Targeting of many polypeptide proteins to the inner membrane of prokaryotes occurs via an essential signal recognition particle-like pathway. FtsY, the *Escherichia coli* homolog of the eukaryotic signal recognition particle receptor α-subunit, binds to membranes via its amino-terminal AN domain. We demonstrate that FtsY assembles on membranes via interactions with phosphatidylethanolamine and with a trypsin-sensitive component. Both interactions are mediated by the AN domain of FtsY. In the absence of phosphatidylethanolamine, the trypsin-sensitive component is sufficient for binding and function of FtsY in the targeting of membrane proteins. We propose a two-step mechanism for the assembly of FtsY on the membrane similar to that of SecA on the *E. coli* inner membrane.

In *Escherichia coli*, most integral membrane proteins are targeted to the inner membrane via the signal recognition particle (SRP) (1–4), a particle composed of Ffh and 4.5 S RNA (5, 6). SRP promotes translational targeting of nascent polypeptide chains via an interaction with FtsY, the membrane-associated SRP receptor (7, 8). Although the targeting steps are distinct from those of the SecB secretory pathway (9, 10), both pathways converge at a common translocation pore in the inner membrane, the trypsin-sensitive component is sufficient for binding and function of FtsY in the targeting of membrane proteins. We propose a two-step mechanism for the assembly of FtsY on the membrane similar to that of SecA on the *E. coli* inner membrane.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Growth Conditions—Strains W3899, AD90, and AD93 (27) were cultured in LB medium supplemented with 50 mM magnesium chloride. Plasmids pMAC988, pMAC1252, and pMAC1253, which encode full-length FtsY and the polypeptides FtsYA (amino acids 1–197 of FtsY) and FtsYAN (amino acids 1–284 of FtsY), respectively, have been described previously (21). Plasmid pMAC141, encoding FtsYNG (amino acids 198–497 of FtsY), was created from plasmid pMAC1310 (21) using the technique described (28) to remove the portion of the construct encoding the fusion partner gPa. A derivative of plasmid pHD83 containing the AcrB576-AP fusion cloned into the BamHI site (2) was first digested with SacI. To introduce a second HindIII site into the plasmid, the complementary oligonucleotides 5′-CATCGTAAATCGGAAAGCTTGTAGACT-3′ and 5′-AAGCAGCTT-TCCGACTTACGGATGACT-3′ were then ligated to the linearized DNA. The resulting plasmid was digested with HindIII, and the fragment containing the AcrB576-AP fusion was ligated into the HindIII site of pUC19-FtsY(WT) or FtsY(G385A) to make plasmids pHD83 (19) and the amino-terminal domain of SRa. No homolog of SRβ has been identified in the *E. coli* genome sequence. Although SRA and FtsY are homologous over two-thirds of their lengths, the amino-terminal domains are highly divergent (14, 15). Instead of the SRβ-binding domain found in SRA, FtsY has a highly negatively charged A region at the amino terminus (arbitrarily defined as amino acids 1–196) (20) that, together with the central N region (amino acids 197–280), forms the minimum domain sufficient for assembly on the *E. coli* inner membrane (21). Mutations that abolish attachment of FtsY to the *E. coli* inner membrane interfere with protein targeting and cell viability (8, 22).

Both bilayer and non-bilayer phospholipids have been implicated in the assembly and activity of several components of the secretory pathway in *E. coli*, including SecA (23, 24) and the translocon (25). Recent investigations have demonstrated the presence of a region in the carboxyl-terminal region of FtsY that may be regulated by binding to anionic phospholipids (26). It was also speculated that a second lipid-binding site exists in the amino-terminal region of FtsY (26). Here we show that the previously defined membrane-binding domain of FtsY binds liposomes containing the zwitterionic phospholipid phosphatidylethanolamine (PE) independent of a protein receptor. Surprisingly, in the absence of PE, SRP-dependent protein targeting remains functional, and the FtsY membrane assembly domain binds to *E. coli* inner membrane inverted vesicles (INV) via an interaction with a trypsin-sensitive component. This suggests that, similar to SecA of the general secretory pathway (23, 24), membrane assembly of FtsY involves interactions with both a specific lipid and a membrane protein. Based on these results and previously published data, we propose that FtsY binding to the membrane occurs initially through phospholipid binding, followed by targeting to translocation sites via an interaction with a membrane protein.

