A Site-specific, Membrane-dependent Cleavage Event Defines the Membrane Binding Domain of FtsY*

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Targeting of many polytopic proteins to the inner membrane of prokaryotes occurs via an essential signal recognition particle-like pathway. Unlike the general secretory pathway, the proteins involved in this pathway and their activities appear in many respects to mirror closely those of their eukaryotic homologues. However, the *Escherichia coli* signal recognition particle receptor, FtsY, differs significantly at the amino terminus from the eukaryote homologue α-subunit of the signal recognition particle receptor. In addition, there is no prokaryote homologue of the transmembrane β-subunit of the receptor. Therefore, FtsY must assemble on the membrane in a unique manner. Using assays designed to accurately discriminate membrane-bound proteins from aggregated material, we found that in contrast to a previous report, only amino acids 1–284 of FtsY are necessary and sufficient for membrane assembly. These amino acids together constitute a *bona fide* membrane binding domain that includes both the regions originally designated A and N based on sequence comparisons. Furthermore, we found that a membrane-bound factor mediates specific cleavage of some membrane-bound FtsY molecules between the N and G regions previously believed to be functionally linked to generate a novel membrane-bound isoform composed of only the AN domain.

In mammalian cells, membrane and secretory proteins are targeted to the endoplasmic reticulum cotranslationally via the signal recognition particle (SRP) pathway (reviewed in Ref. 1). SRP is a cytoplasmic ribonucleoprotein particle composed of six polypeptides associated with an RNA scaffold. The targeting process begins with recognition of a hydrophobic signal sequence on a nascent secretory or integral membrane polypeptide by the 54-kDa protein of SRP (SRP54) resulting in the binding of SRP to both the ribosome and nascent polypeptide as well as a concomitant slowing of translation elongation. The ribosome nascent chain complex is targeted to the translocon, the aqueous pore through which proteins are translocated cotranslationally (reviewed in Ref. 3).

Homologues of the eukaryotic SRP pathway have been identified in many prokaryotes (4–10). In *Escherichia coli*, Ffh and 4.5 S RNA form a SRP-like complex that interacts with nascent secretory and membrane proteins (11, 12). This particle promotes cotranslational targeting of nascent chains via an interaction with FtsY, the SRα homologue (13). Recent data suggest that this pathway may be of particular importance for membrane assembly of hydrophobic inner membrane proteins (14–17). Although the targeting steps are distinct from those of the more ubiquitous Sec secretory pathway, at least some of the translocation apparatus is the same (18).

An interesting divergence between the eukaryotic and prokaryotic SRP pathways is in the membrane assembly of the receptors. In eukaryotes, SRβ, the transmembrane β-subunit of the SRP receptor, anchors SRα on the endoplasmic reticulum membrane through an interaction with the amino-terminal domain of SRα (19). No homologue of SRβ has been identified in the *E. coli* genome sequence. Furthermore, the amino-terminal domains of FtsY and SRα are highly divergent (6, 7), suggesting that FtsY assembles on the membrane in a different manner. Because FtsY is believed to shuttle proteins to the membrane that are not efficiently inserted by the general secretory pathway and because FtsY is an essential gene in *E. coli*, it is likely that membrane assembly of FtsY is tightly regulated.

A highly negatively charged region has been identified at the amino terminus of FtsY (amino acids 1–196) that is not found in other eukaryotic or prokaryotic homologues (20). In addition to this "A" region, central N (amino acids 197–280) and carboxy-terminal G (amino acids 292–497) regions have been identified. The G region is a GTP binding domain that together with the GTPase domains of SRP54, SRα, Ffh, and their homologues constitute a specific subfamily of Ras-related low molecular weight GTPases. The N region is found amino-terminal of the G region in all SRP family GTPases and has been assumed to have a role in GTPase activity (21). The amino-terminal A region, by analogy with the membrane assembly domain of SRα, was expected to be involved in membrane assembly.

Based on this supposition, the membrane assembly properties of the FtsY A region, NG region, and G region were each analyzed (22). Surprisingly, all three polypeptides fractionated with membranes after centrifugation, leading the authors to conclude that each independently binds to the *E. coli* inner membrane (22).

**Here we used a gel filtration chromatography-based assay to unambiguously distinguish membrane bound from aggregated FtsY. Using this assay, we determined that neither the A region nor the NG region were sufficient for membrane association, but the AN region of FtsY is both necessary and sufficient for membrane assembly. Furthermore, we found specific cleavage of some membrane-bound FtsY molecules to generate**
a novel membrane-bound isoform of FtsY confirming the only the A and N regions confirming that AN constitutes the membrane binding domain of FtsY.

