Features of Transmembrane Segments That Promote the Lateral Release from the Translocase into the Lipid Phase†

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ABSTRACT: Topogenic sequences direct the membrane topology of proteins by being recognized and decoded by integral membrane translocases. In this paper, we have compared the minimal sequence characteristics of helical-hairpin, reverse signal-anchor, and stop-transfer sequences in bacterial membrane proteins that use either the YidC or SecYEG translocases for membrane insertion. We find that a stretch composed of 3 leucines and 16 alanines is required for efficient membrane-anchoring of the M13 procoat protein that inserts by a helical hairpin mechanism, and that a stretch composed of only 19 alanines has a detectable membrane-anchoring ability. Similar results were obtained for the reverse signal-anchor sequence of the single-spanning Pf3 coat protein and for stop-transfer segments engineered into leader peptidase. We have also determined the contribution to the apparent free energy of membrane insertion of M13 procoat for all 20 amino acids. The relative order of the contributions is similar to that determined for a stop-transfer sequence in the mammalian endoplasmic reticulum, but the absolute difference between the contributions for the most hydrophobic and most hydrophilic residues is somewhat larger in the *E. coli* system. These results are significant because they define the features of a membrane protein transmembrane segment that induce lateral release from the YidC and Sec translocases into the lipid bilayer in bacteria.

Membrane proteins are assembled by the action of sophisticated translocation and membrane-insertion machineries. In bacteria, there are three such machineries known. The SecYEG translocase facilitates the translocation and membrane insertion of the majority of inner membrane proteins (*1*, *2*, *3*). The second translocase, the Tat machinery, operates in a radically different manner from the Sec translocase and is responsible for translocating folded exported proteins that typically have bound metal cofactors (*4*). The third translocase, YidC, functions in the insertion and assembly of proteins into the inner membrane (*5*−*7*). YidC can function on its own, or together with the SecYEG translocase. In the "YidC only" pathway, YidC catalyzes the insertion of a subset of inner membrane proteins (*8*). In conjunction with the SecYEG translocase, YidC has been implicated in the membrane insertion, lateral integration, folding, and assembly of sec-dependent inner membrane proteins (*5*).

The information that specifies membrane protein insertion is located within topogenic sequences in the polypeptide chain (*9*). Cleavable signal peptides (SPs) and uncleaved signal-anchor (SA) sequences are topogenic elements that initiate translocation of carboxyl-terminal regions across the membrane (*10*−*12*). Stop-transfer (ST) sequences halt translocation that was initiated by a preceding signal peptide (*13*). Reverse signal-anchor (rSA) sequences initiate translocation of an amino-terminal domain (*14*). Finally, some proteins require two closely spaced hydrophobic domains that insert as a helical hairpin (*15*). It is believed that these different topogenic sequences are recognized and decoded by the translocase machineries. While it is clear that the hydrophobic character and flanking positively charged residues are important for the function of topogenic sequences (*16*, *17*), there is a lack of quantitative understanding of the membrane insertion process.

In the case of membrane proteins inserted by the SecYEG apparatus, there must be lateral transfer of the hydrophobic domains into the lipid bilayer after insertion into the Sec complex (*3*). The high-resolution structure of the archaeal Sec61 translocon provides a clue to how this may occur (*18*). The structure reveals that the Sec61α-subunit (homologous to SecY in bacteria) forms the core of the protein-conducting channel. This region consists of two pseudosymmetrical halves (transmembrane segments 1−5 and 6−10) with transmembrane segments 2b and 7 forming a 'lateral gate' where hydrophobic domains of the inserting membrane protein can bind and then exit from the channel to enter the lipid phase. The sequence characteristics required for lateral release of a ST sequence from the ER translocon have been studied in detail using leader peptidase as a model protein (*19*).

In contrast, very little is known about how topogenic sequences are decoded by the YidC machinery. YidC, a 60