Jonathan S. Millman‡, Hai-Yan Qi§, Felicia Vulcu‡, Harris D. Bernstein§, and David W. Andrews¶

From the ‡Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada and the §Genetics and Biochemistry Branch, NIDDK, National Institutes of Health, Bethesda, Maryland 20892-1810.

Received for publication, December 15, 2000, and in revised form, May 14, 2001.

Published, JBC Papers in Press, May 15, 2001, DOI 10.1074/jbc.M011331200

*This work was supported by a grant and a scientist award from the Medical Research Council of Canada (to D. W. A.) and by a studentship from the Natural Sciences and Engineering Research Council of Canada (to J. S. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Dept. of Biochemistry, McMaster University, 1200 Main St. W., Hamilton, Ontario L8N 3Z5, Canada. Tel.: 905-525-9140 (ext. 22075); Fax: 905-527-9033; E-mail: andrewsd@mcmaster.ca.

¶The abbreviations used are: SRP, signal recognition particle; SRA and SRβ, α- and β-subunit of the SRP receptor, respectively; PE, phosphatidylethanolamine; INV, inner membrane inverted vesicle; AP, alkaline phosphatase; WT, wild-type; FBST, *Propionibacterium shermanii* transcarboxylase; CRMs, canine rough microsomes; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(N-morpholino)propanesulfonic acid; FG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine.

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Membrane—INVs were isolated from E. coli strain W3899 or AD93 as described previously (21). To inactivate surface proteins on the INVs by proteolysis, INVs were adjusted to 10 mM calcium chloride (to stabilize the membranes) and treated with 50 μg/ml trypsin or 100 μg/ml proteinase K for 1 h at 24 °C. Following incubation and other proteases with an inhibitor mixture (containing 20 μg/ml each chymostatin, antipain, leupeptin, and pepstatin and 40 μg/ml aprotinin) and 1 mM phenylmethylsulfonyl fluoride, vesicles were adjusted to 500 mM potassium acetate and collected by centrifugation over a 0.5 mM sucrose cushion at 70,000 rpm (215,000 × g) in a Beckman TLS100.2 rotor. As an alternative means of inactivating surface proteins, separate aliquots of INVs were treated with 5 mM N-ethylmaleimide at 37 °C for 30 min at pH 7.5 and collected as described above. Lipidophilic liposomes were generated using purified lipids (Avanti Polar Lipids, Inc.) in 50 mM Tris acetate (pH 7.5) by passing the suspension through a 0.1-μm filter 10 times using a mini-extruder (Avanti Polar Lipids, Inc.). Microsomes (CRMs) were isolated from canine pancreas as described (19).

CONSTRUCTION OF ADO204 AND ORIENTAL—Plasmids were transferred into E. coli strain DH5α using SP6 polymerase, and then polypeptides were synthesized from unpurified transcription products and labeled with [35S]methionine using a rabbit reticulocyte lysate translation system. Translation products (20 μl) were incubated for 45 min at 24 °C with 1 μl of canine pancreatic microsomes, inverted vesicles, or phospholipid liposomes. To assess targeting to microsomes and inverted vesicles, the mixture was loaded onto a 0.8-ml column of Sepharose CL-2B equilibrated with 50 mM Tris acetate (pH 7.5) and 1 mM DTT in a 1-ml syringe. The column was eluted with the same buffer; fractions (two drops each) were collected; and 8-μl samples were analyzed by SDS-PAGE. The included and excluded volumes of the Sepharose CL-2B column were calibrated as described (19).

Binding to liposomes was assayed by liposome flotation. The mixture was adjusted to a final sucrose concentration of 1.6 M, and 50 μl was overlaid with sucrose steps of 100 μl (1.25 M sucrose) and 50 μl (0.25 M sucrose) in 50 mM Tris acetate (pH 7.5) and 1 mM DTT. Following centrifugation, in a Beckman TLS100 rotor at 100,000 rpm (435,000 × g) for 90 min, the gradient was divided into 40-μl fractions, and the pellet was solubilized in 40 μl of 10 mM Tris acetate (pH 7.5) and 1% SDS and boiled for 5 min for proteinase K digestion as described previously (2). Cell-free translation products were incubated with canine microsomes (Fig. 1A), and the mixture was loaded onto a 0.8-ml column of Sepharose CL-2B equilibrated with 50 mM Tris acetate (pH 7.5) and 1 mM DTT in a 1-ml syringe. The column was eluted with the same buffer; fractions (two drops each) were collected; and 8-μl samples were analyzed by SDS-PAGE. The included and excluded volumes of the Sepharose CL-2B column were calibrated as described (19).