EXPERIMENTAL PROCEDURES

Materials and General Methods—General chemical reagents were obtained from Fisher, Sigma, or Life Technologies, Inc. SURE™ E. coli cells used for plasmid construction were purchased from Stratagene. Except where specified, restriction enzymes, other molecular biology enzymes, and reagents were from New England Biolabs or MBI Fermentas. RNA guard (an RNase inhibitor) was from Amersham Pharmacia Biotech.

Plasmids—Construction of plasmids, sequencing, and polymerase chain reactions were performed using standard methods (23). Deletion mutants and fusion proteins of FtsY are outlined in Fig. 2 and are described below. Full construction details for the plasmids are available from the authors on request.

FtsY was amplified from genomic DNA isolated from E. coli strain JM109 using the following oligonucleotides: 5′-CGCCCATGGGCAAAGAAA-3′ and 5′-CAGTAGATGGGCTTGGA-3′. To generate the FtsY expression plasmid pMAC897, the full-length coding sequence for FtsY was inserted behind tandem SP6 and tac promoters in the plasmid pSPtac using the restriction enzymes NcoI and BglII. The former specifically recognizes epitopes in the AN binding domain of FtsY, and rabbit IgG were used for immunoprecipitation of FtsY. The latter contains a Shine-Dalgarno sequence to direct bacterial translation. The entire FtsY coding sequence as well as the SP6 and tac promoter regions were excised from pMAC897 and inserted into the BglII site of the plasmid pSPMP366 (described previously) (24). The resulting plasmid, designated pMAC988, contains the coding region of FtsY followed by a sequence encoding the passive passenger protein gPa. In this plasmid, the gPa fusion protein is present between the two coding regions. Plasmids encoding the various FtsY deletion mutants, FtsY, and FtsY-gPa containing the amino-terminal 58, 96, 155, 198, and 231, amino acids of FtsY were generated using the technique described in Ref. 25 to delete the required sites.

Plasmid pMAC1000 encodes the polypeptide FtsYdSRY1 comprising amino acids 41 to 198 of FtsY with the gPa domain at the carboxyl terminus. This polypeptide has a small positively charged region deleted from the amino terminus of FtsY. Plasmid pMAC999 encodes the polypeptide FtsYdSRY2 comprising amino acids 59 to the stop codon of FtsY.

Plasmids pMAC997, pMAC1177, pMAC1178, pMAC1176, and pMAC995 encode polypeptides F56-gPa, F96-gPa, F155-gPa, FtsY-gPa, and FtsY-gPa consisting of the amino-terminal 58, 96, 155, 197, and 231, amino acids of FtsY with the gPa domain at the carboxyl terminus. Plasmid pMAC1310 encodes the polypeptide FtsYNg-gPa consisting of amino acids 198 to the final amino acid of FtsY with the gPa domain at the carboxyl terminus.

Plasmid pMAC1252 and pMAC1253 encode polypeptides FtsYA and FtsYAN-gPa consisting of the amino-terminal 197 and 284 amino acids of FtsY, respectively, followed by Leu-Gln-Asp-Pro-Arg-stop codons.

Antibody Generation and Purification—Polyclonal antisera against FtsY was raised in rabbits immunized with bacterially expressed fusion protein. Plasmid pMAC1042 encodes amino acids 41 to the stop codon of FtsY fused to the carboxyl terminus of glutathione S-transferase in the vector pMAC241, a modification of pGEX2T (Amersham Pharmacia Biotech) with an enhanced polylinker. The fusion protein was purified using a glutathione-Sepharose column. Antibodies specific to FtsY were purified from serum as described (26).

Immunoprecipitations and Western Blots—Affinity purified anti-FtsY antibody and rabbit IgG were used for immunoprecipitation of FtsYAN-gPa. The former specifically recognizes epitopes in the AN region of this polypeptide derived from FtsY, whereas the latter is bound by gPa, which contains the IgG binding region of Staphylococcus aureus protein A (24). Following membrane targeting, fractions eluted at 4°C from the column were dialyzed with 1 ml of buffer A (100 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1% Triton X-100). Affinity purified IgG against FtsY (3 μl of 0.1 mg/ml) or 3 μl of buffer was added following a 2-h incubation at 4°C. Protein A-agarose (Bio-Rad) was added to the fractions incubated with 5 μl of FtsY antibody. 3 μl of IgG-Sepharose were added to the other fractions. Following incubation for 2 h at 4°C, the beads were washed 3 times with 1 ml of buffer A with the 2-tube rack with 1 ml of buffer A without Triton X-100. To release the bound protein, the washed beads were incubated in 50 μl of SDS-PAGE loading buffer for 5 min at 80 °C, and 8 μl were analyzed by SDS-PAGE.

