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DROPPING OUT AND OTHER FATES OF TRANSMEMBRANE SEGMENTS INSERTED BY THE SecA ATPase

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Abstract

Type-II single-span membrane proteins, such as CadC or RodZ, lacking a signal-sequence and having a far-downstream hydrophobic segment, require the SecA secretion motor for insertion into the inner membrane of *Escherichia coli*. Using two chimeric single-span proteins containing a designed hydrophobic segment *H*, we have determined the requirements for SecA-mediated secretion, the molecular distinction between TM domains and signal peptides, and the propensity for hydrophobic *H*-segments to remain embedded within the bilayer after targeting. By means of engineered *H*-segments and a strategically placed SPase I cleavage site, we determined how targeting and stability of the chimeric proteins are affected by the length and hydrophobicity of the *H*-segment. Very hydrophobic segments (e.g. 16Leu) are stably incorporated into the inner membrane, resulting in a C-terminal anchored membrane protein, while a 24L construct was not targeted to the membrane by SecA and remained in the cytoplasm. However, a construct carrying preMalE at the N-terminus led to SecA targeting to SecYEG via the native signal sequence and stable insertion of the downstream 24L *H*-segment. We show that the RseP intramembrane protease degrades weakly stable *H*-segments and is a useful tool for investigating the borderline between stable and unstable TM segments. Using RseP⁻ cells, we find that moderately hydrophobic sequences (e.g. 5Leu+11Ala) are targeted to SecYEG by SecA and inserted, but subsequently drop out of the membrane into the cytoplasm. Therefore, the free energy of transfer from translocon to bilayer is different from the transfer free energy from membrane to water.

Keywords

single-span membrane proteins; protein targeting; membrane protein stability; RseP intramembrane protease; protein secretion

Introduction

CadC or RodZ (Figure 1a) are unusual Type-II (N_{in}-C_{out}) single-span membrane proteins (MPs), because they lack an N-terminal signal-sequence (S-S) and have a far-downstream

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transmembrane (TM) segment. We have shown that both proteins require the SecA secretion motor for targeting and insertion into the inner membrane of *Escherichia coli* [1, 2]. CadC activates the *cadBA* operon during low-pH stress [3–5] while RodZ, plays an important role in the maintenance of the rod shape of *Escherichia coli* [6, 7]. Targeting and TM insertion are determined solely by hydrophobic segments that are more than 100 residues downstream from the N-terminus (over 150 residues for CadC and 100 residues for RodZ). In contrast, most Type-II MPs have their signal-anchor sequences at, or very close to, the N-terminus.

In an earlier study of the dimerization of the CadC sensor domain [1], which is required for activating the *cadBA* operon, we developed a tripartite single-span chimera CadC-*H*-RodZ in which the periplasmic domain of RodZ replaced the periplasmic CadC sensor domain. The two domains were linked by a hydrophobic *H*-segment of the form GGPG-*H*-GPGG to serve as the single TM helix. The purpose of the GGPG/GPGG sequences was to isolate the hydrophobic TM domain from the surrounding sequence [8]. We added a signal peptidase cleavage site (*c/v*) [9–12] of the form *c/v* = -AXA- following the *H*-segment. The inclusion of a T7 tag upstream from *H* and a His₆ tag downstream at the C-terminus (Figures 1b & 1c) allowed us to track the insertion and membrane topology of the chimera using Western blots [13].

The C-*H*R chimeras have proven useful for examining SecA-dependent targeting of proteins to the SecYEG translocon for insertion. The presence of a cleavage site allowed the cellular location of the tagged fragments (periplasm, cytoplasm, or membrane) to be determined in order to verify their N_{in}-C_{out} topology. We found that targeting by SecA to the SecYEG translocon could be easily judged by whether or not SPase I cleaved the periplasmic domain. For example, SPase I cleavage of a polyleucine construct revealed that the construct was targeted and inserted into the inner membrane, because the CadC fragment was located in the membrane fraction as a C-terminal anchored membrane protein while the RodZ fragment was found in the periplasmic fraction. A polyalanine construct, on the other hand, was not cleaved and was found solely in the cytoplasm, indicating that it was not even recognized by SecA. These results suggested to us a simple means for examining in greater detail the requirements for *H*-segment recognition by SecA. They further suggested a means of determining *H*-segment stability in the membrane to answer an important question: Are there *H*-segments that are sufficiently non-polar to be recognized by SecA and inserted via SecYEG but not sufficiently ‘greasy’ to remain in the membrane after SPase I cleavage? Our results also suggested that we could determine the rules governing recognition of far-downstream *H*-segments by SecA to answer the question of whether the rules for N-terminal signal sequence recognition apply to far downstream hydrophobic segments.

We present in this paper answers to these questions obtained using the chimeras shown in Figure 1b. The C-*H*R construct is the same as used earlier [1]. The C-*H*F construct is similar except that the C-terminal fragment is CusF (without its natural signal sequence), which is the periplasmic copper chaperone of the *E. coli* CusCFBA copper-transporting efflux system [14]. Our experience so far is that the complete mature domain of almost any periplasmic protein is suitable for constructing this class of chimeras. What is important is that the protein form a stably folded domain. Early experiments revealed that fragments of exported proteins that do not form stable folds are rapidly degraded. We show below that C-

H-R or *C-H-F* constructs with very long and hydrophobic *H*-segments are not recognized by SecA and therefore not targeted to SecYEG for secretion or insertion, consistent with earlier studies of signal peptides by Debra Kendall and her colleagues [15, 16]. We wished to learn whether secretable proteins carrying long and greasy downstream *H*-segments could nevertheless be inserted by SecYEG via the SecA pathway even if recognition of the substrate did not depend on the *H*-segment. For this purpose, we created the ssM-*H-R* construct (Figure 1b and 1c) containing the immature form of the periplasmic maltose binding protein MalE [17] (preMalE) at the N-terminus and RodZ at the C-terminus. We show below that ssM-*H-R* can be targeted successfully by SecA regardless of the *H*-segment structure. For example, as revealed by signal sequence cleavage, the *H*= 16 Ala construct is secreted through SecYEG while the *H*= 16Leu or 24Leu constructs are partitioned into the membrane by SecYEG.

Results

The fates of long and short H-segments of varying hydrophobicity

Figure 2 shows an immunoblot (T7 antibody) of *C-H-F* constructs expressed in *E. coli* BL21 cells grown in SOC media at 37° C. The *H*-segment contained 16 residues composed primarily of Ala and Leu ranging A₁₅L₁ to A₁₀L₆. SecA recognition and SecYEG-guided insertion of the chimera—zcleaved by SPase1—requires at least 4 leucines (lane *H4*) for complete insertion, but partial insertion occurs with 3 leucines (lane *H3*). A single Leu-to-Pro substitution at the center of the A₁₀L₆ construct to give A₁₀P₁L₅ prevents recognition of the construct. These results are entirely consistent with early secretion studies of PhoA containing artificial N-terminal signal sequences [18].