For light scattering measurements, at time 0, 10 μg of myelin basic protein, FtsYAN-gPa, or FtsYA-gPa, or buffer alone was added to 1 ml of 0.01 mg/ml liposomes in a 1-cm path length cuvette using a remote sample injector and a cell equipped with a magnetic stirrer. A 550-nm wavelength was directed through the sample, and photons scattered at 90° to the sample were collected with a 1-s integration time (collection time of 1 s/point) through a 1-μm slit using a photomultiplier and recorded using FeliX™ software (Photon Technology International). For graphical comparison, counts obtained using purified proteins at the indicated concentrations in buffer without lipids were subtracted from the values obtained in the presence of liposomes.

Membrane Protein Insertion Assay—To monitor membrane protein insertion, AD90 cells that were freshly cured of plasmid pDD72 as previously described (27) and W3899 cells were both transformed with a plasmid from the pJH10–13 series. Cells containing plasmid pJJ10 or pJJ11 were grown overnight at 37 °C in MOPS minimal medium supplemented with all the amino acids except cysteine and methionine and 50 μg/ml ampicillin, washed, and added to fresh medium at A550 = 0.05. When the cultures reached A550 = 0.3, 2 mM isopropyl-β-D-thiogalactopyranoside was added to cells containing pJJ11. After an additional incubation of 40 min, cells were radiolabeled, converted to spheroplasts, and subjected to proteinase K digestion as described previously (2). AP-containing polypeptides were immunoprecipitated with a polyclonal antiserum (5 Prime—3 Prime, Inc.) and resolved by SDS-PAGE and fluorography. In all of the cultures, the trans, AN, and dIII sites were transcribed in opposite directions.

RESULTS

Membrane Binding of FtsY Does Not Require a Specific Protein Receptor—We have previously demonstrated, using a column chromatography-based assay, that the AN domain of FtsY (amino acids 1–284) specifically interacts with E. coli INVs, whereas the A region alone (amino acids 1–197) is unable to bind INVs (21). Similarly, deletion of as few as 19 amino acids from the amino terminus abolishes binding (21). Thus, the AN domain is the minimum membrane-binding domain of FtsY.

To further investigate membrane binding of FtsY, we compared binding of full-length FtsY, FtsYAN, and, as a negative control, FtsYA synthesized in reticulocyte lysate to INVs and canine microsomes (Fig. 1). As expected, reticulocyte-translated FtsY and FtsYAN, but not FtsYA, bound INVs and therefore eluted in the excluded fractions when subjected to gel exclusion chromatography (Fig. 1A, fractions 3 and 4, arrowheads). As seen previously, a fraction of membrane-bound FtsY was cleaved between the N and G regions (Fig. 1A, arrow). Unexpectedly, full-length FtsY eluted in the excluded volume when incubated with canine microsomes (Fig. 1A, upper panel, +CRMs, fractions 3 and 4). Since the exclusion limit of this matrix is sufficient to retain cytosolic proteins and large aggregates of FtsY within the included volume, this indicates that FtsY can also bind to the eukaryotic endoplasmic reticulum membrane. As seen previously, a large fraction of the FtsY synthesized in vitro folded such that it would not bind to INVs, and although aggregated, it was recovered in the included fractions (Fig. 1A, upper panel, +INVs, fractions 5–12). Similarly, these molecules did not bind to microsomes (Fig. 1A, upper panel, +CRMs, fractions 5–12).

When FtsY bound microsomes, it was not cleaved (Fig. 1A, upper panel, +CRMs, fractions 3 and 4; the migration position of the cleavage product is indicated by the arrow). This confirms our prior suggestion that the proteolytic activity that cleaves FtsY resides on E. coli inner membranes (21).

Binding of FtsY and FtsYAN to microsomes was assayed to determine if binding of FtsY requires the same minimum domain required for binding to the E. coli inner membrane. The A region of FtsY eluted entirely within the included volume following Sepharose CL-2B chromatography (Fig. 1A, middle panel, +CRMs, fractions 3–12), demonstrating that it does not bind to microsomes. However, FtsYAN bound to microsomes as efficiently as it bound to INVs, confirming that the A and N regions together fold into a domain that binds to membranes (Fig. 1A, lower panels, compare fractions 3 and 4). The relatively high efficiency of binding of FtsYAN to vesicles in vitro compared with full-length FtsY is likely due to decreased aggregation when the G region is absent (21). Thus, the AN

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domain is sufficient for binding to either eukaryotic microsomes or *E. coli* inner membranes. We have previously demonstrated that the NG domain of FtsY does not bind INVs (21).

