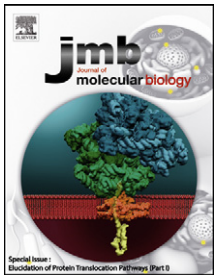

SecA Drives Transmembrane Insertion of RodZ, an Unusual Single-Span Membrane Protein

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http://dx.doi.org/10.1016/j.jmb.2014.05.005
Edited by I. B. Holland

Abstract

The transmembrane (TM) helices of most type II single-span membrane proteins (S-SMPs) of *Escherichia coli* occur near the N-terminus, where the cell's targeting mechanisms can readily identify it as it emerges from the ribosome. However, the TM helices of a few S-SMPs, such as RodZ, occur a hundred or more residues downstream from the N-terminus, which raises fundamental questions about targeting and assembly. Because of RodZ's novelty and potential usefulness for understanding TM helix insertion *in vivo*, we examined its membrane targeting and assembly. We used RodZ constructs containing immunotags before the TM domain to assess membrane insertion using proteinase K digestion. We confirmed the N_{in}-C_{out} (type II) topology of RodZ and established the absence of a targeting signal other than the TM domain. RodZ was not inserted into the membrane under SecA depletion conditions or in the presence of sodium azide, which is known to inhibit SecA. Insertion failed when the TM proton gradient was abolished with Carbonyl cyanide m-chlorophenyl hydrazone. Insertion also failed when RodZ was expressed in SecE-depleted *E. coli*, indicating that the SecYEG translocon is required for RodZ assembly. Protease accessibility assays of RodZ in other *E. coli* depletion strains revealed that insertion is independent of SecB, YidC, and SecD/F. Insertion was found to be only weakly dependent on the signal recognition particle pathway: insertion was weakly dependent on the Ffh but independent of FtsY. We conclude that membrane insertion of RodZ requires only the SecYEG translocon, the SecA ATPase motor, and the TM proton motive force.
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Introduction

The mechanisms of membrane protein (MP) assembly and protein secretion of Gram-negative bacteria converge at the SecYEG translocon complex (protein-conducting channel) located in the inner membrane (Fig. 1). The extensive literature on these mechanisms has been recently reviewed [8–11]. Secreted proteins and proteins destined for the outer membrane are driven post-translationally through the SecYEG channel by the ATP-powered SecA translocase. These secreted proteins are generally identified biologically by a hydrophobic N-terminal signal sequence that is cleaved by signal peptidase during secretion through the SecYEG channel. In contrast, multi-span MPs with short interhelical loops are assembled co-translationally at SecYEG after delivery of active ribosomes by two essential components of the signal recognition particle (SRP) pathway [8–11]: Ffh, which is the protein component of the SRP protein–RNA complex, and surface-active FtsY, which partitions into the inner membrane from the cytoplasm [12] to receive the ribosome/nascent chain complex from the SRP and transfer it to SecYEG. In some cases, assembly is aided by another inner MP, YidC, which can directly contact SecYEG [13]. An exception to this picture occurs whenever a multi-span MP has long interhelical connecting loops: If the loops exceed about 30 amino acids in length, then SecA is required to transport the loops across the membrane, presumably through the channel [5,14,15].

Much of our understanding of the long-loop SecA intervention rules comes from extensive studies of

two imitation type II single-span membrane proteins (S-SMPs), Momp2 and LepΔH_2 (Fig. 1c), which have their N-termini in the cytoplasm and their C-termini in the periplasm (N_{in}-C_{out}). A common feature of these model S-SMPs is that the hydrophobic transmembrane (TM) segment occurs near the N-terminus,

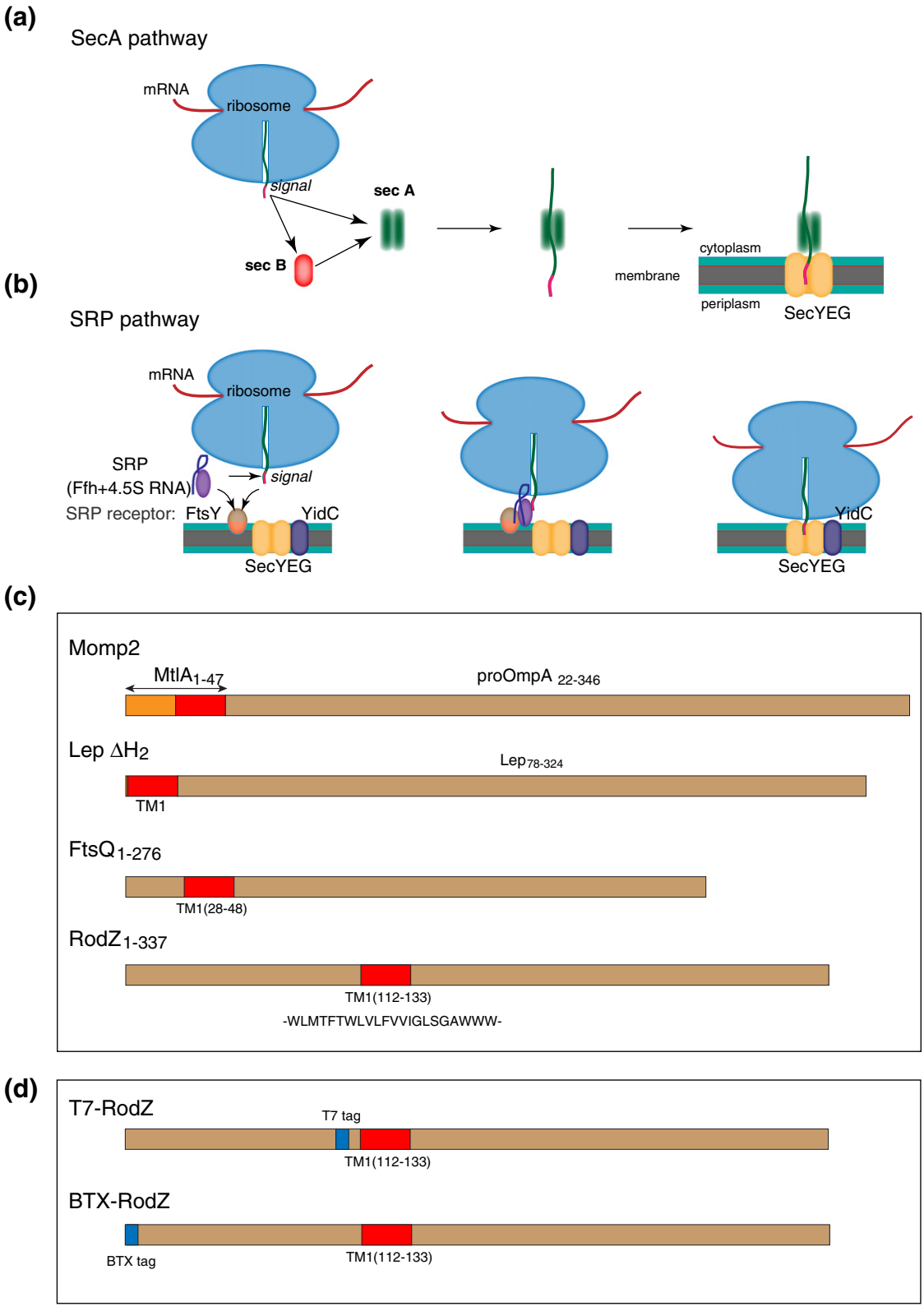


Fig. 1 (*legend on nextpage*)

acting, in effect, as a non-cleavable signal sequence. Momp2 [4] is a chimeric proOmpA in which the cleavable signal sequence is replaced by the first 47 residues of the multi-span MtlA mannitol permease. Within these 47 residues is the first TM helix of MtlA. Because OmpA is a secreted protein driven by SecA, it was not surprising to find that Momp2 insertion required SecA [4,16]. However, in addition, targeting to SecYEG required SRP. In a much earlier study using variants of *Escherichia coli* double-span leader peptidase (Lep), Andersson and von Heijne found that a deletion mutant of Lep lacking TM2 (called H2) strictly required SecA for assembly based upon inhibition of SecA by sodium azide [5]. The insertion of the native protein FtsQ, which has an N-terminal TM segment, is also SecA dependent but requires SRP and a TM proton motive force (PMF) as well [6,17]. In addition, FtsQ was found to interact with YidC during insertion [7]. These studies together make a strong case that SecA is required for the assembly of type II S-SMPs with TM segments near the N-terminus. But suppose the TM segment is far downstream from the N-terminus, could a TM segment occurring a hundred or more residues downstream affect the insertion pathway?