C-H-F constructs such as these could be used for exhaustive studies of signal sequence recognition by SecA, but here we are concerned with the conditions for SecA-based recognition of TM segments. Because polyleucine segments form the most stable stop-transfer sequences [16], we examined the fate of polyleucine *H*-segments ranging in length from 10 to 16 leucines, using anti-T7 antibodies to identify the location of cleaved fragments produced by SPase I. Figure 3 shows that *H*-segments shorter than 13L are not seen in the inner membrane fraction; all are located in the soluble fraction. For 13L, the fragments are found equally in the soluble and membrane fractions. All fragments containing 14 or more Leu are found exclusively in the inner membrane fraction. Figure 3 shows that the soluble and the membrane bound fragments differ in size. We hypothesized that the 10L-13L *H*-segment fragments are further cleaved by RseP [19, 20] and that the remaining RseP cleaved signal sequence drops out of the membrane (see Figure 4). We do not know exactly where RseP cleaves, but it seems that it discriminates between ‘signal sequences’ (10L-13L), which are attacked, and TM segments (14L-16L), which are not attacked. This suggested that RseP could be a useful indicator tool for investigating the borderline between stable or unstable TM segments.

To test further the idea that RseP recognizes signal sequences but not TM-like polyleucine segments, we examined the membrane stability of the signal sequences of MalE, OmpA, and PhoA with the CadC cytoplasmic domain as an N-terminal extension (Figure 4), which allowed us to track the signal sequence after SPase I cleavage. As expected from their

similarity to the *C-HR* construct, SecA targeted all of the constructs to SecYEG as determined by SPase I cleavage. The cleaved T7-labeled fragments revealed two bands on the immunoblots resulting from cleavage by SPase I (left-hand lanes labeled RseP⁺). These bands must arise from post-cleavage processing by the site-2 intramembrane metalloprotease RseP system [19, 20], as proven by the presence of a single-band in RseP⁻ mutants (right-hand single bands).

Using the RseP⁻ strain and T7-labeling of immunoblots, we looked for the location of the cleaved fragments produced in experiments such as those of Figure 2. We used *C-HR* constructs containing 16 residues in the *H*-segment ranging from A₁₁L₅ to A₆L₁₀. Figure 5 shows the distribution of the fragments between soluble (periplasm + cytoplasm) and insoluble (membrane) fractions. As indicated by the lack of cleavage product (lane *HI*), the A₁₅L₁ protein is not processed. For the A₁₁L₅ construct, on the other hand, the soluble and insoluble fractions contained about equal quantities cleaved fragments. Importantly, as the number of leucines increased, there was a progressive shift of cleaved material in the soluble fraction to the insoluble fraction; virtually all of the fragments were found in the insoluble (membrane) fraction for A₈L₈ and beyond. We interpreted this to mean that *H*-segments containing fewer than about 8 leucines are not stably bound to the membrane and consequently “drop out” into the cytoplasm after cleavage. These and the results of Figure 2 suggest that in a 16-residue Leu+Ala *H*-segment, 4 Leu are sufficient for complete partitioning from translocon (SecYEG) to membrane while about 8 leucines are required to keep the tail-anchored fragment in the membrane.

The fates of very long hydrophobic *H*-segments

To this point, we have described experiments in which the longest Leu/Ala *H*-segments were 16 residues. Because it is known from studies of artificial signal sequences that polyleucine segments longer than about 20 residues are ineffective in targeting and secretion PhoA [18], we created *C-HR* constructs containing polyleucine *H*-segments with lengths of 16 to 24 residues (Figure 6a). As expected, a 16L segment was readily targeted to SecYEG, inserted into the membrane, and cleaved by SPaseI. But as the number of leucines in the segments increased, there was steady decline in recognition by SecA as indicated by the diminishing amounts of cleaved RodZ. No cleavage products were apparent for 22 or more leucines, and it appears that SecA begins having difficulty recognizing *H*-segments longer than 16L. This suggested that SecA could not recognize TM segments longer than 22 residues. Figure 6c reveals, however, that the substitution of arginines for two leucines (positions 10 and 15) can ‘rescue’ the 24L construct; making the sequence less hydrophobic converts it into a SecA target. This implies that the length of the segment is not the critical issue. These very long segments are, of course, unusual and one would not expect to encounter such highly hydrophobic segments in nature. Interestingly, although we expected these constructs to produce insoluble proteins, that was not the case. As shown in Figure 6b (see also Figure S3), the *C-24L-RodZ* protein is found mostly in the cytoplasmic fraction. This could be because it is misfolded or is stabilized or ‘protected’ in some way by chaperones.

Because there is no insertion of the long greasy segments, it is clear that the signal recognition particle (SRP/ffH) co-translational pathway is not being utilized despite the

greasiness of the *H*-segment. This is consistent with our earlier finding [2] that single-span membrane proteins with far downstream TM segments are recognized and inserted via SecA. The data of Figures S1 and S2, carried out using depletion strains under the control of arabinose, confirm the central importance of SecA in the insertion process. In addition, the depletion of SPase I clearly shows that the introduced cleavage site (-AXA-) is exclusively used by this protease. Notice in Figure S2, particularly, that ffH depletion had only minor effects on targeting and secretion, as we observed earlier [2] for the targeting and insertion of RodZ. Although ffH can enhance the SecA pathway for very hydrophobic signal sequences, there is little doubt that SecA is necessary and sufficient for the targeting and secretion of proteins [21]. Because the 24L construct is found only in the cytoplasm, it is extraordinarily unlikely that the SRP pathway is involved.

Figure 6 shows rather dramatically that SecA cannot insert C-*H*-R constructs across the inner membrane if the *H*-segments are very long and greasy. Two possibilities are that the long segments cannot bind to SecA for recognition or that, even if recognized, SecYEG is incapable of inserting them. The experiment of Figure 6 was designed to eliminate the possibility that SecYEG cannot manage the insertion of a very long *H*-segment (24L). We hypothesized that a natural secreted protein (ss-MalE) at the N-terminus of the construct would force the construct into the 'regular' SecA pathway independent of the late-occurring *H*-segment. For this purpose, we used the ssM-*H*-R construct (Figure 1b and 1c) consisting of T7-tagged preMalE and an *H*-segment followed by the His₆-tagged C-terminal sequence of RodZ. Figure 7 shows that the 24L construct is readily inserted into the membrane as is the 16L construct, consistent with earlier work in eukaryotes using an in vitro expression system [22]. The 16A construct, however, passed through SecYEG as a secreted protein. These results are consistent with the idea presented earlier that as the 'secreted' protein passes through the translocon, the very greasy *H*-segment partitions into the membrane from the translocon. Importantly, the ssM-16LR construct contains two SecA targets (the MalE signal sequence and the 16L *H*-segment). We could not detect any competitor effect, which would have been indicated by the presence of two different topologies. The data show that the first-occurring signal sequence is exclusively recognized.

Discussion

We have explored the rules *E. coli* follows for targeting and secretion/insertion of model Type-II single-span membrane proteins that have a far-downstream hydrophobic segment but lack an N-terminal signal sequence (Figure 1a). The results confirm earlier work on the targeting and membrane insertion this class of single-span membrane proteins into the *E. coli* inner membrane by the SecA secretion motor [1, 2]. To examine more thoroughly the requirements for SecA recognition and SecYEG-guided membrane insertion, we created several chimeric proteins of the form C-*H*-X in which C is the cytoplasmic domain of CadC, *H* is a hydrophobic sequence of the form GGPG-*H*-GPGG [23], and X is the periplasmic domain of either RodC (R) or CusF (F) (Figure 1b and 1c). We explored initially the *H*-segment requirements for SecA identification and processing for 16-residue *H*-segments comprised of leucine and alanine residues (Figure 2). The results showed that at least four Leu leucines are required for membrane partitioning, although partial partitioning occurs with three leucines. This is consistent with earlier studies on SecA recognition of N-terminal

signal sequences using PhoA as a model [18]. We conclude that the rules for identification of signal sequences by SecA is independent of location within the proteins. N-terminal extension of the signal sequence has no influence of the secreted C terminus.

