basal expression levels. These results indicated that the observed interaction between Arg\(^{198}\) and Asp\(^{479}\) is not essential for NG+1 function, but might contribute to the stability of the NG+1 structure. The observation that the functional mutant D479R could not be crystallized, due to aggregation of the purified protein at elevated concentrations, might further support this

\(^7\) R. Parlitz and I. Sinning, unpublished data.

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FIGURE 2. Comparison of the structures of FtsY truncation mutants NG and NG+1. A, the structure of the NG domain of FtsY (1FTS) (14). B, the structure of NG+1 with the extension at the N terminus marked in red (current study, 2QY9). C, structure of NG+1 showing the nucleotide-binding pocket. GTP is placed according to Montoya et al. (14). The conserved residues Arg\(^{198}\) (N terminus) and Asp\(^{479}\) (G5 closing loop) show a polar interaction. D, the space-filling model of NG+1 with the N-terminal and C-terminal helices marked in red and green, respectively. The color code is as in A and B. The figure was generated using PyMOL (45).

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not shown), we investigated f-Met processing of selected mutants. Briefly, NG, NG+1, and several of the active and non-active mutants were purified using a C-terminal His\(_6\) tag, and the purified proteins were analyzed by N-terminal sequencing. As shown in Table 1, NG+1 and the functional constructs retained their N-terminal methionine. In contrast, the inactive mutants lost this methionine (NG, NG+1(F196A)). Together, the results suggest that the length of the N terminus of NG might be important, as well as a large hydrophobic residue at position 196. To evaluate these conclusions and investigate possible structural requirements at the N terminus of NG, we crystallized NG+1.

**Structural Differences between NG and NG+1**—In the crystal structure of NG from *E. coli* FtsY the N terminus is not ordered (14). For a direct assessment of the observed differences between NG and NG+1, we crystallized and solved the structure of NG+1 at 1.9-Å resolution (supplemental Table S2). A comparison with the NG structure (1FTS (14)) revealed that, unlike NG (Fig. 2A), the N terminus of NG+1 is well ordered and forms a regular \(\alpha\)-helix (Fig. 2B). Apart from this N-terminal peptide, the overall structures of NG and NG+1 are identical (the root mean square deviation between the 295 C\(_\alpha\) atoms of residues 201–495 is 0.54 Å). Interestingly, the structure of NG+1 also reveals a polar interaction between Arg\(^{198}\) and Asp\(^{479}\) (Fig. 2C). Arg\(^{198}\) is located at the ordered N terminus of NG+1, and Asp\(^{479}\) is part of the closing loop (G5) of the

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**Note:** The reference to R. Parlitz and I. Sinning, unpublished data, indicates that the authors have access to additional unpublished information to support their conclusions.
notion, although additional studies are required to establish the structural role of the Arg<sup>198</sup>–Asp<sup>179</sup> interaction.

The N Terminus of NG+1 Forms a Class A Amphipathic Helix—A more detailed analysis of the N-terminal sequence of NG+1 reveals the presence of a class A amphipathic helix (Fig. 4). Class A amphipathic helices have a distinct charge distribution on their polar face, containing a negative charge that is typically flanked by positive charges. The positive charges interact preferentially with the negatively charged head groups of anionic phospholipids, thereby anchoring the helix to the membrane (32). The polar face of the N-terminal helix in NG+1 contains the characteristic positive charges (Arg<sup>198</sup>, Lys<sup>200</sup>, Arg<sup>210</sup>, Lys<sup>205</sup>, and Lys<sup>207</sup>) as well as a negative charge (Glu<sup>208</sup>). The hydrophobic face is mainly formed by Met<sup>195</sup>, Phe<sup>196</sup>, Leu<sup>199</sup>, and Leu<sup>203</sup>. The inactive mutants (Table 1) have significantly reduced hydrophobicity at their N termini (Fig. 4). NG lacks both Met<sup>195</sup> and Phe<sup>196</sup>. The F196A mutant lacks Met<sup>195</sup>, and the alanine at position 196 cannot compensate for the loss of the aromatic side chain. The F196K mutant should contain Met<sup>195</sup> according to the rules of f-Met processing (33), but replacing Phe<sup>196</sup> by a lysine destroys the amphipathic character of the helix. All in vivo active mutants have Phe<sup>196</sup> replaced by larger hydrophobic residues, in addition to Met<sup>195</sup> (as shown for F196I).