S-SMPs are abundant in all branches of life [18]. Based upon a recently published database of S-SMPs [19] and a hydropathy analysis using MPEx (*M*embrane *P*rotein *Ex*plorer) [20], there are several S-SMPs whose TM segments occur a hundred or more residues downstream from the N-terminus. Of 94 *E. coli* S-SMPs identified in the database, we found six qualifying proteins by MPEx analysis (exclusive of C-tail anchored proteins). The proteins identified are CadC, RodZ, DamX, GspL, RseA, and YjhP. Two of these are of particular interest to our laboratories in the context of S-SMP assembly and stability: RodZ, which plays an important role in maintenance of the rod shape of *E. coli* [21,22], and CadC, which activates the CadBA operon during low-pH stress [23–25]. We report here the results of a comprehensive study of the *in vivo* assembly of RodZ, a 337-residue protein with a TM domain located at residues 112–133. We find that, indeed, SecA drives the insertion of RodZ via the SecYEG translocon complex. The only other requirement for insertion is the TM PMF, as observed for FtsQ [6]. Unlike FtsQ, SRP is not strictly required for the assembly of RodZ. Because of the relative simplicity of its assembly requirements, RodZ is an ideal S-SMP for studies of SecA-driven insertion of TM helices.

Results

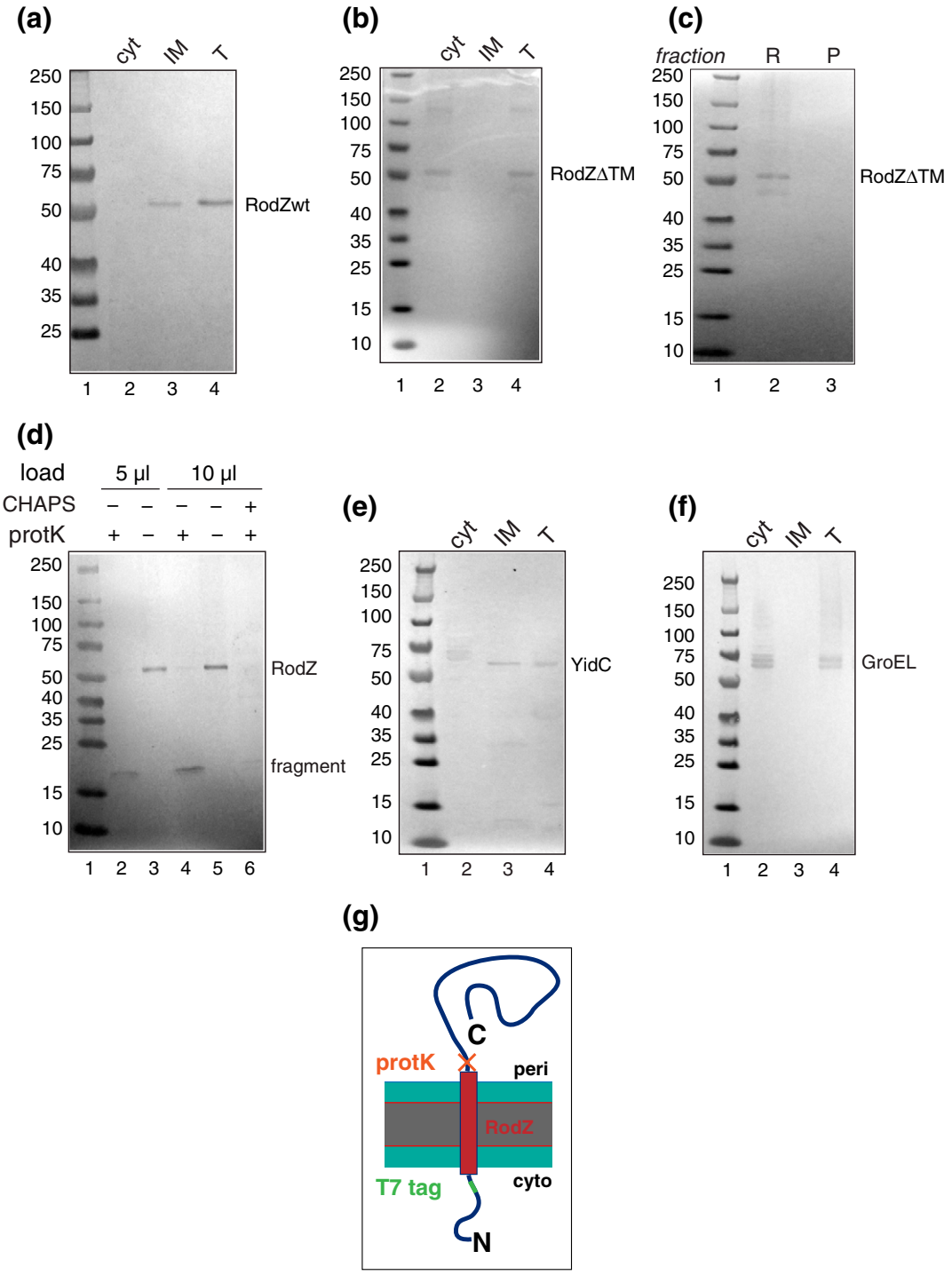
RodZ is a single-span membrane protein with N_{in}-C_{out} topology

An analysis of the RodZ sequence using the SPOCTOPUS software [26] revealed the absence of an N-terminal signal sequence and the presence of a downstream hydrophobic segment that could serve as an internal signal/stop-transfer sequence. MPEx [20] showed the most likely TM segment as residues 112–133 (22 amino acids; Fig. 1c). To visualize RodZ in Western blots, we incorporated a T7 tag (MASMTGGQQMG) between amino acids Ala95 and Pro96 by mutagenesis using overlap extension (Fig. 1d). We first expressed T7-RodZ to verify its presence in the inner membrane. The cell culture was subjected to DNAse I treatment followed by several freeze–thaw cycles prior to separating different subcellular fractions [27]. RodZ appeared only in the inner membrane (IM) and total fraction (T) (Fig. 2a). We then generated a TM-deleted version of RodZ (RodZΔTM). RodZΔTM appeared in the cytoplasmic (cyt) and T fractions, but not in the IM fraction (Fig. 2b). Furthermore, we determined that RodZΔTM was not secreted into the periplasm, as judged by the absence of a Western-blot signal in the periplasmic 'P' fraction (Fig. 2c). These results are consistent with residues 112–133 forming the transmembrane domain required for stable insertion of RodZ into the inner membrane. Because targeting of both membrane and secreted proteins invariably involves recognition of a hydrophobic amino acid sequence by the targeting/assembly components of the cell (see Introduction), the TM domain is likely the targeting signal. However, further studies are

Fig. 1. Targeting and membrane insertion of MPs in *E. coli* and some S-SMPs relevant to this study. (a) Schematic representation of the SecA protein secretion/membrane insertion pathway. (b) Schematic representation of the SRP pathway. The majority of secreted *E. coli* proteins are synthesized as preproteins with a cleavable signal peptide at their N-terminus. These preproteins are targeted to the SecYEG translocon post-translationally by the cytosolic ATPase SecA, sometimes helped by the molecular chaperone SecB [1]. The integral MP YidC associates with SecYEG but is not essential for insertion or secretion of many proteins. Many multi-span inner MPs are targeted in a co-translational manner to the SecYEG translocase by the SRP (Ffh) and its receptor (FtsY) [2]. In the SRP-mediated targeting pathway, the SRP–ribosome nascent chain complex is targeted to FtsY at the membrane and then translocated via SecYEG [3]. (c) Examples of S-SMPs. Momp2 [4] and LepΔH_2 [5] are model type II S-SMPs. FtsQ is one of the few native type II MPs whose biogenesis has been studied in much detail [6,7]. The three proteins all have TM segments near the amino-terminal end of the protein. The TM segment of RodZ, however, appears far downstream from the amino-terminus. (d) RodZ constructs used in the present study for determinations of RodZ topology. Both constructs carry immunotags on the N-terminal side of the single TM segment to allow determination of the location of intact and proteolytically cleaved protein.

required to establish unequivocally that the TM domain also serves as the targeting signal.