Binding of FtsY to eukaryotic microsomes via an interaction with a specific protein is unlikely, as there is no eukaryotic homolog for the FtsY membrane-binding domain. Therefore, it is possible that FtsY binding to INVs is also independent of a specific protein receptor. To determine whether a protein on the cytoplasmic face of the *E. coli* membrane is required for binding FtsY, proteins on the surface of the INVs were inactivated either by proteolysis or by alkylation of free sulfhydryl groups (cysteine) with *N*-ethylmaleimide (Fig. 1B). Alkylation of INVs with *N*-ethylmaleimide had no effect on binding of FtsYAN (Fig. 1B, fractions 3 and 4). Following incubation of FtsYAN with either trypsin- or proteinase K-treated vesicles, the vast majority of the protein continued to elute in the excluded volume following Sepharose CL-2B chromatography (Fig. 1B). Taken together, these results strongly suggest that FtsY binds to non-proteinaceous membrane component(s). Membrane phospholipids are therefore an obvious potential binding site for FtsY.

The AN Domain of FtsY Binds to Phospholipid Liposomes via a Specific Interaction with PE—To investigate the possibility that FtsY binds to *E. coli* membranes through an interaction with phospholipids, a liposome flotation assay was employed (Fig. 2). The gradients in Fig. 2 were fractionated such that the floated vesicles were recovered in the 0.25 m sucrose step (lanes 1, 2, 7, and 8, arrowheads). In the absence of liposomes, full-length FtsY was found in dense fractions (Fig. 2A, upper panel, lanes 10–12). When incubated with liposomes, some of the full-length FtsY migrated into the 0.25 m sucrose step (Fig. 2A, compare lanes 1 and 2 with lanes 7 and 8), confirming that it bound to liposomes. Similarly, FtsYAN also fractionated at the 0.25 m sucrose step only when incubated with liposomes (Fig. 2A, compare lanes 1 and 2 with lanes 7 and 8). Compared with these molecules, only a small fraction of FtsYA migrated at the 0.25 m sucrose step in the presence of liposomes (Fig. 2A). Furthermore, under these conditions, the NG domain failed to migrate into the 0.25 m sucrose step in the presence of liposomes (Fig. 2A). Thus, the AN domain specifically mediates binding of FtsY to membranes via an interaction with one or more species of *E. coli* phospholipid. FtsY is not cleaved in this assay, even after binding to liposomes (Fig. 2A, upper panel), confirming that the protease responsible for specific cleavage of FtsY (Fig. 1) is not present in the reticulocyte lysate.

Using the flotation assay described above, lipid binding of the AN domain of FtsY was examined for lipids with different head groups (Fig. 2B). The inner membrane of *E. coli* contains four predominant phospholipids: PE, phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidic acid (PA). Although the ratios of these species vary between strains of *E. coli*, a typical wild-type strain (W3899) has a composition of 76% PE, 14% PG, 10% CL, and trace amounts of PA (30). Therefore, we investigated the binding of FtsYAN to liposomes prepared from different combinations of these lipids.

Since, in some instances, it was not possible to generate liposomes using a single lipid, we used PG as a “background” lipid in which to assess binding. PG was used for this purpose because, unlike PE, it was possible to generate liposomes from pure PG as well as other lipids in combination with PG. In each case, the liposomes were made from equal amounts of PG and the lipid being tested.

In flotation assays, ~12% of FtsYAN bound to liposomes generated from pure PG (Fig. 2B). This did not differ significantly from the amount of FtsYA (used as a non-membrane binding control) that bound to pure PG liposomes (Fig. 2B). A similar amount of FtsYA bound to liposomes composed of both PE and PG, whereas ~50% of FtsYAN bound to liposomes containing PE (Fig. 2B). FtsYAN failed to bind above base-line levels to liposomes containing mixtures of either PA or CL with PG (Fig. 2B). Thus, it is likely that in *vivo* membrane assembly of FtsY involves binding to PE.