Multiple sequence alignments of FtsY show that a motif of 13 amino acid residues, which corresponds to the amphipathic helix observed in the NG+1 structure, is conserved in eubacteria, archaea, and chloroplasts (Fig. 5A). Yet we noticed that the negative charge (Glu<sup>208</sup>) is not fully conserved. It was proposed that, although the negative charge is not essential for interaction with lipids, when present, it enhances binding of the amphipathic helix to membranes by repulsion, pushing the helix further down in between the head groups and the carbon chains of the lipid layer (32). Interestingly, a similar functionally important motif (termed membrane-targeting sequence (MTS)) was described previously for the conserved cell division determinant MinD (34, 35). MinD is an ATPase closely related to FtsY, because both are members of the SIMIBI class of NTP-binding proteins (36). Notably, certain MTS sequences are similar to the amphipathic helix of FtsY (Fig. 5B). However, unlike in FtsY, MinD proteins harbor the amphipathic helix at their C termini.

Interaction with LUVs—The results described above strongly suggest that the N-terminal helix of NG+1 is involved in the documented interaction of the receptor with lipids (21). To test this possibility, we prepared LUVs consisting of 70% phosphatidylethanolamine and 30% phosphatidylglycerol to mimic the composition of E. coli membranes. Various FtsY constructs were purified and incubated with the LUVs, which were then purified by floatation centrifugation. The LUV-associated proteins were analyzed by SDS-PAGE and quantified by densitometry (see “Experimental Procedures”) (Fig. 6). FtsY binds to LUVs efficiently (~30%), unlike the A domain alone, which shows only little association with LUVs (~8%) (Fig. 6A), indicating that
The observed N-terminal helix of NG and NG

Discussion). These results confirm the hypothesis that the apparently affects the capacity of the receptor to bind lipids (see experiment (16). These results suggest that, despite the observation of 30% FtsY bound to IMVs in an in vitro translation (21), in which however lipid binding was assayed only qualitatively by Western blotting and the composition of the liposomes was different (see “Experimental Procedures”). The observed membrane interaction of full-length FtsY correlates nicely with the previously observed interaction of 30% FtsY bound to IMVs in an in vitro translation experiment (16). These results suggest that, despite the observation that the A-domain alone does not interact with LUVs, it apparently affects the capacity of the receptor to bind lipids (see “Discussion”). These results confirm the hypothesis that the N-terminal helix of NG+1 is required for interaction with lipids. The observed in vivo inactivity of the NG mutants lacking Met195 and Phe196 (Table 1 and Fig. 7) supports the importance of the hydrophobic face of the helix for this interaction.

To examine whether this segment is an autonomous lipid-binding element, we attached the conserved N-terminal peptides from NG and NG+1 to E. coli Trx, a soluble protein that does not bind to LUVs. The hybrids were then tested for their ability to bind LUVs. Fig. 6A shows that Trx and NG-Trx did not associate with liposomes, whereas NG+1-Trx bound efficiently. Taken together, these studies demonstrate that FtsY contains a transplantable, bona fide membrane-interacting sequence in the A/N-domain interface and that the hydrophobic residues at the N terminus of NG+1 are important for efficient interaction.

The Lipid-binding Amphipathic α-Helix Is Essential for Function of FtsY—The results described above clearly show that, unlike in NG, the identified N-terminal amphipathic helix of NG+1 enables the receptor to interact with liposomes in vitro. To address the physiological relevance of these results further (see also Ref. 15) and to examine whether this helix is required for the in vivo activity of NG+1 and FtsY, we performed the following mutagenesis experiments. In FtsY the helix was deleted (Phe196–Lys207), and in NG+1 the positively charged face of the helix was neutralized by replacing the corresponding four basic residues (Arg198, Lys200, Lys205, and Lys207) by leucines. The mutated FtsY (FtsY1quadro) and the A-domain with LUVs. The N-terminal peptide representing the amphipathic helix of NG and NG+1 were fused to Trx (the sequences are shown below the flotation). Purified proteins incubated with LUVs were subjected to flotation ultracentrifugation, and fractions were collected from the top. Samples were separated by SDS-PAGE, stained with Coomassie, and quantified by densitometry. Percentage values given represent protein found in the top fraction (bound to the liposomes). All constructs bound with a reproducibility of ±2% to the liposomes, except NG, which showed a much greater variation. This is due to the tendency of this protein to aggregate. Aggregates are found in the bottom fraction.

DISCUSSION

We have previously shown that the FtsY-truncated mutant NG+1 is fully active in vivo (15) and that shortening its N terminus by a single residue abolishes its function. We have now

| Membrane targeting sequence | FtsY | MinD L. monocytogenes | MinD B. subtilis | MinD E. coli | MinD N. gonorrhoeae |
|-----------------------------|------|-----------------------|-----------------|---------------|---------------------|
| **E. coli**                  | 192-202 | **KEGGFRALKRSLK**  | 252-265 | 253-266 | 153-270 | 261-271 |
| **L. monocytogenes**         | 192-202 | **KEGGFRALKRSLK**  | 252-265 | 253-266 | 153-270 | 261-271 |
| **B. subtilis**             | 192-202 | **KEGGFRALKRSLK**  | 252-265 | 253-266 | 153-270 | 261-271 |
| **E. coli**                  | 192-202 | **KEGGFRALKRSLK**  | 252-265 | 253-266 | 153-270 | 261-271 |
| **N. gonorrhoeae**          | 192-202 | **KEGGFRALKRSLK**  | 252-265 | 253-266 | 153-270 | 261-271 |

**FIGURE 5.** The N terminus of *E. coli* NG+1 is conserved in FtsY homologs and MinD proteins. **A**, alignment of FtsY sequences from various origins. **B**, alignment of the N-terminal sequence of NG+1 with the MTS sequences of several MinD proteins. Identical residues are shown in blue.