Using a FLAG epitope, Shiomi *et al.* demonstrated the membrane localization of RodZ and determined its likely topology using a PhoA fusion approach [28]. We confirmed the topology of RodZ using proteinase K (protK) digestion in which spheroplasts of BL21 cells harboring the pET21-T7-RodZ were treated with protK followed by Western blotting. The cytoplasmic fragment of T7-tagged RodZ confirmed the N_{in}-C_{out} topology of RodZ (Fig. 2d). In Fig. 2d (lanes 2 and 3 and lanes 4 and 5), the samples were loaded in different volumes to make sure of complete protK digestion of the protein and for better visibility



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of the digested fragment. The fragment ran at around ~17 kDa, consistent with protease digestion of the RodZ periplasmic domain. RodZ is therefore a type II S-SMP. This fragment could be completely digested by protK when the detergent 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonic acid was present (lane 6).

To validate the DNAse I separation protocol, we used the MP YidC and the cytoplasmic protein GroEL as loading controls. YidC was found only in the inner membrane and total fractions (Fig. 2e) while GroEL was observed only in the cytoplasmic and total fractions (Fig. 2f). Figure 2g summarizes our finding that RodZ is a type II S-SMP.

RodZ translocation is mediated by SecYEG translocon but is independent of YidC insertase

SecYEG mediates membrane insertion of most inner MPs [10], but there are exceptions [30]. We therefore verified the dependence of RodZ insertion on SecYEG using a RodZ construct with an α-bungarotoxin (BTX) binding tag (WRYYESSLLPYPD) [31,32] inserted near the amino-terminus between Gln2 and Thr3. Because SecE is required to stabilize the SecY protein, SecE depletion results in SecY depletion as well [33]. To test SecY dependence, we used the arabinose-dependent SecE depletion strain CM124 that has the *secE* gene under the control of araBAD promoter [34]. This cell line, harboring a pLZ1-BTX-RodZ vector, was grown under SecE expression and SecE depletion conditions in M9 minimal media supplemented with arabinose or glucose, respectively. IPTG (1 mM final concentration) was added to the CM124 cells (bearing the RodZ expression vector) 3 min before the cells were labeled with [^{35}S]methionine for 1 min. Figure 3a demonstrates the dependence of RodZ insertion on SecYEG: protK treatment of spheroplasts yielded a cytoplasmic BTX-RodZ fragment for the SecE expression condition but not for the SecE depletion condition. As a positive control, we demonstrated that translocation of proOmpA was inhibited under SecE depletion conditions (Fig. S1a). Together, these data show that membrane insertion of RodZ depends upon SecYEG.

The insertion of some MPs requires YidC and SecYEG [10,29]. To see if this is the case for RodZ, we used the YidC depletion strain JS7131 that contains *yidC* gene under the control of the araBAD promoter. The depletion strain containing pMS119-BTX-RodZ vector was grown in the presence of arabinose to express YidC or in glucose to deplete YidC. Following protK treatment of spheroplasts, the membrane translocation of the C-terminal domain of RodZ remained unaffected by YidC depletion conditions, as indicated by complete cleavage of full-length RodZ by protK (Fig. 3b). As a positive control, the YidC-dependent MP Pf3-p2 [35] was fully protected from proteolytic cleavage in spheroplasts upon YidC depletion (Fig. 3c), while mature OmpA was fully digested (Fig. S1b). We conclude from these experiments that the membrane insertion of RodZ does not require YidC but does require SecYEG.

SecA is required for membrane insertion of RodZ

Based upon the studies of the assembly of Momp2 [4,16] and LepΔH_2 [5] discussed earlier, we fully expected SecA to be essential for the assembly of RodZ. As a first test of this expectation, we grew cells in the presence of sodium azide, which is known to inhibit protein translocation in *E. coli* by

Fig. 2. RodZ is a type II S-SMP as determined by cell fractionation and immunoblotting. In the immunoblots shown, the total fraction (T) was obtained by solubilizing whole cell pellets with SDS loading buffer. The cytoplasmic (cyt) and inner membrane (IM) fractions were obtained by subjecting the cell suspensions to 10 freeze–thaw cycles in the presence of lysozyme and DNAse I. The suspensions were then centrifuged at 13,000*g* in a microfuge. The supernatant, consisting of the cytoplasm and periplasm, is taken as the cyt fraction. The pellet, consisting of inner and outer membranes, was resuspended in buffer containing detergent, which solubilizes the inner membrane. The suspension was again spun at 13,000*g*; the clear supernatant contained the solubilized IM proteins. Molecular mass markers (in kilodaltons) are shown in lane 1 of all blots. (a) This Western blot for T7-RodZ shows that wild-type RodZ is found in the T and IM fractions but is absent in the cyt fraction. Although RodZ has a molecular mass of 36 kDa, it migrates anomalously in SDS gels with an apparent molecular mass of ~55 kDa [28]. (b) The TM segment of RodZ is the insertion signal. This Western blot (T7 antibodies) shows that the expression of RodZΔTM, which is a T7-RodZ construct lacking the TM domain, is found only in the cyt and T fractions; it is absent in the IM fraction. (c) There is no signal in RodZΔTM for secretion. Secretion of RodZΔTM was tested using spheroplasts prepared by the osmotic shock method. The cells were spun at 13,000*g* for 15 min. The supernatant was used as the periplasmic fraction "P" and the pellet fraction (R) was used as non-periplasmic proteins. (d) RodZ is a TM protein oriented with a N_{in}-C_{out} topology. Spheroplasts of BL21 cells harboring pMS119-T7-RodZ were treated with or without protK (lanes 2 and 3 and lanes 4 and 5). In the presence of protK (protK+), only a fragment with the T7 tag corresponding to the cleaved N-terminal domain is observed, whereas in the absence of protK (protK−), only full-length protein is observed. In the presence of detergent, 1% 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonic acid, the fragment is digested by protK (lane 6). (e and f) Control experiment showing the efficacy of our separation protocol for isolating the cyt and IM fractions. Western blots using YidC antibodies show that the inner MP YidC [29] is found only in the IM and T fractions (e). Western blots using GroEL antibodies show that cytoplasmic protein GroEL is found only in the cyt and T fractions (f). (g) Cartoon of the topology of RodZ.