To detect FtsY by immunoblotting, 5 μl of E. coli inner membrane inverted vesicles (INVs) containing approximately 15 μg of protein were solubilized in 50 mM Tris-Cl, pH 8.0, 1% SDS and then analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose using a semidry transfer apparatus ( Hoeffer Instruments).

Cell-free Translation Systems—An S30 lysate was prepared from E. coli strain MRE600 (27) as described previously (28). To remove any remaining chromosomal DNA, micrococcal nuclease was added to a final concentration of 25 units/ml of lysate along with 1 mM CaCl2. The reaction was stopped after incubation for 30 min at room temperature by adding EDTA to a final concentration of 4 mM. A membrane-free S170 extract was obtained by centrifugation of 175 μl of S30/tube in the A-100/30 rotor of an Airfuge (Beckman) at 4 °C for 15 min at 28 p.s.i. (170,000 × g) and collecting the top 125 μl. Membrane-free ribosomes were isolated as described previously (29).

A typical 20-μl-coupled transcription and translation reaction contained 35 mM Tris acetate, pH 8.0; 190 mM potassium glutamate; 30 mM ammonium acetate; 2 mM DTT; 12 mM Mg(OAc)2; 40 μM each of 19 amino acids (-methionine); 2 mM ATP; 0.5 mM each of CTP, UTP, and GTP; 20 mM phosphoenolpyruvate; 1 mM isopropyl-1-thio-β-galacto-pyranoside; 0.1 mg/ml E. coli RNA; 35 mg/ml polyethylene glycol 8000; 20 μg/ml follicin acid; 12 μCi of L-[35S]methionine; 1 μg of plasmid DNA; 6 μl of S170; and 0.2 μl of membrane-free ribosomes. Incubations were at 37 °C for 45 min.

Transcripts for cell-free translations in rabbit reticulocyte lysate were generated with SP6 polymerase as described previously (30). Translations performed in rabbit reticulocyte lysate and labeled with [35S]methionine were described previously (31). Radiolabeled translation products were separated by SDS-PAGE, visualized by phosphorimaging using a Molecular Dynamics PhosphorImager 473, and quantified using the Imagequant software from Molecular Dynamics.

Vesicles—As a starting material for isolation of INVs, crude inverted vesicles were prepared as described in Ref. 28. 2-ml aliquots were then loaded on a sucrose step gradient consisting of 2.02 (10 ml), 1.44 (13 ml), and 0.77 μl (13 ml) sucrose steps in 50 mM triethanolamine-acetate, pH 7.5, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. Following centrifugation at 25,000 rpm for 18 h in the SW28 rotor in an Ultracentrifuge (Beckman), INVs were removed from the 0.77/1.44 ml-coupled transcription and translation reaction products were separated by SDS-PAGE (32), visualized by phos-
FtsY Membrane Binding and Cleavage

**Results**

**Membrane Binding of FtsY—Attempts to determine the membrane binding domain of FtsY using a simple pelleting assay similar to that used in Ref. 33 did not clearly distinguish membrane-bound FtsY from large aggregates. Greater than 50% of FtsY pelletted both in the presence and absence of membranes (data not shown). That this consisted largely or exclusively of aggregated protein was confirmed by Sepharose S200 size exclusion chromatography. Therefore, to assay for stable membrane binding, translations of FtsY in S170 extract were incubated with or without INVs and then fractionated by gel exclusion chromatography using Sepharose CL-2B, a resin with a large enough exclusion limit to retain FtsY aggregates (Fig. 1). Vesicles and vesicle-associated proteins eluted as a broad peak in the included volume (fractions 5–12). Full-length FtsY (arrowheads) and a 53-kDa species (dots) eluting with membranes in the excluded fractions are marked. The migration positions of molecular mass markers are indicated (in kDa) to the left of the panels.

**Membrane Binding of FtsY—**Attempts to determine the membrane binding domain of FtsY using a simple pelleting assay similar to that used in Ref. 33 did not clearly distinguish membrane-bound FtsY from large aggregates. Greater than 50% of FtsY pelletted both in the presence and absence of membranes (data not shown). That this consisted largely or exclusively of aggregated protein was confirmed by Sepharose S200 size exclusion chromatography. Therefore, to assay for stable membrane binding, translations of FtsY in S170 extract were incubated with or without INVs and then fractionated by gel exclusion chromatography using Sepharose CL-2B, a resin with a large enough exclusion limit to retain FtsY aggregates (Fig. 1). Vesicles and vesicle-associated proteins eluted in the excluded volume (fractions 3 and 4, marked with arrowheads in Fig. 1). The included volume (fractions 5–12) contains cytosolic proteins.