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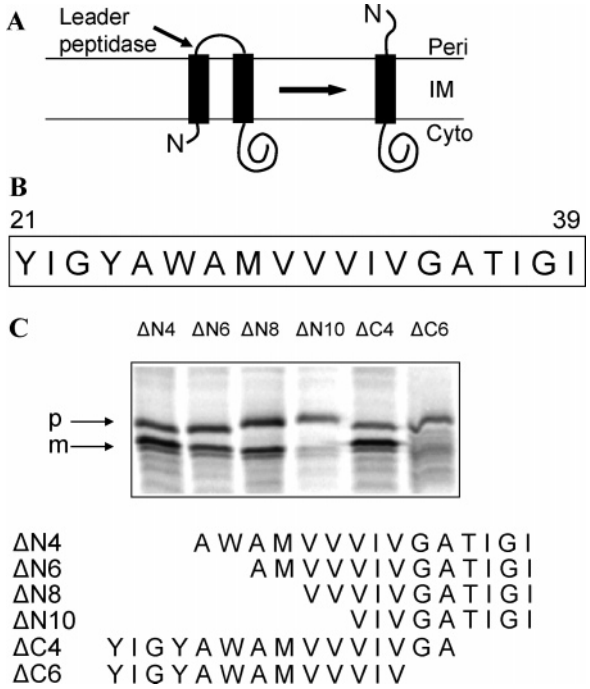

FIGURE 1: Deletion analysis of the PCLep TMH. A. Membrane topology of PCLep. Following membrane insertion, PCLep is cleaved to the mature protein CLep by leader peptidase. B. Amino acid sequence of the PCLep TMH. C. PCLep constructs with amino- or carboxyl-terminal deletions within the transmembrane domain were analyzed for signal peptide processing. An overnight MC1060 cell culture was back-diluted 1:50 into fresh LB, grown to $A_{600} \sim 0.6$ and transferred to M9 medium. After 30 min in M9 medium, cells were treated with IPTG (1 mM) to induce expression of PCLep and labeled with [^{35}S]-methionine for 30 s. PCLep was immunoprecipitated, and analyzed by SDS-PAGE and phosphorimaging.

kDa protein with six transmembrane segments (*20*), interacts with the hydrophobic regions of its substrates during membrane insertion (*21*−*25*). In the YidC family of proteins, the five transmembrane segments in the carboxyl-terminal region are conserved (*26*, *27*) and may provide a platform for binding the hydrophobic regions of the substrates (*28*).

The best-studied substrates that insert by the 'YidC only' pathway are the M13 procoat and the Pf3 coat proteins (*7*). M13 procoat is made with an N-terminal cleavable SP and a C-terminal transmembrane helix (TMH, see Figure 1A). The protein inserts into the membrane as a helical hairpin with both the SP and the TMH promoting translocation of the intervening periplasmic region (*15*). The single-spanning Pf3 coat protein lacks a SP but nevertheless inserts across the membrane and translocates its amino-terminus to the periplasm (Figure 7A). The topogenic sequence within Pf3 coat is defined as a rSA sequence (*29*).

In this paper, we have examined the features of the M13 procoat TMH and the Pf3 rSA sequence that are necessary to induce lateral release from the YidC translocase into the lipid bilayer. Using systemically designed peptide segments, we find that a stretch of 3 leucines and 16 alanines can replace the normal M13 coat TMH. A less hydrophobic TMH comprising 19 alanines still has detectable membrane-insertion activity. Interestingly, when the TMH is replaced by a stretch of 19 serines, the protein switches insertion pathway and is completely translocated into the periplasm by the Sec rather than the YidC translocase; the 19-serine stretch does not anchor the protein in the membrane. In agreement with the result for M13 procoat, we find that an 18-residue stretch of 3−4 leucines and 14−15 alanines is sufficient to ensure efficient integration of both the rSA sequence of the YidC-dependent Pf3 coat protein and a ST sequence engineered into the Sec-dependent leader peptidase. Finally, we have estimated the contribution to the apparent free energy of membrane insertion of the M13 procoat TMH for each of the 20 amino acids.

EXPERIMENTAL PROCEDURES

Strains, Plasmids and Growth Conditions. The *Escherichia coli* YidC depletion strain JS7131 [MC1060: Δ*yidC, attB::R6Kori, ParaBAD-yidC*$^+$ (Spec)] was from this laboratory. To express PCLep and Pf3Lep mutants in the MC1060 or JS7131 strains, the constructs were cloned into the pMS119 vector, which contains the IPTG-inducible *tac* promoter and the *lacI*^{q}. Trans-[^{35}S]-label, a mixture of 85% [^{35}S]-methionine and 15% [^{35}S]-cysteine, 1000 Ci/mmol, was from ICN.

To deplete the JS7131 strain of YidC protein, the overnight cell culture [grown in Luria Broth (LB[1]) with 0.2% L-arabinose (ara)] was washed with LB, diluted 1:50 into LB containing 0.2% glucose (glc), and grown to $A_{600} \sim 0.4$. The cells were then pelleted and resuspended in M9 minimal medium, and the culture was incubated at 37 °C for 30 min.