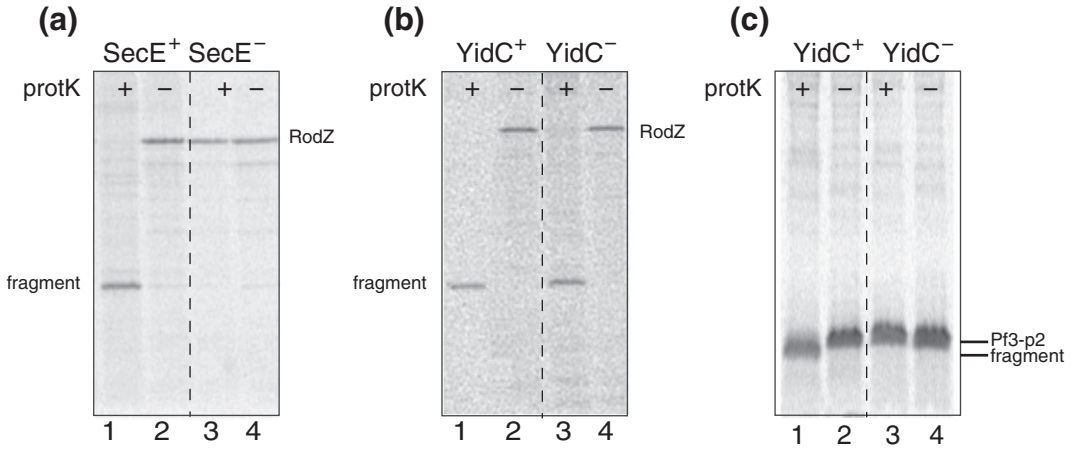


Fig. 3. Translocation of RodZ depends on SecYEG but not on YidC insertase. (a) RodZ incorporation in the membrane is blocked under SecE depletion conditions. A CM124 strain expressing pLZ1-BTX-RodZ was grown to mid-log phase under arabinose ($SecE^+$) or glucose ($SecE^-$) conditions. Cells were pulse labeled with [^{35}S]methionine and analyzed by the protK assay, as described in Materials and Methods. (b) RodZ membrane translocation is independent of YidC. The YidC depletion strain JS7131 bearing the pMS119-BTX-RodZ plasmid was grown for 3 h in LB media supplemented with 0.2% (m/V) arabinose or 0.2% (m/V) glucose. The membrane insertion of RodZ protein in the YidC expression and YidC depletion conditions was analyzed by proteinase accessibility assay as described in Materials and Methods. (c) Control experiment showing the effectiveness of YidC depletion. The YidC dependence of membrane insertion of YidC-dependent Pf3-p2 protein [35] was tested in JS7131, as described in Materials and Methods. Membrane translocation of Pf3-p2 was inhibited under YidC-depleted conditions, as judged by the absence of a proteolytic fragment under protK+ conditions (compare lanes 1 and 3). See also Fig. S1.

blocking SecA ATPase function [36]. We used MC1060 cells harboring the pMS119-BTX-RodZ vector at mid-logarithmic growth phase and treated with or without 3 mM sodium azide (final concentration) for 5 min before pulse labeling with [^{35}S] methionine for 1 min. The spheroplasts of the cell culture were then analyzed using the protK accessibility assay. Figure 4a shows that the sodium azide treatment blocked the membrane insertion of RodZ (lane 3), as evident from the absence of proteolytic fragment compared with cells not treated with azide. Likewise, the export of SecA-dependent proOmpA was strongly inhibited in the cells treated with sodium azide (Fig. S2a).

To confirm further the involvement of SecA in the biogenesis of RodZ, we transformed the SecA depletion strain of *E. coli* EO527 with the plasmid (pMS119-T7-RodZ) encoding T7-tagged RodZ. The expression of the *secA* gene in this strain is under the control of the arabinose-inducible araBAD promoter, which causes SecA depletion when the cells are grown in the presence of glucose rather than arabinose. We grew EO527 cells in LB media supplemented with 0.2% (m/V) of arabinose or glucose and induced expression of RodZ using 1 mM IPTG induction for 90 min. The protK mapping assay of spheroplasts gave a clear answer for $SecA^+$ and $SecA^-$ conditions. For $SecA^+$, RodZ was inserted into the inner membrane with its C-terminus in the periplasm, judging by the ~17-kDa proteolytic fragment (Fig. 4b, lane 1). In contrast, for $SecA^-$, no ~17 kDa proteolytic fragment was detected (Fig. 4b, lane 3). As a protocol control, we used translocation of maltose binding protein (MBP) and showed that it was impaired under SecA depletion conditions because it accumulated in a precursor form (Fig. S2b). As a final test of the SecA requirement, we used a dominant-negative SecA system. We coexpressed RodZ with the dominant-negative SecA(R642E) under T7 promoter control by transforming BL21 cells with the pET21-T7-RodZ-SecA(R642E) vector [37]. Figure 4c shows that membrane insertion of RodZ is inhibited, consistent with R642E abolishing SecA functionality. As a positive control, periplasmic secretion of mature MBP is shown in the presence of wild-type SecA while it accumulates in the precursor form in SecA(R642E) (Fig. S2c).

SecB is a chaperone that sometimes works with SecA to keep secreted proteins in an export-competent state [38,39] (Fig. 1a). To determine if SecB is required for RodZ insertion, we used the *E. coli secB* null strain CK1953 [40]. RodZ was expressed in CK1953 harboring the pMS119-BTX-RodZ vector and the spheroplasts were subjected to the protK assay. Figure 4d shows that RodZ was digested by the externally added protK completely in the *secB* null strain just as in MC1060 ($secB^+$), indicating that RodZ membrane insertion is independent of SecB. Our control experiments showed that export of SecB-dependent proOmpA was strongly inhibited in the *secB* null strain as expected (Fig. 4e).

PMF is required for RodZ membrane insertion

The PMF dependence of RodZ membrane insertion was examined using Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a protonophore that equalizes the proton concentration across the inner membrane. MC1060 cells expressing RodZ were treated with 50 μM CCCP for 45 s before labeling with [^{35}S] methionine. The membrane insertion of RodZ was blocked upon treatment with CCCP to abolish the PMF (Fig. 4f, lane 3). As a positive control, we confirmed that proOmpA, the precursor form of OmpA, accumulates when CCCP is present (Fig. S2d). This experiment shows that RodZ membrane insertion requires a TM proton gradient.

The multi-span SecD/F complex, which conducts protons across the inner membrane, can stimulate SecA-driven protein secretion [11,41]. The physiological function of SecD and SecF, however, is not entirely clear because they are not required to maintain a PMF [42]. Nevertheless, we asked if SecD/F might also be required for RodZ membrane insertion. To deplete SecD/F, we used a JP325 strain in which expression of *SecD/FYajC* is under the control of arabinose-inducible araBAD promoter [43]. The SecD/F depletion strain JP325 containing pMS119-BTX-RodZ was grown under SecD/F expression (0.2% m/V arabinose) or SecD/F depletion (0.2% m/V glucose) conditions. To induce the expression of BTX-RodZ, we added IPTG 3 min before the cells were labeled with [^{35}S]methionine and analyzed for membrane insertion using the protease accessibility assay. We found that the membrane insertion of RodZ was unaffected by SecD/F depletion, as shown by the presence of a proteolytic fragment after protK digestion of spheroplasts (Fig. 4g, lane 3). The membrane translocation of proOmpA was inhibited under SecD/F depletion conditions, which served as a positive control for our experiments (Fig. 4h).

SRP facilitates but is not absolutely required for membrane incorporation of RodZ

Although insertion of the model S-SMP Momp2 and FtsQ requires SecA, targeting to the inner membrane also requires the SRP [4,16,17]. In *E. coli*, SRP is composed of Ffh and 4.5S RNA [44] (Fig. 1b). Thus, to determine whether SRP is required for the membrane insertion of RodZ, we used the Ffh depletion strain WAM121 with the *ffh* gene under control of the araBAD promoter [45]. WAM121 cells harboring the pMS119-BTX-RodZ vector were grown in the presence of arabinose or glucose, to achieve Ffh expression or Ffh depletion conditions, respectively. After IPTG induction (1 mM final concentration) for 3 min, cells containing the RodZ expression vector were pulse labeled by [^{35}S]methionine for 1 min, chased for 6 s or 2 min, and then converted to spheroplasts for protease accessibility analysis. Figure 5a shows that 80% of the C-terminal tails of RodZ are fully digested by externally added protK at the early chase point but is nearly fully digested at the long chase time. This is in contrast to the Ffh+ conditions, in which RodZ is fully inserted at both chase points. It thus appears that Ffh is necessary for efficient insertion of RodZ. We validated our protocols by using TatC-P2 [33] (Fig. 5b) and proOmpA (Fig. S3a and b) as controls [33].