Using polyleucine *H*-segments, we then examined the question of how many leucines are required for an *H*-segment to be partitioned stably into the membrane rather than being attacked by RseP. Figure 3 shows that complete membrane integration requires 14 leucines with partial integration occurring for 13 leucines. This is the border between TM segments and signal sequences. This finding is consistent with similar results obtained by Jaud et al. [24] using an *in vitro* eukaryotic system. Those authors established that polyleucine sequences containing 8 or fewer leucines were thermodynamically too costly to insert into the membrane as result of the extreme hydrophobic mismatch between the 40 Å-thick lipid bilayer and an 8-residue helical segment (12 Å length).

Given that SecA is responsible for targeting of our chimeric proteins to SecYEG, we wondered if SecA could process them if the *H*-R segments were replaced by native secreted proteins. Figure 4 shows that indeed the H-segments could be replaced by the signal sequences of MalE, OmpA, and PhoA, confirming that SecA can identify 'normal' N-terminal signal sequences placed downstream from the N-terminus. However, whereas processing of polyleucine *H*-segments by SPase I resulted in a single species of cleaved product (Figure 3), cleavage of the less hydrophobic native signal sequences resulted in two cleaved species (Figure 4). This is a result of further cleavage on the cytoplasmic membrane surface by the RseP system [19, 20], because RseP⁻ cells produced only a single fragment (Figure 4). Unlike the more polar signal sequences, polyleucine *H*-segments result in very stable fragments not recognized apparently by RseP. These results provide strong support for the idea that RseP plays a major role in disposing of cleaved signal sequences [20]; cleaved fragments that are not stable in the membrane are cleaved further by RseP and consequently drop into the cytoplasm where they can be hydrolyzed by cytoplasmic enzymes.

Because RseP cleaved less stable TM segments such as N-terminal signal sequences, we examined the fate of our 16-residue Ala/Leu transmembrane segments after SecA insertion into the membrane (Figure 2). Examination of the processing of the Ala/Leu segments containing from 5 to 10 leucines, showed that fragments of segments containing 5 leucines appeared in the both the soluble and insoluble (membrane) fractions whereas segments with 10 leucines were found solely in the insoluble fraction (Figure 5). As the number of leucines was increased, there was a steady shift of the fragments toward the insoluble fraction; the major break point occurred at 7–8 leucines. This is an important result, because it shows that the hydrophobicity requirements for partitioning a segment from translocon to membrane are different than for partitioning between membrane and the cytoplasm; 4 leucines are sufficient to guarantee translocon-to-bilayer partitioning of the *H*-segment whereas 7 or so are required to prevent the CadC-*H* protein from dropping out into the cytoplasm. This implies that translocon-to-bilayer partitioning is not equivalent to water-bilayer-partitioning. This is perhaps not surprising in the light of the studies of Capponi et al. [25] who showed using molecular dynamics simulations that water behaves quite anomalously within the translocon. This result also means that proteins such as CadC and RodZ with moderately

non-polar *H*-segments may be stable in the membrane only because of the insolubility of their cytoplasmic and periplasmic domains in the membrane phase.

To establish the criteria for SecA identification of far-downstream TM segments, we determined that it was difficult for SecA to identify polyleucine segments composed of more than 16 leucines; there was a steady decline in the processing of polyleucine segments as the number of leucines increased (Figure 6). For segments of 22 leucines or longer, the *C-H-F* constructs were not processed at all. This raised the question of whether the failure was due to the inability of SecA to recognize the segment or the inability of SecYEG to incorporate/secrete a highly hydrophobic segment across the membrane. To answer that question, we created the preMalE-*H-R* construct (Figure 1b and 1c). The data of Figure 7 show that SecA recognized the preMalE signal sequence and initiated secretion across the membrane. For *H* composed of 16 alanines, the construct was completely secreted. For segments composed of 16 or 24 leucines, however, the greasy segment partitioned into the membrane to form a single-span membrane protein. We conclude that the failure of SecA to target *C-H-R* constructs with long polyleucine *H*-segments (24L) was due to failure of SecA to recognize the segments.

Monné et al. [22, 26] examined the consequences of placing helix-breaking residues into very long poly-leucine segments inserted via the signal recognition particle (SRP) pathway using a dog pancreas microsome system. They showed, for example, that the introduction of a single proline or arginine into the middle of a long poly-Leu segment could cause a topology reversal that led to the insertion of the segment as a hairpin rather than a single-TM segment. We never observed such a phenomenon, probably because the SRP pathway allows greater folding flexibility than the SecA pathway. The formation of a hairpin in our system would require either that SecA reverse its direction of transport at some point or that the soluble periplasmic domain pass back across the inner membrane. Both possibilities seem unlikely and were in fact never observed.

Finally, we confirmed that secretion/insertion of our *C-H-X* is due to the SecA system (Figures S1 and S2), in agreement with Zhou et al. [21] who showed that the SecA pathway is both necessary and sufficient for secretion, although the SRP pathway can enhance the SecA pathway for very hydrophobic sequences.

Typically [27], the first step in protein secretion by SecA is assumed to be insertion into the translocon of a hairpin-like structure comprised of the signal sequence and the adjacent mature sequence such that the N-terminus of the signal faces the cytoplasm (Figure 8a). Thinking about the experiments presented here and our earlier work on RodZ and CadC, we wondered about how SecA could manage secretion of proteins carrying a far-downstream transmembrane segment. It is difficult to visualize how SecA could secrete chimeras like ours that have a folded domain at the N-terminus. Consider the *C-H-R*. The 154-residue CadC cytoplasmic domain must emerge from the ribosome long before the appearance of the *H*-segment. It seems likely that the domain is folded before the construct is recognized by SecA. We suggest in Figure 8b–d a plausible scheme in which the direct interaction the *H*-segment with the membrane bilayer plays a dominant role. We suggest that, with the intracellular CadC domain folded, SecA binds to the *H*-segment and transports it in some

uncertain manner to the vicinity of a SecYEG translocon. If, as seems likely, the *H*-segment has a higher affinity for the membrane bilayer than for SecA, then the segment will spontaneously transfer to the membrane interface. We suggest that because the free energy of the peptide is likely higher in the surface-bound state than in a transmembrane state [28, 29], the segment should spontaneously partition across the membrane carrying its C-terminal domain through the translocon. Thus, we suggest, the secreted protein threads the translocon. This scenario helps explain the presence of positive charges at the N-terminus of signal sequences, which are often dispensable and are required mostly for short less-hydrophobic segments [30, 31]. The positive charge interacting with the negatively charged membrane may anchor the N-terminus of the sequence at the interface to assure the correct topology.