To determine if FtsY binding to liposomes containing PE depends on a direct interaction with the ethanolamine head group, we performed competition experiments by adding ethanolamine to the binding reaction. We found that, in flotation assays, increasing concentrations of ethanolamine reduced the amount of FtsY that bound to liposomes. Since, in some instances, it was not possible to generate liposomes using a single lipid, we used PG as a “background” lipid in which to assess binding. PG was used for this purpose because, unlike PE, it was possible to generate liposomes from pure PG as well as other lipids in combination with PG. In each case, the liposomes were made from equal amounts of PG and the lipid being tested.

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to liposomes containing PE (Fig. 2B).

To ensure that binding of FtsYAN to membranes did not simply reflect liposome aggregation, light scattering measurements were taken for liposomes composed of 50% PE and 50% PG incubated with an equimolar amount of FtsYAN-gPa, FtsYA-gPa, or myelin basic protein (Fig. 3). FtsYAN-gPa has been shown to bind INVs (21) and liposomes (see Fig. 4). Fusion to gPa permits purification of the fusion proteins following expression in E. coli by virtue of the affinity of gPa for immunoglobulins.

As has been described previously (30), addition of myelin basic protein to liposomes leads to an immediate and extensive increase in light scattered due to aggregation of liposomes. The scattering observed upon addition of myelin basic protein plateaued and then decreased slowly due to the settling of aggregated liposomes from solution (Fig. 3). Upon addition of either FtsYAN-gPa or the non-binding control FtsYA-gPa to identical liposomes, no difference in light scattering was observed compared with the liposomes alone (Fig. 3). This suggests that binding of FtsYAN to membranes cannot be accounted for by induced liposome aggregation.

FtsY Undergoes a Conformational Change upon Binding Liposomes—We have previously demonstrated that FtsY and FtsYAN-gPa are cleaved carboxyl-terminal to the N region upon membrane binding (21). Although the identity of the protease(s) responsible for this cleavage in vivo is unknown, we found that this site was sensitive to cleavage by trypsin at a concentration of 20 pg/μl even in the absence of membranes (Fig. 4, lane 9). However, when FtsYAN-gPa was bound to liposomes and isolated by blotting, the protein that cofractionated with liposomes in the top fraction of the sucrose gradient was cleaved carboxyl-terminal to the N region by trypsin at a concentration of 2 pg/μl (Fig. 4, lane 2). FtsYAN-gPa that was not associated with liposomes and hence eluted in the denser fractions still required a concentration of 20 pg/μl for cleavage (Fig. 4, lanes 5 and 6). Thus, it is apparent that a conformational change that makes the protease-sensitive site more accessible occurs when FtsYAN-gPa binds liposomes.

PE Depletion Does Not Block Assembly of Inner Membrane Proteins—The E. coli strains AD90 and AD93, which are unable to synthesize PE (27), were used to further investigate the role of PE in membrane binding of FtsY. These strains can be grown in the presence of divalent cations, resulting in E. coli with an inner membrane composed of 46% PG, 50% CL, and 4% PA (31).

Binding of FtsYAN to phospholipid vesicles containing 46% PG, 50% CL, and 4% PA was no higher than that observed in the absence of membranes (Fig. 2B, “AD93”). Addition of magnesium ions at a concentration of 50 mM (similar to the concentration of Mg\(^{2+}\) in the growth medium used for PE-depleted strains) had no effect on binding of FtsY (data not shown). Thus, in AD90 or AD93 cells grown in Mg\(^{2+}\), FtsY cannot bind to the inner membrane via an interaction with phospholipids. We therefore presumed that, in these strains, the assembly of inner membrane proteins that are targeted by SRP would be reduced or eliminated completely. One such protein is the multi-spanning transmembrane protein AcrB (2). To analyze the insertion of AcrB into the cytoplasmic membrane, we utilized an AP fusion to the amino-terminal 576 amino acids of AcrB (AcrB576-AP) that requires the SRP pathway for membrane insertion (2). Proper insertion of the fusion protein and transport of the AP domain into the cytoplasmic membrane were assessed by a protease protection assay (32). When such fusion proteins were properly inserted into the inner membrane, the AP domain folded into a protease-resistant conformation that was cleaved from the remaining protein by the protease and that could be immunoprecipitated and observed by SDS-PAGE (Fig. 5A, AP).