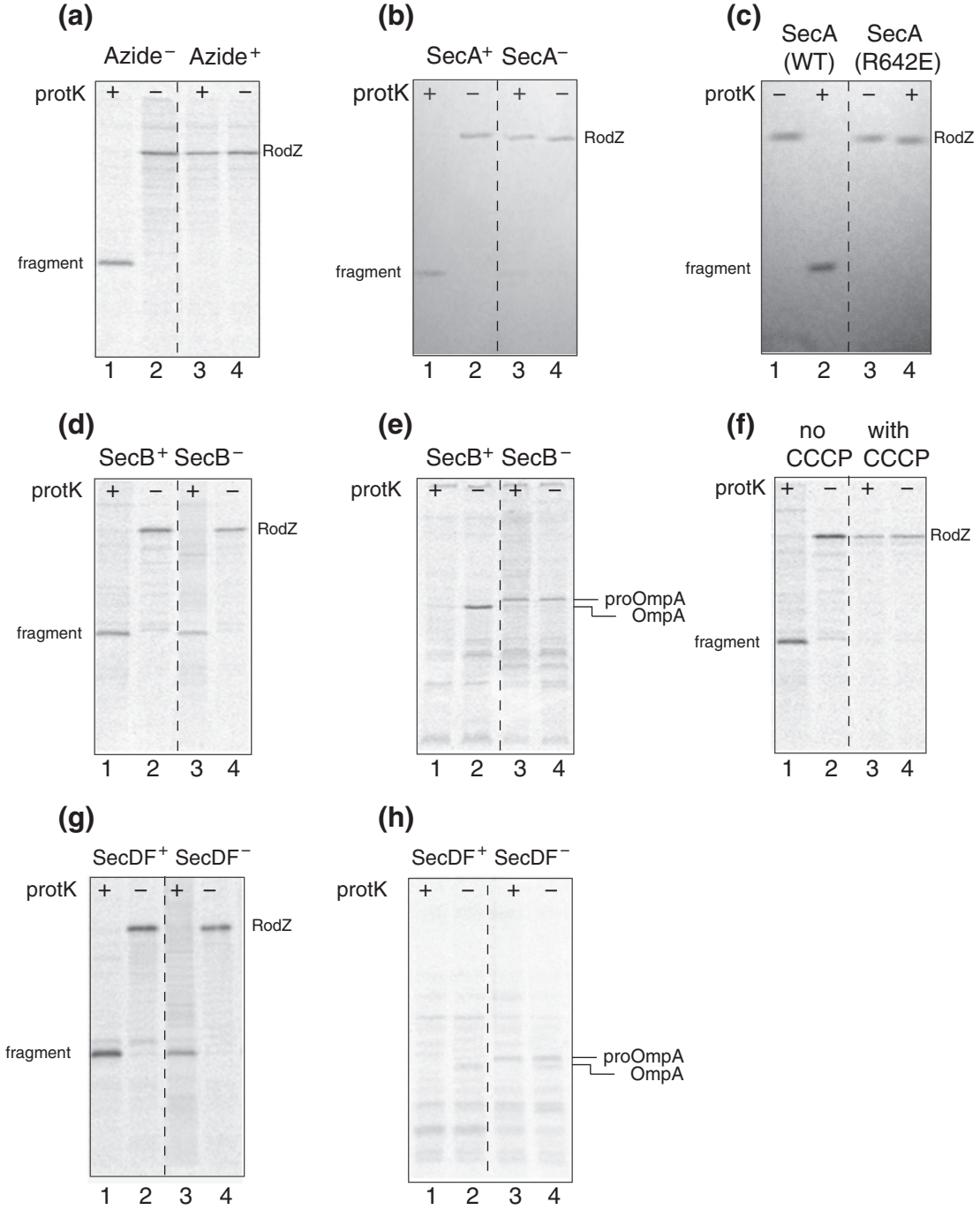
The insertion of multi-span MPs via the SRP pathway also requires the SRP receptor, FtsY (Fig. 1b). To examine more closely the participation of the SRP pathway in RodZ assembly, we used the *E. coli* FtsY depletion strain IY26 that harbored the pMS119-BTX-RodZ vector in which *ftsY* gene expression is under the control of an arabinose-inducible araBAD promoter. To deplete FtsY, we grew the cells bearing the RodZ expression vector in the LB medium supplemented with 0.2% (m/V) glucose. The cells were pulse labeled for 1 min and then analyzed using the protease accessibility assay. Figure 5c shows that FtsY depletion does not inhibit membrane insertion of RodZ (compare lanes 1 and 3). We used TatC-P2 [33] (Fig. 5d) and proOmpA expression as positive and negative controls for FtsY depletion (Fig. S3c and d). Overall, our results show that although Ffh has a small favorable effect on RodZ insertion, FtsY is not required at all. This implies that full engagement of the co-translational pathway (Fig. 1b) is not essential for RodZ insertion.

Discussion

Most studies of S-SMP assembly have used chimeric or mutant model proteins [4,16,46]. As far as we can establish, our examination of RodZ biogenesis presented here is the first complete exploration of the membrane insertion mechanism of a native S-SMP. The study was motivated by the observation that the single TM helix of RodZ is more than a hundred residues downstream from the amino-terminus, which is unusual; the TM segment of most S-SMPs occurs early in the sequence near the N-terminal. The results presented here show that membrane targeting of RodZ does not require SecB or full engagement of the SRP pathway; Ffh seems to enhance insertion but, oddly, FtsY is not required. Membrane insertion does not require YidC or SecD/F; SecA and the TM PMF are sufficient for the insertion of RodZ via SecYEG. In retrospect, this might have been anticipated by the observation that SecA is required for the TM transport of long interhelical loops of multi-span MPs or long C-terminal domains [4,5,15,17].

The partial engagement of the SRP may be related to the hydrophobicity of the RodZ TM segment.

Extensive studies of the effect of N-terminal signal sequence hydrophobicity on the targeting and export of thioredoxin-1, physiologically a cytoplasmic protein, suggest that hydrophobicity is an important determinant in pathway selection [47,48]. Thioredoxin, which folds rapidly in the cytoplasm, can nevertheless by exported to the periplasm by adding an N-terminal signal sequence that can target the protein to the SRP pathway so that secretion occurs co-translationally, thus preventing cytoplasmic folding. Schierle *et al.* found that fusion of the MalE signal sequence did not lead to thioredoxin export whereas fusion of the DsbA signal sequence caused efficient export [48]. Huber *et al.* subsequently showed that the hydrophobicity of the signal sequence affects pathway selection, with high hydrophobicity favoring the SRP pathway [47]. These results raise the possibility that the hydrophobicity of the RodZ TM segment determines the extent of engagement of the SRP pathway.



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One might think of RodZ as a protein containing the first TM helix of a multi-span MP with a long interhelical loop, but without the other spans. However, unlike a multi-span MP, RodZ does not absolutely require the SRP pathway. Our results are similar to those of Newitt *et al.* [49] who showed that the insertion of a YfgA (now RodZ) chimera was affected, but not prevented, when the SRP pathway was disrupted. The differences between their study and ours are that they used a RodZ-phoA fusion protein with phoA fused after the TM segment at position 139 and they disrupted the function of the SRP pathway by inducing the dominant lethal *ftsY* allele (G385A). It is not clear why we did not observe that the insertion of RodZ was affected by FtsY depletion (Fig. 5c). One possibility is that induction of the FtsY G385A mutant is a more effective way of disrupting the SRP pathway or the different results may have to do with the fact that we used the native RodZ protein. In any case, RodZ does not appear to require the SRP pathway strictly because Ffh depletion caused only a minor effect on its insertion while Ffh depletion caused a strong effect on the SRP-dependent TatC-P2 membrane insertion. Interestingly though, FtsY depletion had no effect of RodZ insertion while it partially inhibited TatC-P2 insertion (Fig. 5d, lane 3).