As expected in the absence of INVs, FtsY synthesized in S170 extract fractionated almost exclusively in the included volume of a CL-2B column (Fig. 1A, lanes 5–12). After incubation with INVs most of the full-length FtsY still fractionated in the included volume (Fig. 1B, lanes 5–12), likely representing FtsY molecules present as aggregates that do not assemble onto membranes. Nevertheless, a fraction of the full-length FtsY eluted in the excluded volume with membranes, in contrast to the incubations without INVs, demonstrating that some of the FtsY in S170 extracts binds to inverted vesicles (Fig. 1B, lanes 3 and 4, arrowheads). Although the background is increased, it is clear that when translated in S30 lysate, which contains endogenous membranes, almost half of the full-length FtsY eluted in the excluded fractions (Fig. 1C). This increase in binding may be because of the high concentration of vesicles present in S30 lysate, as well as the elimination of the incubation time in the absence of membranes during which aggregation may occur.

In addition to the band corresponding to the previously described 92-kDa migration product of full-length FtsY (34), a second band migrating as a 53-kDa species was observed when FtsY was incubated with membranes. This product eluted entirely in the excluded volume (Fig. 1B, lanes 3 and 4, dots). This band is not a result of membrane-dependent alterations in translation, such as premature termination or internal initiation, because membranes are added after translation was terminated and following removal of ribosomes from the extract. Although FtsY has a predicted mass of approximately 54 kDa, it migrates on SDS-PAGE with an apparent weight of 92 kDa (34). The 53-kDa product could therefore represent full-length FtsY with an as yet unidentified modification removed or a proteolytically processed form of FtsY. As shown below, this band results from post-translational cleavage of FtsY between the N and G regions. After correcting for the number of methionine residues in each of these proteins, the cleaved product accounts for approximately 75% of the membrane-bound FtsY.

**Regions of FtsY Required for Membrane Binding—**It has previously been demonstrated that the GTPase domain located in the carboxyl-terminal two-fifths of FtsY interacts directly with the E. coli SRP particle (35, 36). Therefore, it was predicted that the amino-terminal region would be involved in membrane binding. To determine which sequences in the amino-terminal region of FtsY were necessary for membrane binding, a set of plasmids was made (Fig. 2) encoding FtsY molecules with deletions of the amino-terminal 19 and 57 amino acids (SRY1 and SRY2). To identify FtsY sequences sufficient to bind INVs, test sequences were fused to gPa; a protein domain previously demonstrated to have no intrinsic targeting or membrane binding activity (24). A series of plasmids were constructed encoding the amino-terminal 58, 96, 155, 197, and 284 amino acids of FtsY as well as amino acids 198–497 of FtsY fused to gPa. The latter three constructs have regions fused to gPa that correspond to the A region, AN regions, and NG regions of FtsY, respectively. Constructs encoding only the amino-terminal 197 or 284 amino acids of FtsY were also tested.
Deletion of amino acids 1–19 or 1–58 of FtsY resulted in polypeptides (FtsYdSRY1 and FtsYdSRY2, respectively) that did not bind INVs and therefore, fractionated identically by gel filtration chromatography in the presence and absence of INVs (Fig. 3A, compare lanes 1–10 with lanes 11–20). This demonstrates that the extreme amino terminus of FtsY is necessary for membrane binding. To determine how much of the amino-terminal region of FtsY was sufficient for membrane assembly, constructs containing amino-terminal segments of the A domain of increasing size fused to gPa were fractionated in the presence or absence of membranes (Fig. 3B). Surprisingly, the elution patterns for all of these molecules are similar with and without INVs (Fig. 3B, compare lanes 1–10 with 11–20 for FtsY 58-gPa, FtsY 96-gPa, FtsY 155-gPa, and FtsYA-gPa). Therefore, fusions containing part or all of the A region of FtsY did not bind to INVs.

A single prominent band was obtained when a construct corresponding to the A region fused to gPa (FtsYAN-gPa) was expressed in vitro and incubated with INVs. In contrast with the A region fusions, essentially all of this translation product fractionates with membranes in the excluded volume (Fig. 3B, lanes 1–2, dots). However, this band migrated at 53 kDa rather than the 92-kDa position observed for full-length FtsYAN-gPa in the absence of membranes (Fig. 3B, lanes 14–20). Unlike full-length FtsY where 75% of membrane-bound product was a 53-kDa species, in reactions containing FtsYAN-gPa essentially all of the membrane-bound protein migrated at 53 kDa.