Generation of Mutants. The TMH deletions were made by site-directed mutagenesis PCR. To generate the PCLep mutants with artificially designed TMH segments, a new SpeI site was introduced into the gene upstream of the PCLep TMH, and a new KpnI site was introduced into the downstream region. Double-stranded oligonucleotides with SpeI and KpnI overhangs were prepared and purified by IE HPLC by Integrated DNA Technologies, Inc. After endonuclease digestion and dephosphatase treatment, the SpeI/KpnI-restricted vector was purified from an agarose gel, followed by ligation with the artificial oligonucleotides. The Pf3Lep mutants with the artificially designed rSA sequences were generated by the same method, except that an EcoRI site was introduced into the gene at the 3′ end of the TM segment. Double-stranded oligonucleotides to make the artificially designed rSA sequences were designed accordingly with SpeI and EcoRI overhangs.

Signal Peptidase Processing Assay and Proteinase K Protection Assay. Prior to labeling, 1 mM IPTG was added to the cultures for 5 min to induce the expression of the plasmid-encoded proteins. Cells were then labeled with trans-[^{35}S]-methionine (100 μCi/mL cells) for 30 s, and, where appropriate, chased with non-radioactive methionine for various times. To analyze the signal peptidase processing of PCLep mutants, labeled cells were precipitated with trichloroacetic acid (TCA) and immunoprecipitated with anti-Lep antisera. For analysis of the membrane anchoring of the PCLep and Pf3Lep artificially designed TMH segments, radiolabeled cells were converted to spheroplasts and incubated in the presence or absence of proteinase K (final concentration, 0.5 mg/mL) for 60 min on ice. The samples were then precipitated with TCA.

[1] Abbreviations: ara, L-arabinose; ECL, enhanced chemiluminescence; glc, glucose; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria broth; M9, minimal medium; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

To test the SecA dependency of PCLep, 3 mM sodium azide was added to the culture after IPTG induction of the PCLep mutants, followed by labeling with [^{35}S]-methionine. Radiolabeling was done 5 min after with the addition of azide. OmpA antisera were used to immunoprecipitate the SecA-dependent OmpA protein. The [^{35}S]-labeled proteins were separated by SDS-PAGE and detected by phosphorimaging (*30*).

Activity of PhoA Fusion Proteins. The alkaline phosphatase activity assay was performed in the CC118 strain transformed with the pHA-4 plasmid (*31*) carrying the appropriate Lep-PhoA fusion constructs. Cells were inoculated in a 2.2 mL 96-well growth plate (ABgene) containing 1 mL of Luria Broth (LB) medium supplemented with 100 μg/mL ampicillin in each well and grown overnight at 37 °C. Overnight cultures were diluted 1:100 in 1 mL of fresh medium with antibiotics and grown at 37 °C. When the OD_{600} reached 0.13−0.15, cells were induced with arabinose (to a final concentration of 0.2%) and grown to a final OD_{600} of 0.3−0.5. In order to prevent the spontaneous activation of PhoA localized to the cytoplasm, 1 mM iodoacetamide was added to the cultures for 5 min prior to harvesting and to all buffers used subsequently (*32*). The activity assay was carried out on a 100 μL sample as described (*33*), and the remaining cells were used for Western blotting using a PhoA antiserum. The time of the activity assay was set to 90 min for all samples. Mean activity values were obtained from at least four independent measurements.

RESULTS

The M13 Procoat Protein. The M13 coat protein is synthesized in a precursor form (procoat) with a cleavable SP and has a TMH in the mature domain. It is processed by leader peptidase to mature coat protein during membrane insertion (Figure 1). In our studies, we have used PCLep, a derivative of procoat that contains a large globular domain (*P*2) derived from leader peptidase at the carboxy terminus of the protein (*34*). Use of PCLep makes it possible to monitor the membrane anchoring of the protein by a protease-protection assay (see below).

Deletion Analysis of the PCLep TMH. Using a deletion approach, we first examined the minimum length of the TMH sequence in PCLep necessary for translocation of the amino-terminal region. To examine membrane insertion, we monitored leader peptidase processing of radiolabeled PCLep that converts it to the mature form (CLep). MC1060 cells bearing a plasmid encoding PCLep were pulse labeled with [^{35}S]-methionine for 30 s. Cleavage of PCLep was observed for the ΔN4, ΔN6, and ΔN8 mutants but not for ΔN10 (Figure 1C). Processing was also observed for the ΔC4 but not for the ΔC6 mutant. The results show that a hydrophobic TMH segment of only 11 residues is sufficient for membrane insertion of PCLep.