Presumably SecA mainly targets the native RodZ to SecYEG when it recognizes the late-arriving TM segment. This occurs perhaps post-translationally, but we have no evidence that bears on the question. Based upon recent structural studies showing that SecA binds to the exit tunnel of the ribosome [50,51], it is possible that SecA interacts with the nascent chain in a co-translational manner. If insertion is post-translational, one possibility is that the TM segment first partitions into the membrane interface followed by SecA-guided TM insertion and C-terminal transport through the SecYEG translocation channel. The TM segment partitioning into the membrane may be possible because the TM segment has an extraordinarily favorable free energy of transfer from water to octanol of −18 kcal/mol, based on the Wimley–White whole-residue hydrophobicity scale [52]. Microsecond-scale equilibrium molecular dynamics simulations show, for example, that poly-leucine helices fold into bilayer interfaces as α-helices and never return to the aqueous phase [53]. The possibility of direct first contact of the helix with the membrane is supported by studies of KdpD, which acts as a potassium sensor in *E. coli* [54,55]. Several features of KdpD are significant: The protein is tethered to the cytoplasmic surface of the inner membrane by four TM segments (residues 401–498) with very short interhelical loops that occur far downstream from the amino-terminus, it is targeted to the inner membrane by SRP recognition of a cytoplasmic amphipathic helix (residues 22–48) [56], and membrane insertion does not require SecA, SecE, or YidC [30]. Because KdpD does not apparently engage SecA or the SecYEG channel, a reasonable explanation is spontaneous insertion following targeting by the SRP.

We have not explored the determinants of the topology of RodZ, but we note that a highly positively charge sequence (-KRRKKRD-) immediately precedes the TM segment. This should guarantee N_{in}-C_{out} topology based upon the positive-inside rule [57,58]. Interestingly, though, the topology of leader peptidase in *E. coli* can be inverted simply by lengthening the normally cytoplasmic interhelical P1 loop between the two TM segments to 60–70

Fig. 4. RodZ depends on SecA and PMF for insertion into the membrane. (a) RodZ insertion is inhibited by sodium azide. MC1060 strain expressing RodZ was treated with sodium azide (3 mM final concentration) for 5 min before pulse labeling with [^{35}S]methionine for 1 min. Membrane translocation of RodZ was analyzed by protK mapping as described in Materials and Methods. See also Fig. S2a. (b) RodZ membrane insertion is inhibited under SecA depletion conditions. The SecA depletion strain EO527 containing pMS119-T7-RodZ was grown overnight in LB medium supplemented with 0.2% arabinose. The cells were then grown and Western blotted as described in Materials and Methods. The SecA-dependent MBP was used as a control and the whole cell lysate was analyzed by Western blot using anti-His primary antibody followed by AP conjugated anti-mouse secondary antibody (Fig. S2b). (c) RodZ insertion is inhibited when a SecA dominant negative is expressed using the SecA(R642E) cell line. RodZ carrying a T7 tag before the TM domain was coexpressed with mutant SecA(R642E) on a pET21 plasmid under control of T7 promoter in BL21 cells and induced with 40 μM IPTG at OD_{600} of 0.4 for 90 min, followed by protK treatment (lanes 2 and 4). See also Fig. S2c. (d) RodZ insertion is SecB independent. MC1060 (SecBwt) and CK1953 (SecBnull) transformed with pMS119-BTX-RodZ and the dependence of SecB for RodZ membrane insertion was examined by protK mapping, as described in Materials and Methods. (e) Positive control using proOmpA expression and the protK accessibility assay, demonstrating that our depletion protocol for SecB is effective. OmpA protein was immunoprecipitated and resolved on 15% SDS-PAGE before phosphor-imaging, as described in Fig. S1a. (f) RodZ requires a PMF for membrane insertion (PMF). Cells were treated with CCCP (50 μM final concentration) for 45 s, then pulse labeled with [^{35}S]methionine for 1 min and analyzed by protK mapping as described in Materials and Methods. See also Fig. S2d. (g) RodZ does not require SecD/F for membrane incorporation. JP325 strain expressing pMS119-BTX-RodZ was grown to mid-log phase under arabinose (SecD/F^{+}) or glucose (SecD/F^{-}) conditions before pulse labeling with [^{35}S]methionine for 1 min. To test the membrane translocation of RodZ, we subjected cells expressing the RodZ protein to protK mapping assay as described in Materials and Methods. (h) Positive control using proOmpA expression and the protK accessibility assay, demonstrating that our depletion protocol for SecD/F is effective. OmpA protein was immunoprecipitated and resolved on 15% SDS-PAGE before phosphor-imaging, as described in Fig. S1a.

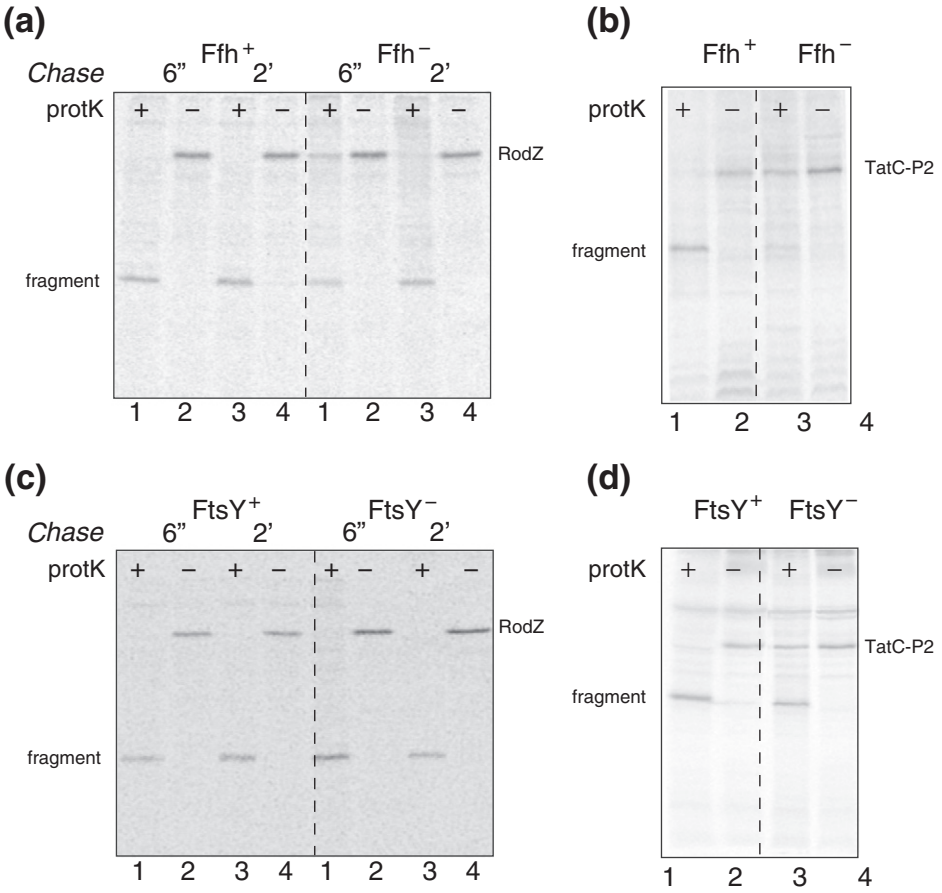

Fig. 5. RodZ insertion does not strictly require the SRP pathway. (a) RodZ incorporation is facilitated by Ffh, but Ffh is not absolutely required. The Ffh depletion strain WAM121 bearing the RodZ expression vector pMS119-BTX-RodZ was grown to mid-log phase in the presence of arabinose (Ffh expression condition) and glucose (Ffh depletion condition), prior to pulse labeling for 1 min and chase at 6 s and 2 min time points. The SRP pathway dependence of RodZ protein insertion was tested by proteinase accessibility assay, as described in Materials and Methods. The protK assay reveals that, under Ffh depletion conditions, some uncleaved RodZ is present after 6″ and 2′ of chase, implying that Ffh affects the insertion of RodZ. (b) Control experiment showing that the Ffh-dependent TatC-P2 does not insert into the membrane under Ffh^- conditions. (c) RodZ membrane insertion is independent of FtsY despite weak dependence on Ffh. To deplete FtsY, we grew the FtsY depletion strain IY26 with the RodZ expression vector pMS119-BTX-RodZ in the LB medium added with 0.2% (m/V) glucose. The cells expressing RodZ protein, grown in the $FtsY^+/FtsY^-$ condition, were pulse labeled for 1 min and then chased with non-radioactive methionine (40 mM final concentration) at 6 s and 2 min. The protein insertion of RodZ in $FtsY^+/FtsY^-$ conditions was examined by proteinase accessibility assay as mentioned in Materials and Methods. (d) A control experiment demonstrating that TatC-P2 [33] insertion is reduced but not eliminated under $FtsY^-$ conditions. Although TatC-P2 insertion is only partially reduced under $FtsY^-$ conditions, the control demonstrates that the strain becomes depleted in the absence of arabinose. See also Fig. S3a–d.

residues from the 39 wild-type residues [5]. This occurs despite the fact the P1 loop is heavily positively charged, suggestive of a significant role for SecA in topology determination.