Materials and Methods

Bacterial strains, plasmids, and materials

All constructs were amplified from chromosomal DNA (*E. coli* K12). We used the restriction sites *NdeI* and *XhoI* for gene insertion into the pET21 vector (T7 promoter/*lac* operator, NOVAGEN). We inserted two additional unique restriction sites (*KpnI* and *BamHI*) to the *cadC* gene to exchange the H-segment using cassette cloning or overlap extension. All constructs were confirmed by sequencing. BL21(DE3) (F⁻ ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) cells were used to express the various CadC constructs, which all carried an internal T7-tag and a C-terminal His₆-tag for Western blot detection [13]. For SPase I depletion studies, we used *E. coli* strain FTL85 in which *lepB* is under the control of AraC [32]. For SecA depletion studies, we used *E. coli* strain EO527 in which *secA* is under the control of AraC. For Ffh depletion studies, we used *E. coli* strain WAM121 in which *ffh* is under the control of AraC. All depletion strains were received from Ross E. Dalbey at the Ohio State University who obtained them from Tracy Palmer (FTL85) and Tom Rapoport (EO527), respectively. For RseP studies we used AD1811 (*rseA*) and AD2328 (*rseA*, *rseP*) cells kindly provided by Prof. Yoshinori Akiyama at Kyoto University.

Growth Conditions

Various CadC-based proteins were expressed from an IPTG-inducible and T7 polymerase-dependent system (pET-vector). We used a standard expression strategy: 1h expression in BL21(DE3) cells (presence of T7 polymerase). Protein expression in Figures 2, 3, 6, 7, S1, and S2 was done using BL21(DE3) cells containing the gene for T7-polymerase (CadC protein is regulated by the T7-promoter and the *lac*-operator). This leads to high protein expression levels even in the presence of small amounts IPTG inducer (10 – 20 μM) and short expression time (1 h). The experiments were done at pH 7 in Luria-Bertani (LB) medium or super optimal broth with catabolite repression (SOC) full media using glucose for repression [33].

SPase I, SecA, and Ffh Depletion Protocols

Depletion experiments. *C-H-R* constructs with *clv* = AQA (modified pET-vector, T7-RNA-polymerase independent system using a T5 promoter sequence, which is recognized by the

wt *E. coli* RNA-polymerase) were transformed in depletion cells. Overnight cultures were grown in SOC media in the presence of 0.02 % arabinose (non-depletion condition). A 400 μ l inoculum from the culture was added to 10 ml fresh SOC media with or without 0.02 % arabinose. After 2 h ($OD_{600} \sim 0.6$) protein expression was induced by adding 10 μ M IPTG. After 0.5 h of protein expression, cells were pelleted and analyzed. (Figures S1 and S2)

RseP deletion experiments

C-H-R constructs with *clv* = AQA (modified pET-vector, T7-RNA-polymerase independent system using a T5 promoter sequence which is recognized by the wt *E. coli* RNA-polymerase.) were transformed in deletion cells AD1811 (*rseA*) (positive control RseP plus condition) or AD2328 (*rseA*, *rseP*) (RseP minus condition; cells are only viable when *rseA* is deleted in addition). A 400 μ l inoculum from an over-night culture was added to 10 ml fresh SOC media. After 1 h ($OD_{600} \sim 0.6$) protein expression was induced by adding 10 – 20 μ M IPTG. After 1 h of protein expression, cells were pelleted and analyzed. Figure 4 and 5.

Cell Fractionation

Cell fractionation was performed by cell lysis using freeze-thaw and DNaseI treatment [33]. The bacterial cells were harvested, centrifuged, and the pellet resuspended in Lysis-Equilibration-Wash buffer (LEW buffer: 50 mM NaH_2PO_4 , 300 mM NaCl, pH 8.0) containing DNaseI enzyme, DNaseI buffer, lysozyme, and phenylmethanesulfonyl fluoride (PMSF). 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 minutes. The cell suspension was centrifuged at 13,000xg for 15 minutes at 4°C, and the supernatant containing the soluble and periplasmic proteins (called the *C/P* fraction) was transferred to a new tube. The pellet was either washed once with 100 mM ice-cold Na_2CO_3 to remove membrane-adherent proteins[34] (CW fraction) or directly resuspended in LEW+1.5% CHAPS to solubilize membrane proteins. The suspension was centrifuged at 13,000xg at 4°C for 15 minutes. The supernatant contains the inner membrane (IM) fraction.

Periplasmic fraction

Cells were grown to mid-logarithmic phase and harvested by centrifugation. Osmotic shock was performed by a method adapted from Neu and Heppel [35] and Thorstenson et al. [36] as follows: Cell pellets were resuspended in 100 μ l osmotic shock buffer (0.5 M sucrose, 0.2 M Tris, 0.5 mM EDTA) and incubated on ice for 15 min, followed by the addition of 400 μ l of 5 mM $MgSO_4$. The cells were incubated on ice for an additional 30 min, followed by pelleting at 13,000xg at 4°C for 15 minutes. The supernatant (periplasmic fraction) and the pellet were mixed separately with SDS sample buffer and analyzed by SDS-PAGE [37].

Protease treatment studies

Cells were grown to mid-logarithmic phase and harvested by centrifugation. Cell pellets were resuspended in 100 μ l osmotic shock buffer (0.5 M sucrose, 0.2 M Tris, 0.5 mM EDTA) and incubated on ice for 15 min. Then, 400 μ l of 5 mM $MgSO_4$ containing prot K (80 ng) was added and the cells incubated on ice for an additional 30 min, followed by pelleting at

13,000xg at 4°C for 15 minutes. The supernatant was discarded, the pellet resuspended in SDS sample buffer, and analyzed by SDS-PAGE [37].

Western Blotting

The pellet was resuspended in SDS sample buffer and analyzed by SDS-PAGE (4–20 %)[37] and then Western blotted using iBLOT from Invitrogen® (Invitrogen Corp., Carlsbad, CA), which guarantees complete protein transfer that is necessary under low-expression conditions. The protein was detected by a T7-tag alkaline phosphatase-conjugated antibody from Novagen® (Novagen (EMD) Biosciences, Madison, WI) or by a His₆-tag antibody from Roche® (Hoffman La Roche, Basel, Switzerland)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Single-span membrane proteins with far downstream TM segments use the SecA pathway
- SecA recognizes signal sequences even if not at the protein's N-terminus
- Moderately hydrophobic sequences drop out of the membrane into the cytoplasm
- Translocon-to-bilayer transfer ΔG s of TM segments differ from membrane-to-water ΔG s
- Bilayer interface is crucial for SecA to thread sequences through the translocon