As expected, in the WT strain expressing only wild-type FtsY, only bands corresponding to AP alone were immunoprecipitated following protease treatment of spheroplasts (Fig. 5A, upper panel, lanes 6–8). This indicates that insertion of the fusion protein into the cytoplasmic membrane in wild-type cells...
conditions under which cell growth is more rapid (33). The 576-amino acid amino-terminal fragment of AcrB was expressed as a fusion to PBST. If the fusion fails to integrate into the cytoplasmic membrane, the 76-amino acid PBST is recognized and biotinylated by cytoplasmic E. coli biotin ligase. The biotinylated protein was detected with horseradish peroxidase-conjugated streptavidin on a Western blot (Fig. 5B). Expression of this protein in wild-type cells together with dominant lethal FtsY led to reduced integration of the fusion protein into the cytoplasmic membrane, as indicated by the recognition of biotinylated AcrB576-PBST with horseradish peroxidase-conjugated streptavidin (Fig. 5B, lanes 3 and 4). When wild-type FtsY was expressed, essentially no fusion product was recognized by horseradish peroxidase-conjugated streptavidin (Fig. 5B, lanes 1 and 2), showing that the fusion protein integrated appropriately into the cytoplasmic membrane. In cells deficient in PE, the protein integrated into cytoplasmic membranes of cells expressing wild-type FtsY (Fig. 5B, lanes 5 and 6), but not dominant lethal FtsY (lanes 7 and 8). Similar to the results with the AP fusion, depletion of PE failed to elicit a decrease in AcrB membrane assembly. It is therefore apparent that, despite the observation that the membrane assembly domain of FtsY binds PE, the presence of this phospholipid in the cytoplasmic membrane is not essential for the activity of the SRP pathway.

**FtsY Membrane Assembly Involves Both PE and a Membrane-Associated Protein**—The absence of an apparent defect in assembly of SRP-dependent inner membrane proteins in strains devoid of PE suggested that FtsY might continue to bind to the cytoplasmic membrane in these cells. This hypothesis is supported by immunoblots of cytosol and INVs prepared from WT and AD93 cells that revealed an identical distribution of FtsY between the cytosol and membrane in both strains (data not shown). Therefore, in the presence of 50 mM Mg\(^{2+}\), FtsY assembles on the inner membrane of AD93 cells that do not contain any PE.

Binding of FtsY to *E. coli* inner membranes lacking PE was confirmed in vitro using a column chromatography assay.

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\(^{2}\) H. D. Bernstein, unpublished data.
FtsYAN binding to vesicles prepared from AD93 cells was the same as that to vesicles prepared from wild-type E. coli (Fig. 6, upper panels, compare lanes 1 and 2 with lanes 11 and 12). Since the presence of divalent cations restores proper protein translocation across PE-depleted vesicles (34), we considered that magnesium or other cations present in in vitro translocations may participate in FtsY binding to PE-depleted INVs. Therefore, in a separate control experiment, translation reactions were depleted of small molecules by passage over a Sephadex G-25 gel or exclusion column, and the INVs were prepared in a buffer that did not contain Mg²⁺. FtsYAN continued to bind INVs in the absence of Mg²⁺ (data not shown), suggesting that some component of INVs in addition to PE binds FtsY.

To examine the potential involvement of an E. coli inner membrane protein in binding FtsY to membranes, we assayed binding of FtsYAN to WT and AD93 vesicles treated with trypsin. FtsYAN bound to control (mock-treated) AD93 and WT vesicles (Fig. 6, middle panels, compare lanes 1 and 2 with lanes 11 and 12) and to trypsinized wild-type vesicles that contained PE (lower panels, lanes 1 and 2). However, when incubated with trypsin-treated vesicles prepared from AD93 cells, FtsYAN was essentially absent from the excluded fractions (Fig. 6, compare middle and lower panels, lanes 11 and 12). Since both PE depletion and protease degradation of INV proteins are required to eliminate binding to INVs, either lipid or a membrane-protein can bind FtsY to membranes. Other explanations such as loss of FtsY binding after trypsin treatment due to a proteolysis-induced physical change (e.g. curvature) in the membranes are unlikely since binding was lost only with INVs that did not contain PE.