The molecular mechanism of SecA transport of long periplasmic domains in multi-span MPs is not known. Although SecA-driven secretion of periplasmic and outer MPs is understood reasonably well, just how SecA engages and transports long periplasmic domains during co-translational assembly of multi-span proteins is a mystery. The insertion mechanism of type II S-SMPs by SecA is equally mysterious. Does SecA recognize the TM segment, carry it to SecYEG, and push it into SecYEG where it partitions between translocon and membrane in the course of secretion of the periplasmic domain? Or is the TM segment delivered by SecA directly to the membrane in the vicinity of SecYEG, where it partitions spontaneously prior to ATP-dependent transport of the periplasmic domain? The molecular targeting process of the RodZ class of proteins is also far from clear. How might targeting and insertion process be affected by the amino acid composition of the TM segment? While it seems

certain that SecA is involved in targeting, are other molecules, such as trigger factor, required? We suggest that RodZ provides a starting point for finding answers to these questions.

Materials and Methods

Bacterial strains, plasmids, and materials

The *E. coli* JS7131 (YidC depletion strain), WAM121 (Ffh depletion strain), CM124 (SecE depletion strain), JP325 (SecD/FyajC depletion strain), EO527 (SecA depletion strain), and IY26 (FtsY depletion strain) were previously described [34,40,45]. In these depletion strains, the araBAD promoter controls the expression of the *yidC*, *ffh*, *secE*, *SecD/FyajC*, *secA*, and *ftsY* genes. SecA(R642E) dominant-negative strain was developed in our laboratory. In this strain, coexpression of RodZ and SecA(R642E) mutant is controlled under the T7 promoter on pET21 vector. BL21 and MC1060 were used as wild-type strains. See Table 1 for more details. Media preparation and bacterial manipulations were performed according to standard methods [65].

Sodium azide, CCCP, phenylmethylsulfonyl fluoride (PMSF), NBT (*n*itro *b*lue *t*etrazolium), BCIP (5-*b*romo-4-*c*hloro-3-*i*ndolyl *p*hosphate), and lysozyme were obtained from Sigma. protK was purchased from Invitrogen. IPTG was purchased from Research Products International Corporation. BTX (Biotin-XX Conjugate) was from Invitrogen. Streptavidin agarose resin was purchased from Thermo Scientific Company. Anti-T7 antibody was from Novagen (#69999). Trans-[^{35}S]methionine was from Perkin Elmer. Phusion PCR kit and restriction enzymes were from New England Biolabs.

RodZ gene was amplified by PCR from *E. coli* K12 strain and subcloned into expression vector pET16b and pET21 using NdeI and XhoI with a stop codon. An 11-amino-acid T7 tag (MASMTGGQQMG) was introduced by mutagenesis by overlap extension between Ala95 and Pro96 for immunodetection of RodZ. These constructs, named as pET16b-T7-RodZ and pET21-T7-RodZ, were expressed in a BL21 cell line. RodZΔTM was made by mutagenesis by overlap extension. Nucleotides from 333 to 400 were not amplified to make the RodZΔTM construct. This construct is called as pET21-T7-RodZΔTM. RodZ was subcloned into pMS119 expression vector using EcoRI and HindIII restriction sites. This construct was named pMS119-T7-RodZ and was expressed in SecA depletion strain EO527. To make a dominant-negative strain, we generated the SecA-RodZ coexpression plasmid named pET21-SecA-T7-RodZ and pET21-SecA(R642E)-T7-RodZ plasmids using the BglII site, in which SecA was added with T7 promoter and terminator. These plasmids were expressed in BL21 cell line. We made one more construct in which we inserted a 13-amino-acid BTX-binding peptide (WRYYESSLLPYPD) near the amino-terminus between 2Gln and 3Thr of RodZ rather than the T7 tag close to the TM domain [31,32]. This construct was called pMS119-BTX-RodZ and was expressed in YidC depletion strain JS7131, Ffh depletion strain WAM121, FtsY depletion strain IY26, and SecD/F depletion strain JP325. It was also expressed in SecB null strain CK1953 and wild-type strain MC1060. The DNA encoding BTX-RodZ was subcloned into a modified pEH1 vector pLZ1 (GenBank Accession #KF918333) to allow protein expression in the SecE depletion strain CM124. The coding regions of all constructs were verified by sequence analysis.

Growth conditions

For Ffh depletion, WAM121 [61], FtsY depletion IY26, SecD/F depletion JP325, and YidC depletion JS7131, we followed the following growth condition protocol: The cells of various *E. coli* strains containing pMS119-BTX-RodZ plasmid were grown overnight at 37 °C in LB medium containing 0.2% (w/v) arabinose, washed in medium

Table 1. Various *E. coli* strains and plasmids used in this study of RodZ biogenesis

Strains[a]	Plasmids[b]	Tag[c]	Mutation	Reference
BL21	pET21-T7-RodZ, pET16b-T7-RodZ, pET21-T7-RodZΔTM pET21-T7-SecA(R642E)RodZ, pET21-T7-SecAwt-RodZ	T7		Novagen #69449-3
MC1060	pMS119-BTX-RodZ	BTX		[59,60]
WAM121	pMS119-BTX-RodZ	BTX	*ffh*	[45,61]
IY26	pMS119-BTX-RodZ	BTX	*ftsY*	[62]
EO527	pMS119-T7-RodZ	T7	*secA*	[63]
JS7131	pMS119-BTX-RodZ	BTX	*yidC*	[29]
CM124	pLZ1-BTX-RodZ	BTX	*secE*	[34]
CK1953	pMS119-BTX-RodZ	BTX	*secB*	[40]
JP325	pMS119-BTX-RodZ	BTX	*SecD/F*	[64]

[a] Strains are as follows: BL21 {*E. coli* B F– *dcm ompT hsdS*(r_B– m_B–) *gal* [*malB*$^+$]$_{K\text{-}12}$(λ^S)}, MC1060 [Δ(*codB-lacI*)3, *galK16*, *galE15*, λ$^-$, *relA1*, *rspL150*, *spoT1*, *hsdR2*, *ara*$^+$], WAM121 [MC4100, *ara*+, *ffh*::*kan*, *attB*::(*Ori*$_{R6K}$, *ParaBAD-ffh*, *tet*)], Y26 [Δ(*araD-araB*)*567*, Δ*lacZ4787*(::*rrnB-3*), λ$^-$, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*, *kan-araC-P*$_{BAD}$-*ftsY*], EO527 [λ$^-$, *IN*(*rrnD-rrnE*)*1*, *rph-1*, λcI857, Δ(*cro-bioA*), *SecM*<>*kan-araC-P*$_{BAD}$], JS7131 [MC1060, Δ*yidC*, *attB*::(*Ori*$_{R6K}$, *ParaBAD*$^-$*yidC*$^+$, (Spec^r)], M124 [*secE19–111 pcnB80 zadL*::(*Tn10 Tc*^{s} *Str*^{r}) *phoA*Δ*Pvu*II, *lac*Δ*X74*, *galE galK*, *rpsL*, *recA*::*cat*, *pCM22* (*ParaBAD-secE*$^+$)], CK1953 [MC4100, *secB*::*Tn5*], JP325 [*ara*Δ714, Δ(*argF-lac*)U169, *rpsL*150, *rel*A1, *thi*, *flb*5301, *deo*C1, *pts*F25, *recA*::*cat*, *tgt*::*kan-araC*$^+$-*P*$_{BAD}$::*yajC-SecD/F*].

[b] Plasmids are as follows: pMS119 (ColE1 origin, Tac promoter, ampicillin resistant) and pLZ1 (RSF origin, T7/Lac promoter, kanamycin resistant).