Determination of the Contribution to the Apparent Free Energy of Membrane Insertion of the 20 Amino Acids. A simple way to quantitate membrane-insertion efficiency in order to be able to compare results obtained with different proteins and translocons is to calculate an apparent free energy of membrane insertion (ΔG_{app}) (*19*). Previously, this has been done for ST-sequence insertion in the mammalian endoplasmic reticulum (ER) (*19*), and we use the same approach here. We estimate the degree of membrane insertion of the PCLep TMH from the relative amounts of processed PCLep (f_p) and uninserted full-length protein (f_u) as measured from SDS-PAGE gels ($f_p + f_u$=1). ΔG_{app} is calculated from the apparent equilibrium constant $K_{app} = f_p/f_u$ between the inserted and uninserted states: $\Delta G_{app} = -RT \ln K_{app}$.

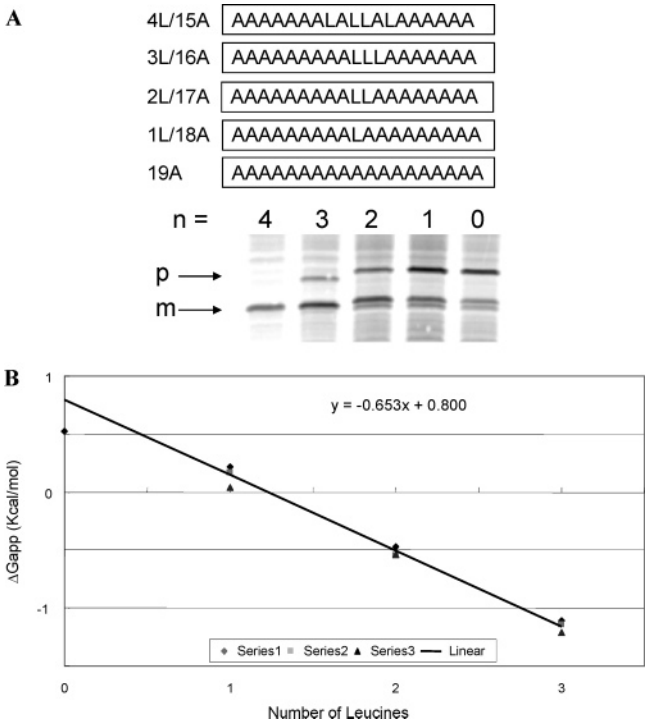

FIGURE 2: Membrane insertion of PCLep with *n*L/(19 − *n*)A TMH segments. A. The signal peptidase processing assay was carried out to analyze PCLep constructs with TMH segments composed of 19A, 1L/18A, 2L/17A, 3L/16A, or 4L/15A as described in Figure 1. The intensity of the precursor and mature protein bands were measured using ImageQuant. B. ΔG_{app} values for the designed TMH segments with 0−3 leucines. The fit was done only with the $n = 1-3$ points where we have three independent trials (marked separately). We do not include $n = 0$ since it is been difficult to get reliable quantitations from the repeat experiments. If we include the $n = 0$ data, the free energy of Ala changes from 0.042 to 0.033 kcal/mol and the free energy of Leu changes from −0.061 to −0.053 kcal/mol.

First, we investigated whether simple, 19-residue long Ala-Leu segments could function in place of the normal TMH of PCLep. We tested TMH sequences with the overall compositions 19A, 1L/18A, 2L/17A, 3L/16A, and 4L/15A. Membrane insertion of PCLep, as indicated by cleavage of the signal sequence, increased concomitantly as the number of leucine residues increased from 0 to 4 (Figure 2A) (note that the lower band in the "mature doublet" is a background band). ΔG_{app} values calculated from quantitation of the mature (m) and precursor (p) bands decrease linearly with the number of leucines (Figure 2B). In the simplest approximation, the total free energy of a *n*L/(19 − *n*)A stretch can be expressed as $\Delta G_{app} = (19 - n)\Delta G_{app}^{Ala} + n\Delta G_{app}^{Leu} = n(\Delta G_{app}^{Leu} - \Delta G_{app}^{Ala}) + 19\Delta G_{app}^{Ala}$. From a least-squares fit to the data in Figure 2B, we find $\Delta G_{app}^{Ala} = 0.04$ kcal/mol and $\Delta G_{app}^{Leu} = -0.6$ kcal/mol, similar to what has previously been found in studies of Sec61-mediated insertion into the ER membrane (*19*). On the other hand, in the ER system, a stretch of 3 leucines and 16 alanines is required for 50%