The avidity of FtsY binding to biological membranes is greater than that to artificial liposomes—Binding of FtsY to both PE and a membrane protein suggests that FtsY binds to INVs in a manner qualitatively different than the manner in which it binds to liposomes. To test this, the avidity of FtsYAN for liposomes and INVs was compared by varying the ionic strength of the incubation buffer and then assaying membrane binding by flotation as described above. Binding of FtsYAN to INVs was unaffected by up to 1 M NaCl (Fig. 7). In contrast, binding to liposomes decreased with increasing salt concentration (Fig. 7, □). The amount of FtsYAN bound to liposomes in 1 M NaCl was 20% of that bound in 0.1 M salt (Fig. 7). Thus, at higher salt concentrations, FtsYAN binds to INVs much better than to liposomes. This difference in binding is likely due to the trypsin-sensitive FtsY-binding protein on INVs.

**DISCUSSION**

The membrane-binding domain of FtsY binds to phospholipids—The ability of the membrane assembly domain of FtsY to bind proteolyzed or alkylated membranes provided the first clear evidence that membrane binding may involve a non-proteinaceous component. The concentration of trypsin used to treat these vesicles was 100 times greater than that previously found to be sufficient to prevent binding of SRα to canine microsomes (35). This clearly shows that SRα and FtsY bind microsomes in a qualitatively different manner, the latter most likely through an interaction with a non-protein component of the membrane.
Our finding that FtsY and FtsYAN bind vesicles made from purified E. coli lipids confirmed that FtsY can bind membranes through an interaction with phospholipids. Concurrent with these experiments, the ability of FtsY to bind liposomes was reported independently by de Leeuw et al. (26). Surprisingly, those authors detected binding of the NG domain of FtsY to liposomes. We found previously that removal of even the amino-terminal 19 amino acids from FtsY completely abolishes binding to INVs (21), and we detected only very inefficient binding to the NG domain of FtsYAN in each fraction were quantified using a PhosphorImager. Percent binding was determined as (radioactive counts in the top two fractions divided by the total radioactivity) × 100. Error bars indicate 1 S.D. calculated from data from three independent experiments.

Unlike the binding of FtsY to INVs (21), full-length FtsY was not cleaved between the AN and G domains when it bound to microsomes or liposomes (Fig. 2). This result, together with the demonstration that cleavage does not depend on the translation extract (21, Fig. 2), extends our previous experiments by demonstrating unequivocally that the protease that cleaves FtsY resides on the E. coli inner membrane.

The cleavage site between the AN and G domains does become more sensitive to proteolytic cleavage upon binding liposomes (Fig. 4), which suggests that FtsY undergoes a conformational change upon binding to lipids. Although a role for this conformational change is unclear, it suggests that FtsY forms specific interactions with liposomes. Furthermore, since the N and G regions of FtsY are quite closely packed together in protein crystals (36), a conformational change upon membrane binding that increases the accessibility of the region between these domains lends greater credence to the observation that the A and N regions together are involved in membrane binding.

**FtsY Binds Membranes through a Specific Interaction with Phosphatidylethanolamine**—Of all the major E. coli lipids, FtsY binds only to liposomes containing PE (Fig. 2B). It also binds the closely related lipid PC (Fig. 2B). Since the A region of FtsY is highly negatively charged and the liposomes to which it binds have the greatest positively charged character of all those investigated, it was of some concern that the binding observed may reflect a simple ion-induced aggregation of liposomes, as has been previously observed (37). This was ruled out by examining liposome aggregation by light scattering following addition of purified protein to liposomes (Fig. 3). Since addition of FtsYAN-gPa to liposomes resulted in no appreciable change in light scattering (Fig. 3), we conclude that the interaction of FtsYAN with liposomes does not result merely from aggregation of the liposomes.

It has previously been observed that full-length FtsY does cause liposome aggregation, leading to the suggestion that there were likely two different lipid-binding sites in FtsY (26). Although two lipid-binding sites in FtsY remains a possibility, our deletion mutagenesis study suggests that only a single domain (AN) binds specifically enough to be physiologically relevant for membrane targeting and assembly. The two sets of results can be reconciled by the observation that FtsY aggregates in solution. Any multimerization (including aggregation) of a protein containing a single lipid-binding domain would result in a protein expected to behave like a multivalent lipid-binding protein. Although we have not assayed oligomerization of FtsY rigorously, gel filtration chromatography revealed that most of FtsY synthesized in vitro forms large aggregates (22). Consistent with multimerization, when FtsY purified from E. coli was analyzed by gel filtration chromatography, it eluted as a single peak with an apparent molecular mass of >200 kDa (38).