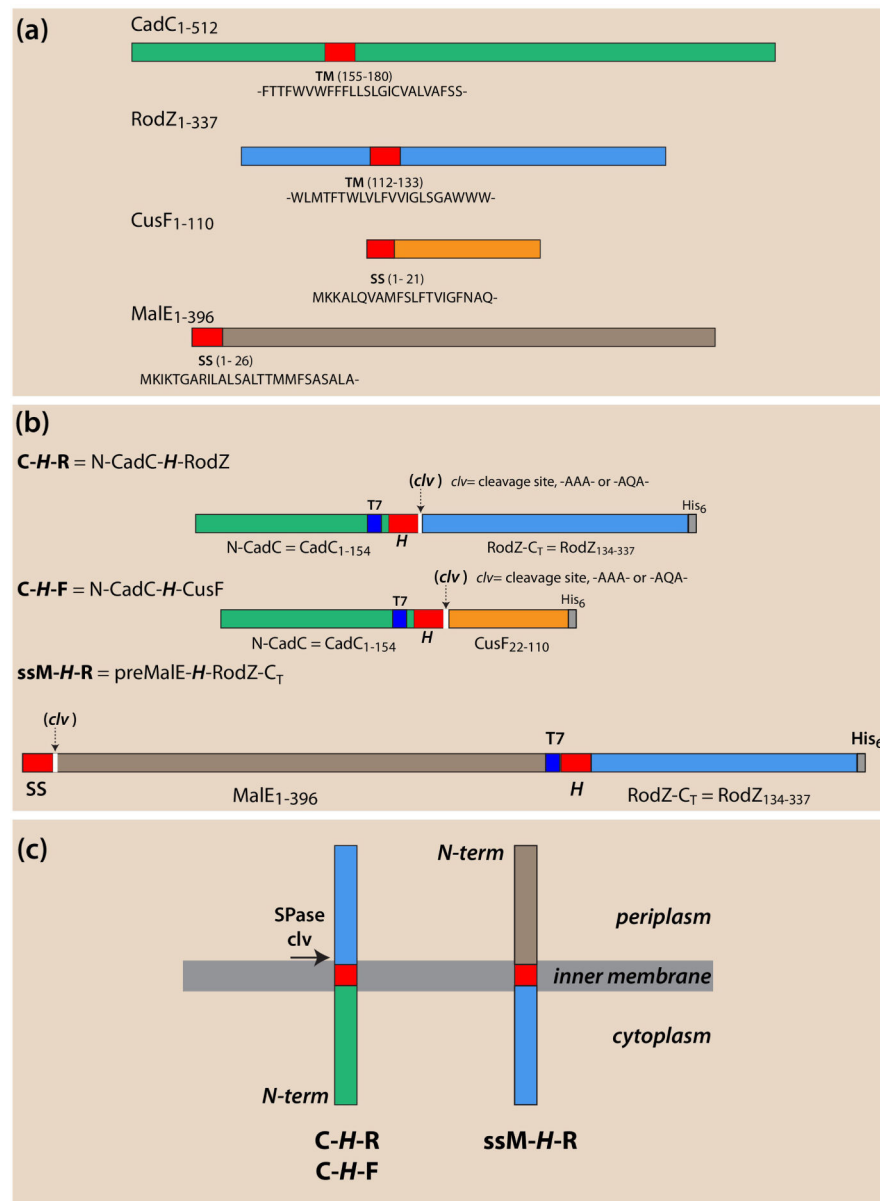


Figure 1. Schematic overview of single-span proteins and chimeras used in this study. Locations of transmembrane (TM) segments [*H*-segments] or signal sequences (ss) are colored red. Location of T7 immuno tags are colored blue, His₆ tags gray, and cleavage sites white. CadC and RodZ non-TM domains are colored green and blue, respectively, CusF orange, and MalE brown. (a) CadC or RodZ are unusual Type-II (N_{in}-C_{out}) single-span membrane proteins (MPs) that lack an N-terminal signal sequence (ss) and have a far-downstream TM segment. CadC activates the CadBA operon during low-pH stress[3–5] while RodZ, plays an important role in the maintenance of the rod shape of *Escherichia coli* [6, 7]. CusF, is the periplasmic copper chaperone of the *E. coli* CusCFBA copper-transporting efflux system[14] and MalE is the well-known maltose binding protein [17]. Both have N-terminal signal sequences (ss) indicated in red. (b) Chimeric proteins composed of foldable fragments

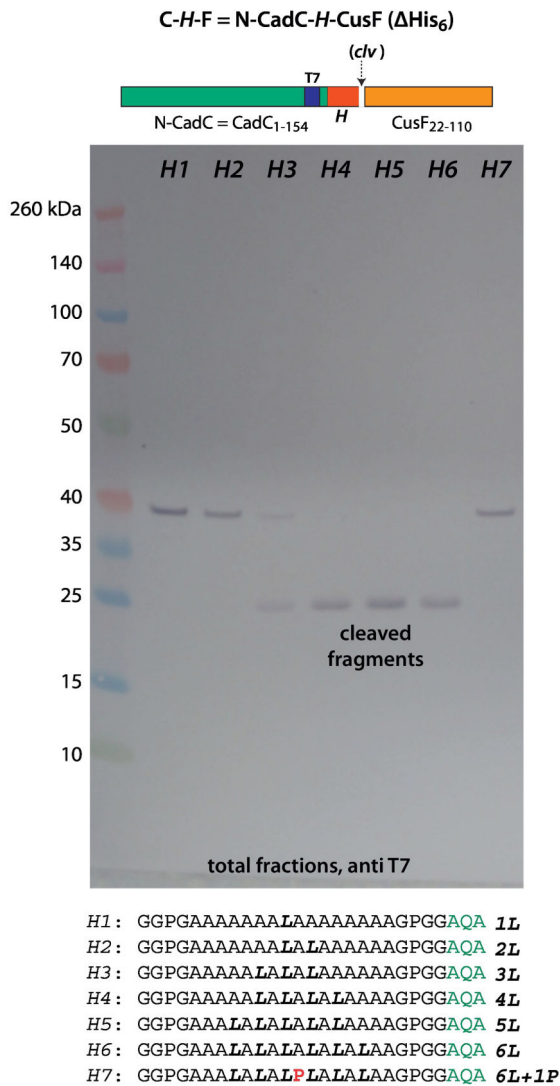
of the proteins in panel **a**. Spase I cleavage sites (*clv*) are inserted immediately following the *H*-segment except for the ssM-*H*-R construct that carries a native cleavable signal sequence. Proteins cleaved by SPase I indicate that the protein has entered the SecA pathway and passed through the SecYEG translocon. **(c)** Schematic overview of the topology of the proteins in panel **b** after SecA targeting and SecYEG membrane incorporation.