[c] Immunotags are as follows: T7, MASMTGGQQMG inserted between residues Ala95 and Pro96; BTX, WRYYESSLLPYPD inserted between Gln2 and Thr3.

lacking arabinose, and back-diluted 1:100 in fresh LB medium containing 0.2% (w/v) arabinose or 0.2% (w/v) glucose. The SecB null strain CK1953 and MC1060 bearing the RodZ expression vector were grown in the LB medium as SecB deletion and SecB wild-type conditions, respectively. When cultures reached an OD_{600} of 0.6, the cells were switched into M9 minimal medium before pulse labeling with [^{35}S]methionine. The SecE depletion strain CM124 containing pLZ1-BTX-RodZ plasmid was grown overnight in M9 minimal medium containing 0.2% arabinose and 0.4% glucose. The cells were back-diluted 1:100 and grown in fresh M9 media containing 0.4% glucose supplemented with or without 0.4% arabinose and were grown at 37 °C for 9 h. The SecA depletion strain EO527 containing the pMS119-T7-RodZ plasmid was grown overnight in LB medium containing 0.2% (w/v) arabinose, washed in medium lacking arabinose, and back-diluted 1:10 in LB medium containing 0.4% (w/v) glucose. When cultures reached an OD_{600} of 0.6, the cells were induced with 1 mM IPTG for 90 min. The BL21 cells containing pET21-SecA(R642E)-T7-RodZ and pET21-SecAwt-T7-RodZ plasmids were grown overnight in LB media. Cultures were back-diluted 1:100 in fresh LB media and were induced with 40 μM IPTG at OD_{600} of 0.4 and grown for 90 min. Where appropriate, ampicillin (100 μg/ml final concentration), kanamycin (50 μg/ml final concentration), or chloramphenicol (50 μg/ml final concentration) was added to the medium.

Cell fractionation

Cell fraction was performed by cell lysis using freeze–thaw and DNAse I treatment. The bacterial cells (containing the overexpressed protein RodZΔTM and RodZwt) were harvested and washed with phosphate-buffered saline buffer to remove residual media. The pellet was resuspended in lysis–equilibration–wash buffer (50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0) containing DNAse I enzyme, DNAse I buffer, lysozyme, and PMSF in it. Thereafter, the cell pellet was subjected to 10 cycles of freeze (liquid nitrogen) and thaw (at 37 °C water bath) followed by incubation at 37 °C for 10 min. The cell suspension was centrifuged at 13,000*g* for 10 min at 4 °C, and the supernatant containing the soluble and periplasmic proteins (cyt fraction) was transferred to a new tube. The pellet was washed twice and resuspended in the lysis–equilibration–wash buffer + 1% Triton X-100 (detergent) to solubilize MPs. The freeze and thaw cycle was repeated for 10 min followed by centrifugation at 13,000*g* at 4 °C for 15 min. The supernatant now contains the inner membrane fraction. Cells expressing MBP were treated by the osmotic shock method [65]. After a high-speed spin, the supernatant contains the periplasmic fraction "P" while the pellet/rest fraction "R" includes the total proteins without the periplasmic proteins. Both fractions were precipitated with 10% (m/V) TCA (*tri*chloroacetic *a*cid) buffer and the MBP protein was visualized by Western blot analysis using anti-His antibody.

Protease treatment studies

For all experiments, cells were grown to mid-logarithmic phase under the various growth conditions. IPTG (1 mM final concentration) was added to cells cultured in M9 minimal media to induce RodZ protein expression, and then pulse labeled for 1 min with trans-[^{35}S]methionine (0.2 mCi/ml final concentration). The azide study involved treatment of cells with sodium azide (3 mM final concentration) for 5 min prior to labeling. For the PMF dependence study, cells were treated with CCCP (50 μM final concentration) for 45 s prior to [^{35}S]labeling the cells. For non-radioactive experiment, the BL21 cells bearing the RodZ expression vector were induced with 1 mM IPTG for 90 min before production of spheroplasts. The unlabeled or radiolabeled cells were converted to spheroplasts. To form spheroplasts, cells were resuspended in 0.25 ml of ice-cold buffer A [0.1 M Tris–acetate (pH 8.2), 0.5 M sucrose, and 5 mM ethylenediaminetetraacetic acid] then treated with lysozyme (80 μg/ml final concentration) and 0.25 ml of ice-cold water for 15 min. We added 150 μl of 0.2 M $MgSO_4$ to stabilize the spheroplasts and cells were spun down then resuspended in buffer B (50 mM Tris–acetate, 0.25 M sucrose, and 10 mM $MgSO_4$). Aliquots of the spheroplast suspension were incubated on ice for 1 h either in the presence or in the absence of protK (0.8 mg/ml final concentration). After addition of PMSF (5 mM final concentration) to inactivate protK, samples were precipitated with TCA (15% final concentration) and washed with ice-cold acetone. For radiolabeled proteins, the cell pellet was solubilized in Tris–SDS buffer [10 mM Tris–HCl (pH 8.0) and 2% SDS]. The TatC-P2 and Pf3-p2 proteins were immunoprecipitated using antiserum to leader peptidase while anti-OmpA antiserum was for OmpA protein immunoprecipitation. For non-radiolabeled proteins, the acetone-washed pellet was then dissolved in 50 μl Hoechst sample buffer (containing 6 M urea) for Western blotting using anti-T7-AP conjugated antibody after SDS-PAGE.

Western blots

T7-RodZ protein was detected by Western blot using the chromogenic method of detection. In this process, spheroplasts treated with protK were solubilized in SDS sample-loading buffer containing 6 M urea, boiled for 10 min, and subjected to SDS-PAGE analysis. The gel was transferred to a nitrocellulose membrane using the iBLOT semi-dry technique (Invitrogen) and then blocked with 1% bovine serum albumin in TBS (*T*ris-*b*uffered *s*aline) buffer for 30 min. The membrane was then blotted with AP conjugated T7 tag antibody (1:10,000 dilution in TBS buffer) for 1 h. Before developing the blot, we washed the membrane three times with TBST (TBS + Tween 20) to remove unbound antibody. For chromogenic development, 5% NBT and 5% BCIP dissolved in buffer A (alkaline pH 9.0) were used to develop the blot. NBT and BCIP yield an intense, insoluble black-purple precipitate when reacted with alkaline phosphatase, which provides a good signal for tagged protein detection.

Pull-down protocol for BTX-tagged protein

E. coli cells were pulse labeled with 0.2 mCi/ml [^{35}S] methionine for 1 min. Following the protK accessibility assay, we pelleted and solubilized the spheroplasts with 100 μl Tris–SDS buffer [10 mM Tris–HCl, pH 8.0, and 2% (w/v) SDS]. The protein lysate was diluted with 1 ml of Triton-IP buffer [10 mM Tris–HCl (pH 8.0), 5 mM

ethylenediaminetetraacetic acid, 150 mM NaCl, and 2.5% (v/v) Triton X-100]. After incubation with 30 μl of streptavidin agarose resin, the supernatant was transferred to a new tube and incubated with 2 μg/ml (final concentration) of BTX (Biotin-XX Conjugate) for 45 min before incubation with 30 μl streptavidin agarose resin for 30 min. The resin was then spun down and washed with Triton-IP buffer for three times and the BTX-tagged RodZ protein was eluted by SDS sample buffer. The protein samples were resolved on 15% SDS-PAGE and visualized by phosphor-imaging, as described earlier [33].

Acknowledgements

This work was supported in part by National Institutes of Health Grant GM074637 (to S.H.W.) and National Science Foundation Grant MCB-1052033 (to R.E.D.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2014.05.005.

Received 24 January 2014;
Received in revised form 16 April 2014;
Accepted 7 May 2014
Available online 15 May 2014

Keywords:
membrane protein assembly;
SecYEG translocase;
SecA ATPase;
single-span membrane proteins

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Abbreviations used:
S-SMP, single-span membrane protein; TM, transmembrane; MP, membrane protein; PMF, proton motive force; SRP, signal recognition particle; protK, proteinase K; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; BTX, α-bungarotoxin; MBP, maltose binding protein.

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