It is also apparent that, although the AN domain is highly negatively charged (net charge of −48) (20), binding to PE cannot be accounted for by a simple electrostatic interaction. The A region alone accounts for a net charge of −47, but does not bind PE/PG liposomes above background (Fig. 2). Thus, the inclusion of PE in liposomes does not enhance FtsYAN binding solely by reducing the electrostatic repulsion between the negatively charged AN domain and negatively charged phospholipid head groups. Rather, these data, together with our results demonstrating that ethanolamine competes for FtsY binding to PE-containing liposomes, suggest that the A and N regions fold into a domain containing a specific binding pocket that allows FtsY to bind PE.

Unlike the lipid interaction observed with the NG domain, which was speculated to involve both an electrostatic interaction and the insertion of the domain into the lipid bilayer (26), we propose here that the interaction of the AN domain with the membrane is a true peripheral interaction involving a specific interaction with the head group of PE (or PC) and a membrane protein. Our conclusion that binding is peripheral to the lipid core of the bilayer is consistent with our observation that FtsY is displaced from liposomes by 500 mM NaCl (Fig. 7), a salt concentration that is unlikely to extract a protein domain from a lipid bilayer.

**FtsY Also Binds a Protein on the Cytoplasmic Membrane**—Similar to depletion or disruption of FtsY function (2, 20), depletion of PE from wild-type E. coli leads to both cell division and protein translocation deficiencies (34, 39). The continued targeting of an SRP-dependent protein to the membrane in cells depleted of PE (Fig. 5) and our identification of a trypsin-sensitive binding site on INVs generated from these cells suggest that the defects seen in PE-depleted cells are not entirely due to the effect on the targeting of FtsY. However, it remains likely that the efficiency of FtsY-mediated targeting drops, contributing to the phenotype that eventually develops.

It is clear that the interaction of FtsY with PE is different from the interaction with the membrane protein. FtsY bound to the cytoplasmic membrane is not extracted with NaCl concentrations up to 1 M, whereas when bound to PE-containing liposomes, FtsY binding decreases with increasing NaCl concentrations (Fig. 7). This quantitative difference in binding shows that FtsY binds to the protein component of the membrane more tightly than to PE. Taken together with the abundance of PE in wild-type E. coli membranes (70%), it seems likely that FtsY would bind to E. coli membranes initially by an
interaction with PE, and it may then be transferred to empty translocation sites via the tighter interaction with a membrane-bound receptor. A precedent for this type of membrane binding exists with SecA, which requires an interaction with both acidic phospholipids and the SecY-SecE complex for high affinity binding to the E. coli inner membrane (23, 24).

The similarity to SecA suggests a strategic advantage of “dual specificity” binding. It is possible that membrane assembly occurs by a two-step process in which the first step (lipid binding) serves to reduce a three-dimensional problem to two dimensions. Initial binding of FtsY to the lipid membrane would be greatly facilitated by an interaction with PE, which constitutes >70% of membrane phospholipid. However, an abundant lipid is unlikely to provide sufficient targeting specificity for FtsY or an FtsY-Ffh-nascent protein complex. Thus, following initial binding, the restriction of FtsY to the two-dimensional surface of the membrane may increase the apparent affinity for the specific membrane receptor, possibly the translocation pore, by the restricted movement mechanism proposed by Kholodenko et al. (40). That no SRP-dependent translocation defect was observed in our assays when cells were depleted of PE is not surprising if this mechanism is presupposed. Under the conditions used in our assays, the initial lipid-binding step does not appear to be essential for targeting FtsY to the membrane, allowing the insertion into the cytoplasmic membrane of SRP-dependent proteins to proceed. However, under conditions of stress or rapid metabolism, the two-step mechanism may confer an advantage to cells and thus has been retained in both the SRP pathway for FtsY and the general secretory pathway for SecA.

The identification of a target membrane via binding to abundant lipids may provide sufficient initial targeting specificity in E. coli because there is only a single target membrane. In contrast, an initial lipid-binding step would impair targeting to the endoplasmic reticulum in eukaryotes because there are several noncontiguous membrane-delimited compartments that would compete for initial targeting. Initial binding to an incorrect membrane in the cell would then severely impair targeting to the correct membrane. This may explain why in eukaryotes membrane assembly of the SRP receptor requires a transmembrane β-subunit not found in prokaryotes.

Acknowledgments—We thank Linda Diehl for excellent technical assistance, Dr. William Dowhan for the gift of strains AD90 and AD93, and Dr. George Haraux for the gift of purified myelin basic protein.

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