**FIGURE 6.** The NG+1 amphipathic helix is an autonomous lipid-binding domain. **A**, interaction of FtsY, NG+1, and the A-domain with LUVs. **B**, interaction of Trx and Trx hybrids with LUVs. The N-terminal peptide representing the amphipathic helix of NG and NG+1 were fused to Trx (the sequences are shown below the flotation). Purified proteins incubated with LUVs were subjected to flotation ultracentrifugation, and fractions were collected from the top. Samples were separated by SDS-PAGE, stained with Coomassie, and quantified by densitometry. Percentage values given represent protein found in the top fraction (bound to the liposomes). All constructs bound with a reproducibility of ±2% to the liposomes, except NG, which showed a much greater variation. This is due to the tendency of this protein to aggregate. Aggregates are found in the bottom fraction.

**FIGURE 7.** The NG+1 construct (NG+1quadro) was very active in vivo. The NG+1 construct bound efficiently, although to a lower extent (~19%), whereas NG binds rather poorly (~5%). This observation seems to contradict our previous study (21), in which however lipid binding was assayed only qualitatively by Western blotting and the composition of the liposomes was different (see “Experimental Procedures”). The observed membrane interaction of full-length FtsY correlates nicely with the previously observed interaction of 30% FtsY bound to IMVs in an in vitro translation experiment (16). These results suggest that, despite the observation that the A-domain alone does not interact with LUVs, it apparently affects the capacity of the receptor to bind lipids (see “Discussion”). These results confirm the hypothesis that the N-terminal helix of NG+1 is required for interaction with lipids. The observed in vivo inactivity of the NG mutants lacking Met195 and Phe196 (Table 1 and Fig. 7) supports the importance of the hydrophobic face of the helix for this interaction.

To examine whether this segment is an autonomous lipid-binding element, we attached the conserved N-terminal peptides from NG and NG+1 to E. coli Trx, a soluble protein that does not bind to LUVs. The hybrids were then tested for their ability to bind LUVs. Fig. 6B shows that Trx and NG-Trx did not associate with liposomes, whereas NG+1-Trx bound efficiently. Taken together, these studies demonstrate that FtsY contains a transplantable, bona fide membrane-interacting sequence in the A/N-domain interface and that the hydrophobic residues at the N terminus of NG+1 are important for efficient interaction.

The Lipid-binding Amphipathic α-Helix Is Essential for Function of FtsY—The results described above clearly show that, unlike in NG, the identified N-terminal amphipathic helix
Lipid-binding Domain of FtsY

![Graph A](image)

**FIGURE 7. The amphipathic helix is important for the receptor function in vivo.** A, expression of and FtsY-complementation by NG+1 and FtsYΔ196–207. B, expression of and FtsY-complementation by FtsY and NG+1/quadro (see text).

demonstrated that truncation, together with in vivo f-Met processing, destroys an amphipathic helix in NG+1. This helix is an essential, autonomous membrane-interacting sequence, thus defining for the first time, a lipid-binding domain in FtsY that is required for function in vivo.

The issue of how FtsY associates with the cytoplasmic membrane has received great attention since its first functional characterization (16). Already then, it was proposed that the receptor has a limited number of binding sites on the membrane (16), implying that lipids may not provide the major docking site of the receptor (see also Ref. 20). This proposal has been confirmed several times by demonstrating that FtsY assembles on membranes via interactions with a trypsin-sensitive component (22, 37). Finally, the most compelling evidence for FtsY-membrane protein association has recently been offered by studies of the interaction between FtsY and the SecYEG complex in *E. coli* (11, 12). Whether the translocon is the only proteinaceous binding site for FtsY remains unknown (38, 39). Nevertheless, in addition to convincing evidence for FtsY-membrane protein interaction, other studies demonstrated unequivocally that the receptor also interacts with lipids, although such interaction probably does not dictate docking of the receptor.8 Detailed characterization of this phenomenon was initially put forth by de Leeuw et al. (21), who demonstrated that FtsY interacts directly with *E. coli* phospholipids, with a preference for anionic ones, and that this interaction stimulates the GTPase activity of FtsY. Moreover, at least one determinant of lipid interaction was identified as the NG domain. In contrast to acidic lipids, other studies proposed that FtsY assembles on membranes via interactions with phosphatidylethanolamine, through the AN domain (37). Regardless of the crucial differences in identifying the specific lipids (see later), the combined studies implied a role for the N-domain in lipid binding. Taken together, the established interactions of the receptor, both with membrane proteins and lipids, raised two major questions as follows: (i) Which structural elements in FtsY are responsible for the association with proteins and lipids? Whereas the mechanism of association with the translocon and/or other membrane proteins remains to be investigated, our work discovered a functionally important lipid-interacting determinant of FtsY at the A/N-domain interface. Notably, the LUV-binding assays indicated that, although the A-domain is not essential, it contributes to this interaction. Possible interpretations are as follows:

First, the positively charged, N terminus of the full-length receptor (16) might be able to enhance lipid binding only in the context of the full-length receptor. Second, the structure of full-length FtsY2 revealed that the helix is in fact longer than seen in NG+1, providing a possible explanation for the enhanced LUV interaction of FtsY, compared with NG+1. (ii) What is the role of the individual interactions? Whereas at this stage one can only speculate on the role of FtsY interaction with membrane proteins, which is probably required for docking, our recent work9 provides direct evidence for a role of lipid binding in regulating downstream events along the SRP pathway, by stimulating the GTPase activity of the membrane-bound FtsY-SRP complex. Such communication between the N-terminal helix of NG+1 and the GTPase G-domain has been suggested on the basis of the *M. mycoides* FtsY-NG structure (29) (see later). Importantly, the idea that the amphipathic helix regulates downstream, membrane-associated steps in the pathway is supported by our results showing that it is not required for membrane targeting and docking of the receptor.9

As shown in Fig. 5A, the amphipathic helix of FtsY is relatively conserved in organisms or compartments lacking a membrane protein homologous to the eukaryotic β-subunit of the receptor (bacteria, archaea, and chloroplasts). Comparison of the NG+1 structure to other documented structures of SRP receptors (PDB code 1vma (29)) suggests that not only the N-terminal sequence of NG+1 is conserved but also its structure. Using the program Amphipaseek (40), we predict that the N-terminal peptide of NG+1 forms an in-plain membrane anchor. For FtsY we propose that lipid interaction initially involves electrostatic attraction between basic residues located at the polar face of the amphipathic helix and negatively charged lipid head groups. In the second step, hydrophobic interactions lead to the insertion of the helix into the membrane. This mode of interaction results in orienting the helix parallel to the membrane surface.

An interesting similarity exists between FtsY and other nucleotide-binding, peripheral membrane proteins in their
Lipid-binding Domain of FtsY

functional interaction with membranes. For example, the peripheral translocon subunit SecA also requires acidic phospholipids for its high affinity binding to the translocon (41, 42). Another example, probably the most striking one, is the previously characterized MTS in proteins of the MinD family. These proteins are structurally related to FtsY, because they are also members of the SIMIBI class of NTP-binding proteins (36). This family contains GTPases and ATPases characterized by the formation of nucleotide-dependent dimers. MinD proteins play a crucial regulatory role in selecting division sites in eubacteria, chloroplasts, and archaea. Apparently, in addition to being related in structure and function, the MTS of MinD proteins show some sequence similarity to the amphipathic helix of FtsY (Fig. 5B). Because the membrane composition in bacteria can vary significantly, the observed variation in the sequences of the membrane-binding helix in FtsY and in MinD (Fig. 5) might correlate with the membrane composition. For the MTS in MinD such correlation was indeed shown (35). The different locations of the membrane-binding domains in these proteins, the N terminus of the NG domain in FtsY and the C terminus of MinD, further support the proposal that the amphipathic helix is autonomous and able to link N- or C-terminally fused proteins to membranes, as shown for MinD (35) and FtsY (the present study). Moreover, as shown previously for FtsY (21) and recently summarized for other peripheral proteins, including MinD (43), activation of these proteins is mediated by membrane domains rich in anionic lipids, in accordance with the positively charged face of their amphipathic helices, thus lending support to the notion that FtsY interaction with acidic lipids (21) rather than neutral ones (37) is essential for the receptor function.

Previously, we reported that the GTPase of FtsY is activated by its interaction with anionic phospholipids (21), but the functional relevance of this stimulation was not understood. Now we localize an autonomous membrane-binding site to a conserved amphipathic helix at the N terminus of NG +1 and in a parallel study we provide evidence that the stimulation of the FtsY GTPase by lipids is required for a later step in the SRP pathway. Productive FtsY-lipid interaction requires a certain length and hydrophobicity of the helix. The motif present in NG +1 represents the minimal regulatory element, because NG is not able to function in vivo. Comparison of the structures of SRP receptors shows that the conserved amphipathic helix in NG +1 packs between the C terminus and motif IV of the G domain, as earlier suggested (29).

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