In the absence of membranes, the intensity of the 92-kDa band representing full-length product is greatly reduced (Fig. 3B, lanes 14–20). We attribute this to two factors. First, the product is dispersed over a larger number of fractions in the absence of membranes (at least 6 included fractions versus 2 excluded fractions). Second and more significantly, the FtsYAN-gPa product is apparently subject to nonspecific degradation if it is not targeted to membranes (see below, Fig. 6). Consistent with this interpretation, control experiments demonstrated that the amount of FtsYAN-gPa is maximal in S170 lysate immediately after transcription-translation and declines thereafter (data not shown). Unlike FtsYAN-gPa, the FtsYNG-gPa fusion is stable in the absence of INVs yet was unable to bind to membranes in vitro (Fig. 3B, compare lanes 1–10 with 11–20).

Because of the altered migration of the membrane-bound form of FtsYAN-gPa, we tested unfused versions of the A and AN sequences of FtsY individually for binding to INVs (Fig. 3C). Consistent with the behavior of the gPa fusions, the A region eluted in included fractions in the presence or absence of INVs (Fig. 3C, compare lanes 1–10 with 11–20 for FtsYA), demonstrating that the A region alone cannot bind to INVs. The AN region eluted in the excluded volume in the presence of membranes, demonstrating that this molecule bound efficiently to INVs (Fig. 3C, compare lanes 1–10 with 11–20 for FtsYAN). It is clear from this data that the AN domain, but not the A domain, of FtsY is both necessary and sufficient to direct membrane assembly. Moreover, consistent with the behavior of FtsYAN-gPa, in the absence of INVs FtsYAN is degraded in lysate (Fig. 3C, lanes 11–20).

Vesicle lift assays were used to confirm these results. Control experiments demonstrated that sealed INVs migrate at the interface of the 0.25/1.25 M sucrose steps (Fig. 4, lanes 2 and 7) after centrifugation (data not shown). Some INVs were also found to pellet in this assay, presumably because they are leaky. Full-length FtsY and the putative cleavage product fractionated at the 0.25/1.25 M sucrose interface only when mixed with INVs (Fig. 4, top panel, compare lanes 2 and 7) confirming that they bound to membranes. Both full-length FtsY and the putative FtsY cleavage product were also observed to pellet in the presence of membranes, as expected for aggregates and molecules bound to leaky INVs (Fig. 4, top panel, compare lanes 5 and 10).

As above, FtsYAN-gPa was cleaved in the presence of membranes (Fig. 4, middle panel). This product fractionated at the 0.25/1.25 M sucrose interface only when membranes were added to the reaction (Fig. 4, middle panel, compare lanes 2 and 7). In contrast, FtsYA-gPa fractionates identically in the presence and absence of membranes, as expected for a protein that does not bind to membranes (Fig. 4, bottom panel).

FtsY Is Cleaved upon Membrane Assembly—The lower mo-
when synthesized in either of the regions was observed using the AN domain (calculated molecular mass 32 kDa) exhibits anomalous migration in SDS-PAGE and is not precipitated with IgG-Sepharose (Fig. 3). We observe a band with an apparent molecular weight of 48 kDa (Fig. 3), suggesting that both the 92- and 53-kDa isoforms of FtsY are present in E. coli. An uncharacterized band (indicated with an x) is also detected using these antibodies. A cross-reacting band of the same apparent molecular weight as that seen here has been observed previously by Lührink et al. (34) using an independent FtsY antibody. We also observe a band with greater migration than the putative cleavage product of FtsY. The intensity of this band relative to both FtsY and the cleaved product is predominantly cytosolic. The migration of this band does not correspond to the migration of the G domain of FtsY, thus the origin of this band is uncertain.

To further establish whether a membrane-bound species of FtsY with an apparent molecular mass of 53 kDa is present endogenously in E. coli, whole cells in mid-log phase and INVs were analyzed by IMAC and by immunoblotting. The migration of the α-FtsY reactive species was compared with the excluded fractions obtained in the presence of membranes from S170 translations of FtsY and FtsYAN (Fig. 5). Several prominent bands were obtained when INVs were analyzed by immunoblotting (Fig. 5, lanes 7 and 8). In addition to full-length FtsY, a 53-kDa band is detected that precisely comigrates with the putative cleavage product of FtsY3 (Fig. 5, compare lanes 1–4 with lane 7), suggesting that both the 92- and 53-kDa isoforms of FtsY are present in E. coli. An uncharacterized band (indicated with an x) is also detected using these antibodies. A cross-reacting band of the same apparent molecular weight as that seen here has been observed previously by Lührink et al. (34) using an independent FtsY antibody. We also observe a band with greater migration than the putative cleavage product of FtsY. The intensity of this band relative to both FtsY and the cleaved product is predominantly cytosolic. The migration of this band does not correspond to the migration of the G domain of FtsY, thus the origin of this band is uncertain.