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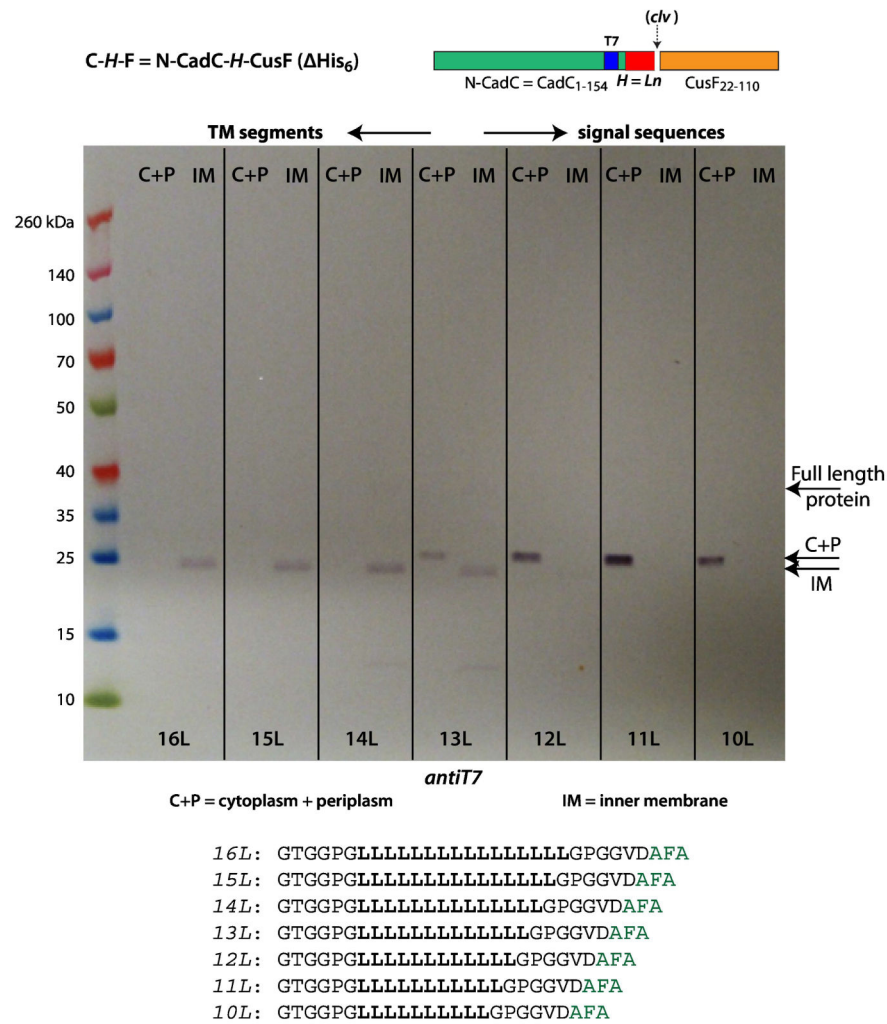
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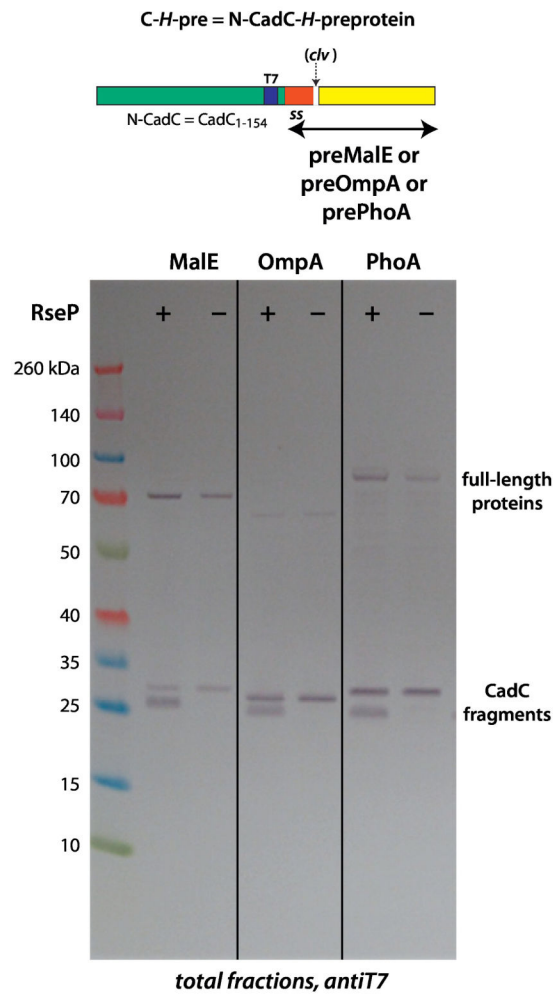
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**Figure 2.**

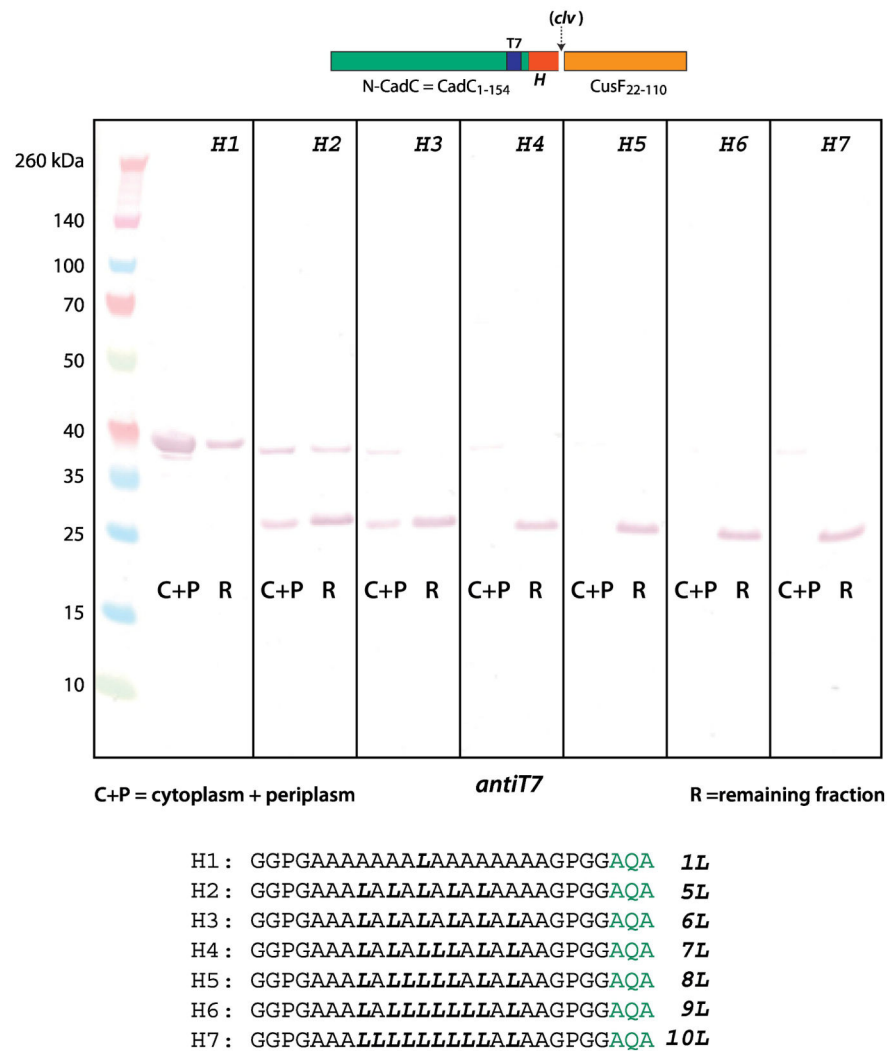
To be recognized by SecA, the 16 residue Ala/Leu *H*-segment must have 3–4 leucines in order to be recognized by SecA. This immunoblot identifies T7 tags, which are contained within the N-terminal domain carrying the *H*-segment. The appearance of the lower molecular weight bands indicates cleavage by SPase I and therefore processing of the construct by SecA. The seven *H*-segments used in the constructs are indicated. The blots show that to be recognized by SecA, the *H*-segment must contain at least 3 leucines (lane *H3*), but for complete processing 4 leucines are required (lane *H4*). A single Leu-to-Pro substitution at the center of the A₁₃L₆ construct to give A₁₃P₁L₅ prevents recognition and secretion of by SecA. The C-H-F constructs were expressed in *E. coli* BL21 cells grown in SOC media at 37° C (see Materials and Methods).