Further evidence that the 53-kDa bands correspond to membrane-dependent cleavage of FtsY between the AN and G sequences was obtained using differential immunoprecipitation of FtsYAN-gPa translation reactions after incubation with and without INVs (Fig. 6). FtsYAN-gPa synthesized in S170 extract was incubated in the presence (top panel) or absence (bottom panel) of INVs and then fractionated by Sepharose CL-2B gel exclusion chromatography. The translation products bound to INVs (excluded fractions 3 and 4) and in the cytosol (included fractions 9 and 10) were identified by immunoprecipitation using either an anti-FtsY antibody (lanes 1–4) to bind the amino terminus or IgG-Sepharose (lanes 5–8) to bind the gPa domain.

The anti-FtsY antibody efficiently precipitates the 53-kDa putative FtsYAN-gPa cleavage product from the vesicle containing excluded fractions (Fig. 6, lanes 1–2, dots). In contrast, this product is not precipitated with IgG-Sepharose (lanes 5–6). Because gPa contains four independent IgG binding domains, this result demonstrates that the 53-kDa putative cleavage product contains less than one-fourth of the gPa portion of FtsYAN-gPa.
FtsY Membrane Binding and Cleavage

Following incubation with INVs a band corresponding to the complete gPa domain is precipitated with IgG-Sepharose from the cytosolic fractions (Fig. 6, top panel, lanes 7–8). This product is not observed in the same fractions without added INVs (bottom panel, lanes 7–8). Thus, the 53-kDa membrane-associated band results from cleavage of FtsYAN-gPa immediately carboxyl-terminal of the AN region, and cleavage releases the gPa domain into the cytosol.

In the absence of INVs, specific cleavage of FtsYAN-gPa was not observed (Fig. 6, bottom panel). Furthermore, without INVs essentially no translation product precipitates from the excluded fractions with either anti-FtsY antibody or IgG-Sepharose, as expected (lanes 1–2 and 5–6). In the included fractions, several bands with greater migration than FtsYAN-gPa are immunoprecipitated with anti-FtsY antibody (lanes 3–4, asterisks). However, these bands are likely to result from relatively nonspecific degradation of FtsYAN. First, none of these bands correspond to the same size as the specific cleavage product observed in the excluded fractions of A. Second, a specific band corresponding to gPa is not precipitated by IgG-Sepharose from the included fractions. Instead IgG-Sepharose primarily precipitates full-length FtsYAN-gPa from these fractions (bottom panel, lanes 7–8). Thus, specific cleavage of FtsYAN-gPa occurs only in the presence of membranes.

Taken together, these data suggest that full-length FtsY as well as FtsY fusion proteins containing both the A and N regions are competent for membrane binding and can be cleaved upon membrane binding. Even though only a small fraction of the total full-length FtsY molecules synthesized in E. coli lysate associated with INVs, the 53-kDa cleavage product, corresponding to the AN membrane binding domain, is efficiently retained on the membrane.

To obtain further evidence that membrane-dependent cleavage is because of a membrane-associated factor rather than a component of the E. coli cytosol, full-length FtsY and FtsYAN-gPa were translated in rabbit reticulocyte lysate, and INVs were then added. After incubation for 45 min at 37 °C the reactions were fractionated by Sepharose CL-2B gel exclusion chromatography as above. As was observed using S170-translated FtsY, most of the full-length FtsY synthesized in reticulocyte lysate did not bind to INVs and therefore fractionated in the included volume (Fig. 7, top panel, lanes 5–12). Moreover, control experiments demonstrated that most of the FtsY synthesized in reticulocyte lysate was present in large aggregates (data not shown). However, a small fraction of the full-length FtsY eluted in the excluded volume with INVs (Fig. 7, top panel, arrowheads). Significantly, these fractions also contained the 53-kDa product expected from cleavage of FtsY between the N and G regions (Fig. 7, top panel, lanes 3–4, dots).

Both membrane binding and cleavage are much clearer for FtsYAN-gPa synthesized in reticulocyte lysate (Fig. 7, bottom panel). In this reaction, essentially all of the full-length translation products fractionate with membranes in the excluded volume (Fig. 7, bottom panel, lanes 3–4, arrowheads). However, most of the molecules are cleaved, and the resulting 53-kDa band that comigrates with AN also elutes with INVs in the excluded volume (Fig. 7, bottom panel, lanes 3–4, dots). The gPa fusion domain that was cleaved from the AN portion behaves as a soluble protein and therefore elutes in the included fractions (Fig. 7, bottom panel, lanes 5–12) as expected. Thus, both FtsY and FtsYAN-gPa are cleaved carboxyl to the AN region only when INVs are added. The most likely explanation of this phenomenon is that cleavage is because of a proteolytic activity associated with INVs, although it remains possible that rabbit reticulocyte lysate contains a similar protease to one found in S170 lysate that performs cleavage only upon association of INVs with the membrane. Furthermore, the AN domain remains tightly bound to the INVs after cleavage, demonstrating that the AN region of FtsY is a bona fide membrane binding domain.

DISCUSSION

We show here that although FtsY molecules synthesized in either E. coli or reticulocyte lysate aggregate, these aggregates can be clearly distinguished from membrane-bound molecules...
using gel filtration chromatography and by floatation in sucrose step gradients. Passing translation reactions incubated with INVs over Sepharose CL-2B (exclusion limit of 40,000 kDa) allow even relatively large protein aggregates to be retained in the included volume, whereas membranes and membrane-bound proteins elute in the excluded fractions (19). Similarly, membrane-bound molecules that float in dense sucrose solutions can be distinguished unambiguously from pelleted aggregates. Moreover, because membranes undergo a very large dilution into buffer in either technique, proteins recovered with vesicles bound stably to membranes.

Although a large amount of aggregated full-length FtsY was found in the included fractions and in the pellet fractions of vesicle lift gradients, FtsY clearly cofractionated with membranes when translation reactions were incubated with INVs (Figs. 1 and 4). Furthermore, FtsY and FtsYAN-gPa also bound to membranes when synthesized in reticulocyte lysate suggesting that cytosolic E. coli proteins are not required for membrane binding (Fig. 7).

Identification of AN as the Membrane Binding Domain of FtsY—To determine which regions of FtsY mediate membrane binding, we constructed plasmids encoding deletion mutants and FtsY-gPa fusion proteins. Analysis of the deletion mutants and fusion proteins demonstrated that both the A and N regions of FtsY together constitute a minimum region of FtsY that is both necessary and sufficient for membrane binding in either E. coli lyse (Fig. 3) or reticulocyte lyse (Fig. 7). That A and N together form a membrane binding domain in FtsY was surprising given the similarity of the N and G regions in FtsY to those in Ffh and SRP54. Indeed the homology of all SRP family GTPases has been interpreted as evidence that a gene duplication event led to the production of the cytoplasmic protein from the receptor or vice versa (37). In SRP54, the N region appears to be involved in efficient signal sequence binding (38). For this reason and because the N region is juxtaposed with the G region in both the membrane-bound receptor molecules and cytoplasmic homologues of SRP pathway GTPases, it has been assumed that the N region is involved in the GTPase domain function of FtsY. This is exemplified in previous structural and biochemical analyses of the N and G regions of FtsY as a single unit (21, 39). Although our data clearly indicate a role for the N region in FtsY membrane binding, it is not clear whether N is involved in the correct folding of the membrane binding domain or if it makes specific contacts with the putative FtsY receptor on the E. coli inner membrane. It is possible that in the cytoplasmic proteins the N domain evolved to function in signal binding, whereas in the E. coli receptor a role evolved for N in membrane receptor binding. The observation that the N region folds into a separate, four helix bundle in crystal structures of the NG regions of both FtsY and Ffh (21, 40) is consistent with this hypothesis. Finally, our data do not rule out an additional function for the N region in FtsY that may involve the G region.

Surprisingly, the FtsYAN-gPa fusion protein bound to INVs much more efficiently than did full-length FtsY. This enhanced efficiency versus wild type might be explained by the observation that unlike the G region, the gPa sequence contains a 23-amino acid linker region amino-terminal of the independently folded IgG binding domains of gPa (24). This spacer may allow the AN and gPa domains to fold more efficiently in the fusion protein reducing aggregation and misfolding thereby leading to more efficient membrane binding. Consistent with this hypothesis, in S170 lyse FtsYAN binds to INVs better than FtsY does. This result also indicates that the low levels of FtsY membrane binding observed above (Fig. 1) are not because of a limiting number of binding sites for FtsY on the membrane, as both reactions contained similar quantities of INVs. Together with the demonstrations that in the absence of INVs FtsY forms large aggregates and that FtsY binds to the endogenous membranes in S30 lysate more efficiently than when INVs are added post-translationally, these results confirm that the low efficiency of FtsY membrane binding is because of misfolding and aggregation.