**Figure 3.**

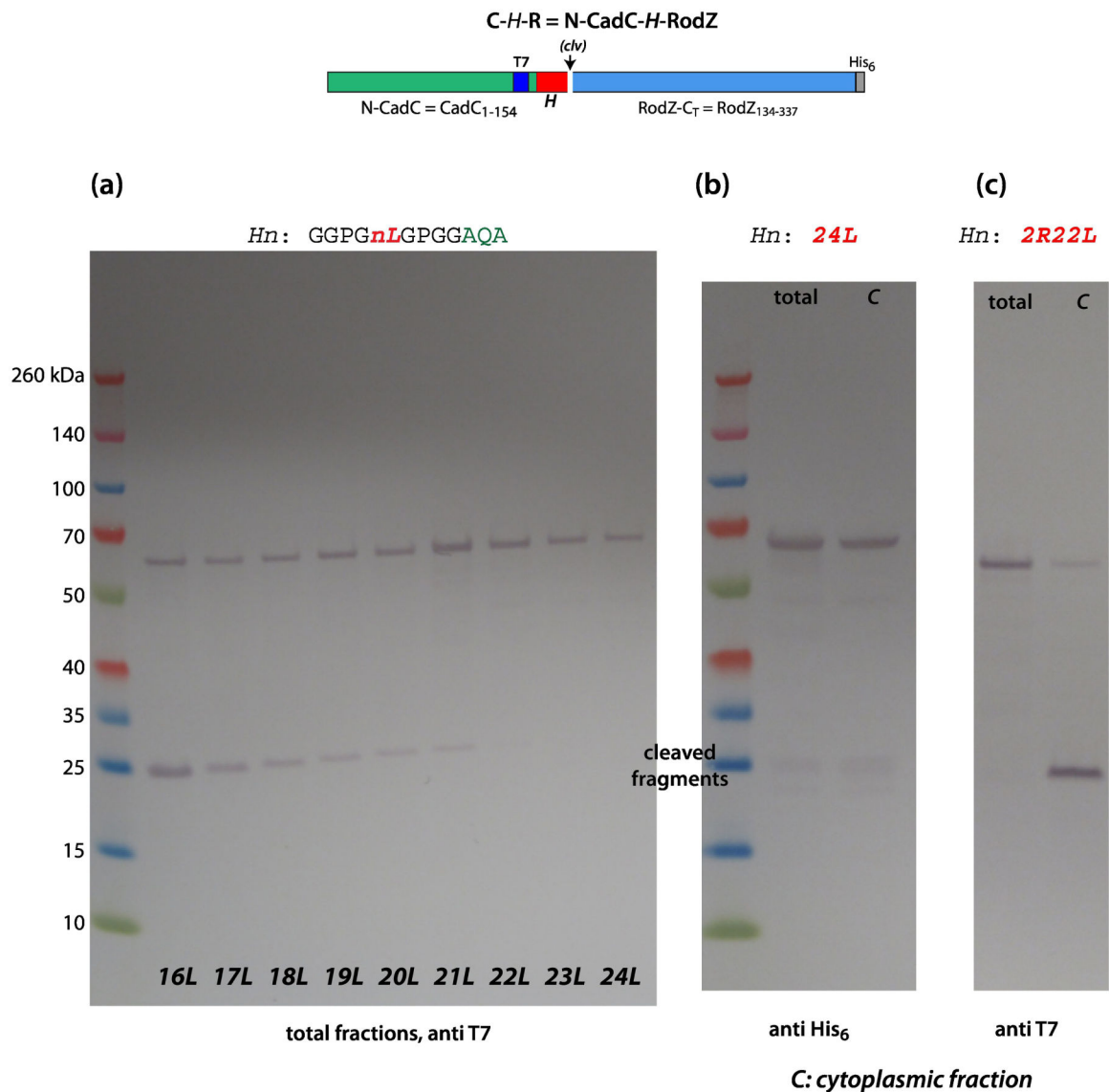
All of the poly-leucine *H*-segments are recognized and processed by SecA, but full incorporation into the inner membrane (IM) requires 14 leucines. This suggests a translocon/membrane partitioning process occurs as the secreted protein passes through the translocon. As in Figure 2, this immunoblot identifies T7 tags, which are contained within the N-terminal domain carrying the *H*-segment. The appearance of the lower molecular weight bands indicates cleavage by SPase I and therefore processing of the construct by SecA. In lanes 10L to 12L, the fragments are found only in the soluble (C+P=cytoplasm plus periplasm) fraction. Beginning with 14L, all fragments are found in the IM fraction. Note the fragment molecular weight differences for lanes 14L-16L compared with lanes 10L-12L. The difference is particularly obvious in 13L, which reveals clearly the transition from the cytoplasm to inner membrane. The difference in molecular weight arises from processing by the RseP intramembrane protease (see Figure 4). The C-H-F constructs were expressed in *E. coli* BL21 cells grown at 37 °C in SOC media augmented with glucose, MgCl₂, and MgSO₄. See Materials and Methods.

**Figure 4.**

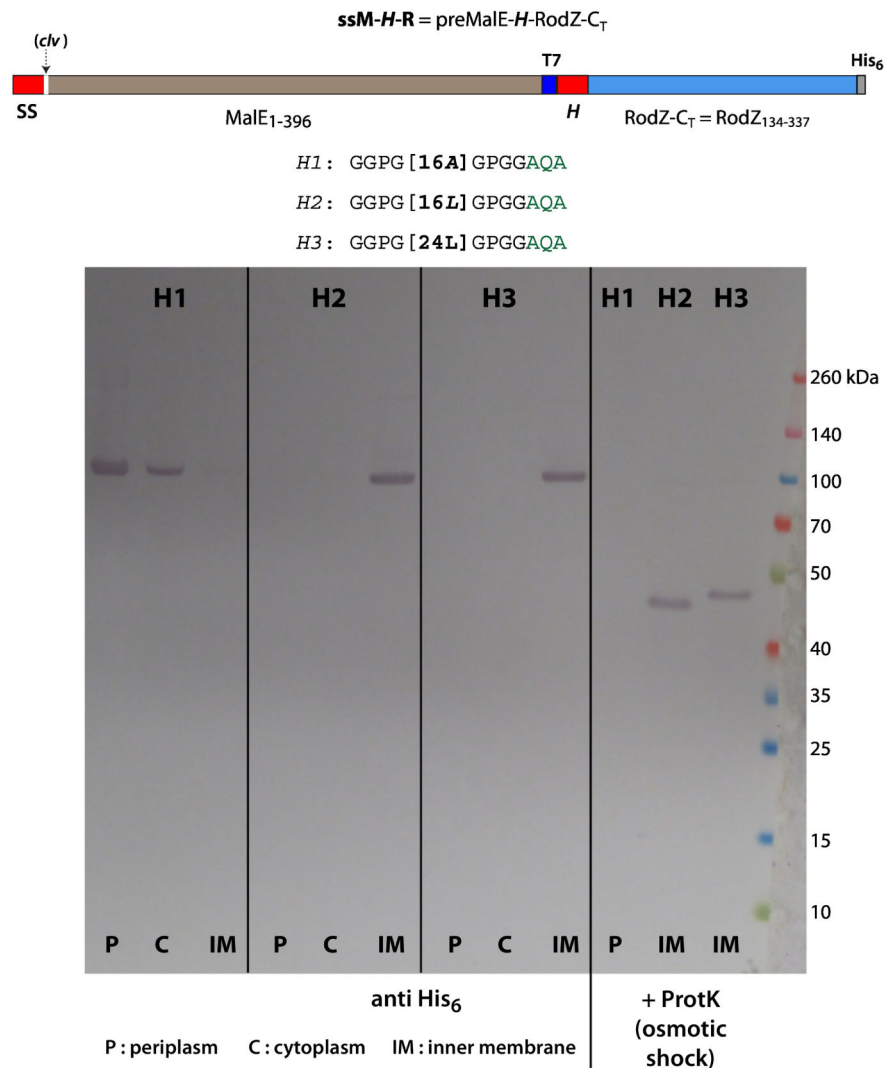
N-terminal-extended native secreted proteins (MalE, OmpA, and PhoA) can serve as SecA targets. SecA targeted all constructs to the membrane, based upon SPase I cleavage. The cleaved T7-labeled fragments revealed two bands on the immunoblots resulting from cleavage by SPase I (left-hand lanes labeled RseP⁺). These bands arise from post-cleavage processing by the site-2 intramembrane metalloprotease RseP system [19, 20], as proven by the presence of a single-band in RseP⁻ mutants (right-hand, single bands). These results indicate that less stable TM segments are attacked by RseP. However, RseP processes membrane embedded sequences only if they have been cleaved initially by SPase I. See Materials and Methods for descriptions of the RseP⁻ cells.

**Figure 5.**

In $RseP^-$ cells, as the number of leucines in Ala/Leu segments increase from 5 leucines to 10, there is a steady shift of the fragments toward the insoluble fraction from the soluble fraction; the major break point occurred at 7–8 leucines. The $RseP^-$ condition prevents further processing of TM segments after SPase I cleavage, which reveals information about the inherent stability of TM segments. The important conclusion from these data is that the hydrophobicity requirements for partitioning a segment from translocon to membrane are different than for partitioning between membrane and the cytoplasm; 4 leucines are sufficient to guarantee translocon-to-bilayer partitioning of the *H*-segment (Figure 2) whereas 7 or so are required to prevent the CadC-*H* protein from dropping into the cytoplasm. See Material and Methods for experimental details.

**Figure 6.**

SecA cannot identify long and highly hydrophobic *H*-segments. **(a)** These data show that SecA identification of polyleucine *H*-segments composed of more than 16 leucines becomes more and more difficult with increasing numbers of leucines, as indicated by the steady decline in the intensity of the cleaved fragments. For segments containing 22 or more residues, the C-*H*-R constructs were not processed at all. **(b)** Remarkably, despite the high hydrophobicity of the polyleucine *H*-segments, the chimeric proteins are found only in the cytoplasmic fraction rather than in inclusion bodies. This might be due to interactions with cytoplasmic chaperones. **(c)** These data show that replacement of two leucines in the 24Leu construct with two arginine (pos. 10 and 15) transform it to a SecA target (left lane without a cleavage site, right lane with a cleavage site). The occurrence of the cleaved fragment (right lane) indicates SecA targeting. These experiments were carried out using BL21 cells carrying a pET21 vector. Cells were grown in SOC media at 37° C. See Materials and Methods.

**Figure 7.**

These results, obtained using the ssM-H-R, construct show that SecYEG can insert proteins containing very long and greasy TM segments. Despite the presence of a long greasy segment far downstream from the N-terminus, SecA identifies the sequence as a target via the preMalE signal sequence at the N-terminal. If the *H*-segment is polyalanine, the protein is secreted and found only in the periplasmic and cytoplasmic fractions. For *H* = 16 and 24 Leu, the protein is found only in the inner membrane fraction, which indicates that SecYEG is capable of incorporating highly hydrophobic segments into the membrane. Osmotic shock followed by proteinase K (ProtK) treatment (right-hand lanes labeled *H1*, *H2*, *H3*) hydrolyzes the periplasmic MalE fragment, leaving the RodZ component as an N-terminal anchored single-span membrane protein. *E. coli* BL21 cells grown at 37 °C in SOC media augmented with glucose, MgCl₂, and MgSO₄. See Materials and Methods.

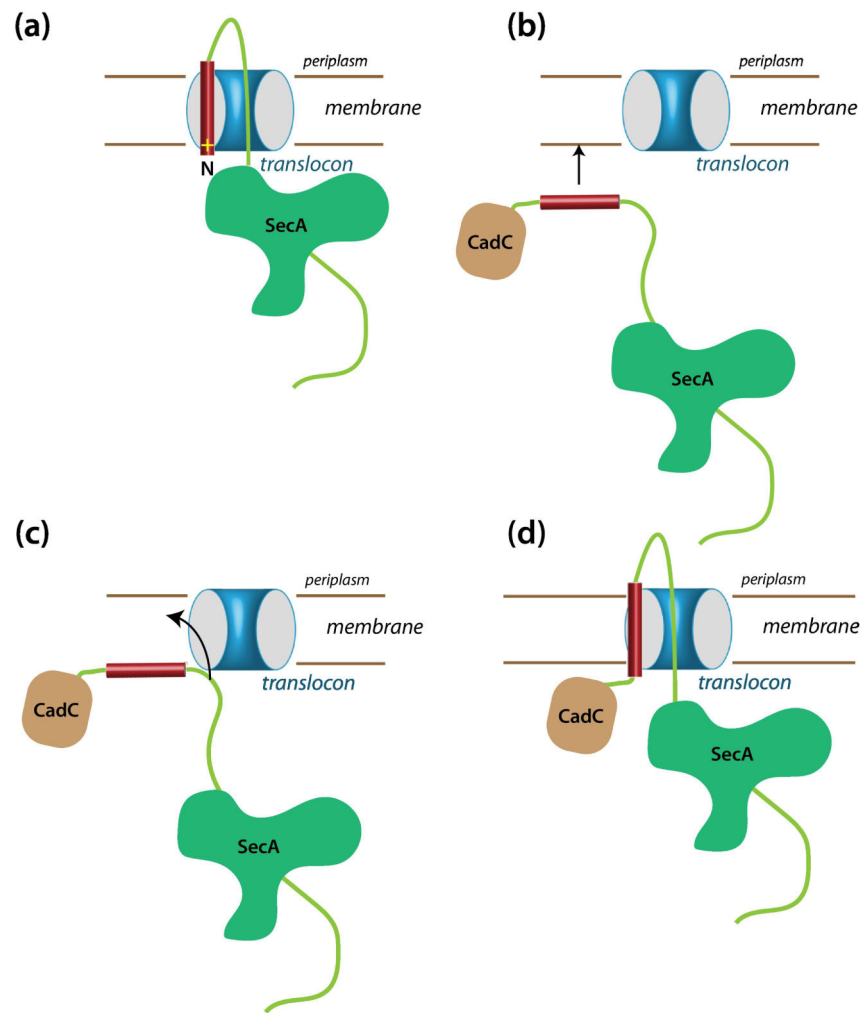


Figure 8. How can a single-span Type II membrane protein with a far-downstream TM segment be inserted into the membrane via the SecYEG translocon? (a) A typical scheme for the insertion into the translocon of a secreted protein carrying an N-terminal signal sequence. Topologically, this scheme seems unlikely to work for proteins with a far downstream TM domain, because the N-terminal domain is likely already folded when the TM domain emerges from the ribosome. We suggest instead the scheme shown in panels b, c, and d. (b) The folded N-terminal domain problem can be avoided if SecA transports the TM segment to the membrane, bringing it into close proximity of SecYEG. (c) If the affinity of the TM segment for the membrane is higher than for SecA, the segment will bind to the membrane. The nearby translocon would provide a pathway across the membrane for the much more polar part of the chain. For a very hydrophobic segment with a low hydrophobic moment [38, 39], a transmembrane configuration likely has a lower free than a surface-bound state. (d) As the TM segment partitions across the membrane, we suggest that this pulls the more polar C-terminal part of the chain into the translocon so that SecA can secrete the remainder

of the chain across the membrane. This scheme works just as well for secreted proteins carrying an N-terminal signal sequence.

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