Membrane-dependent Specific Cleavage—Further support for a membrane binding function for the AN domain comes from the observation that a fraction of the FtsY molecules in E. coli (Fig. 5) and incubated with INVs in vitro (Figs. 1, 3, 4, 6, and 7) are cleaved such that a 53-kDa product remains bound to membranes. That the 53-kDa cleavage product corresponds to the AN domain was demonstrated by differential immunoprecipitation of the membrane-bound and cytosolic products that resulted from incubating the fusion protein FtsYAN-gPa with INVs (Fig. 6). Affinity purified antibodies generated against FtsY precipitated the AN region from membrane-bound fractions, whereas IgG-Sepharose bound to the gPa domain in fractions corresponding to cytosolic proteins.

In the absence of membranes, the full-length FtsYAN-gPa protein behaves as a soluble cytosolic protein (Fig. 6). Although antibodies directed against FtsY also immunoprecipitated a number of bands with greater migration than full-length FtsYAN-gPa from incubations without INVs, the pattern of bands obtained is indicative of relatively nonspecific cleavage and none of the bands obtained migrate at 53 kDa (Fig. 6). Moreover, degradation of cytosolic FtsYAN-gPa does not release intact gPa, as was observed for specific cleavage of FtsYAN-gPa in the presence of membranes. Indeed the major product precipitated with IgG-Sepharose from the cytosolic fractions was full-length FtsYAN-gPa. Thus, we conclude that when FtsYAN-gPa does not bind to membranes it is accessible to proteases that degrade the protein in a relatively nonspecific manner. The same phenomenon is observed when the AN domain alone is incubated in the presence of membranes (Fig. 3). In contrast, membrane-bound AN is protease resistant, suggesting that either the AN domain undergoes a conformational change upon membrane binding or it is stabilized by the association with another molecule on the membrane that masks potential cleavage sites for nonspecific proteases.

The above data demonstrate that upon binding to membranes a site-specific cleavage event occurs that defines the membrane binding domain of FtsY. However, it does not resolve whether the protease is membrane-associated or -free in the cytosol. Membrane assembly of the AN region may result in a conformational change in the protein that exposes a previously inaccessible cleavage site to a soluble E. coli protease. To address this issue, FtsYAN-gPa was translated in reticulocyte lysate prior to incubation with membranes. Membrane assembly of the reticulocyte lysate translation products again resulted in specific cleavage of FtsYAN-gPa into membrane-bound AN and free gPa domains (Fig. 7). In the absence of INVs there is much less nonspecific degradation of FtsYAN-gPa in reticulocyte lysate than in the S170 lysate, probably because the amount of nonspecific protease activity in reticulocyte lysate is less than in the E. coli system. Together, these results suggest that INV-dependent cleavage of FtsY is performed by a membrane-bound protease.

Although cleavage apparently occurs in vivo (Fig. 5), it is unclear at present whether cleavage is physiologically important or is simply a mechanism for dealing with excess FtsY on the membrane. One attractive but speculative possibility is that FtsY might function stoichiometrically rather than catalytically. In this scenario, FtsY could be cleaved subsequent to targeting as a mechanism for ensuring that targeting is unidi-
rectional and to clear the binding site for reuse in future targeting reactions. A candidate for the membrane-associated protease that cleaves FtsY remains uncertain. Preliminary analysis using the membrane-associated protease FtsH whose depletion, like that of FtsY, leads to cell filamentation and protein export deficiencies (41, 42) suggests that FtsH is not involved (data not shown).

Conclusions—We have clearly demonstrated that the AN region of FtsY is required for membrane binding. This suggests that the common practice of conceptually separating FtsY into the membrane binding A region and a GTPase region composed of both N and G sequences must be revisited. Because a structure for full-length FtsY has not been determined it was only possible to determine that AN is a bona fide protein domain by characterizing the biochemical properties of the molecule. Nevertheless, both the protease susceptibility and the membrane anchoring of both FtsY and FtsY fusion proteins strongly suggest that AN is the complete membrane binding domain of FtsY. In addition to revealing new information about the domain organization and functional properties of FtsY, identification of the membrane binding domain is an essential first step in identifying the putative FtsY receptor. A possible physiological role for membrane-dependent cleavage of FtsY and the identity of the protease responsible for membrane-dependent cleavage can also now be elucidated.

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