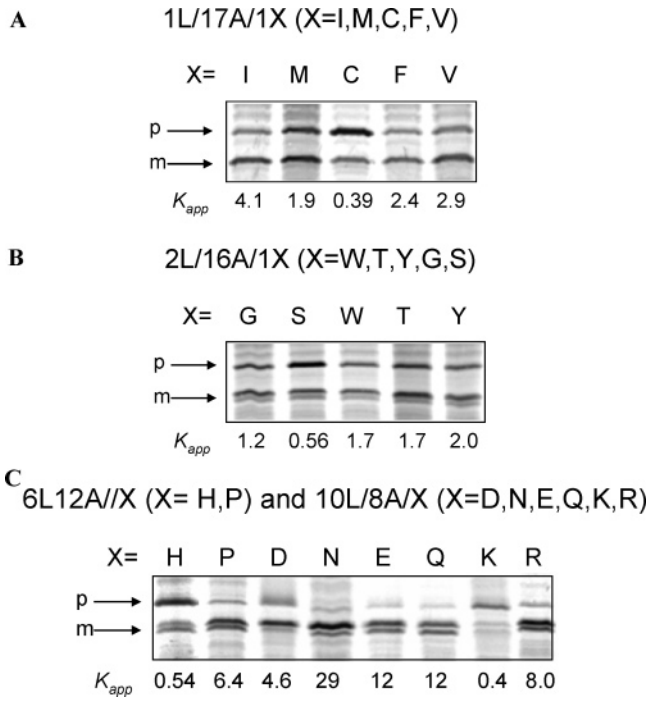

FIGURE 3: Membrane insertion of PCLep with *n*L/(18 − *n*)A/1X TMH segments. Amino acids are separated into three groups according to their predicted hydrophobicity. ΔG^{aa}_{app} values were calculated from the residue-replacement data by comparison to the starting construct. For instance, ΔG^{Met}_{app} was calculated from the 2L/17A and 1L/17A/1M constructs as: $\Delta G^{Met}_{app} = \Delta G^{Leu}_{app} + (\Delta G_{app}$[1L/17A/1M] − ΔG_{app}[2L/17A]). A. TMH segments with the composition 1L/17A/1X (AAAAAAAALXAAAAAAAAA) were used for the hydrophobic amino acids. B. TMH segments with the composition 2L/16A/X (AAAAAAAALXLAAAAAAAA) were used for the polar residue group. C. TMH segments with the composition 6L/12A/X (AAAAAALLLXLLLAAAAAA) and 10L/8A/1X (AAAALLLLLXLLLLAAAA) were used for strongly polar and charged residues.

membrane insertion (ΔG_{app} = 0 kcal/mol) (*19*) whereas for PCLep a stretch of 19 alanines is sufficient for this level of insertion.

To measure the contribution (ΔG^{aa}_{app}) of each of the 20 amino acids to ΔG_{app}, we made the 1L/17A/1X (Figure 3A), 2L/16A/1X (Figure 3B), 6L/12A/X, and 10L/8A/1X series of constructs (Figure 3C). Our results indicate that membrane insertion is promoted by the hydrophobic residues isoleucine, leucine, valine, phenylalanine, and methionine (Figure 4A). Alanine is close to neutral in terms of promoting insertion, whereas the polar residues glycine, serine, threonine, cysteine, tryptophan and tyrosine inhibit membrane insertion to various degrees. Proline, asparagine, glutamine, and all charged residues are strongly inhibitory to membrane insertion.

Figure 4C shows a plot of ΔG^{aa}_{app} values for each of the 20 amino acids measured for the PCLep TMH in *E. coli* vs ΔG^{aa}_{app} values measured for a ST sequence in the mammalian ER (*19*). The two sets of values correlate well (R^2 = 0.93), although the absolute difference between the ΔG^{aa}_{app} values for the most hydrophobic and most hydrophilic residues is somewhat larger for the PCLep TMH than for the ST sequence.

As a control, we used a well-established protease-accessibility assay (*35*) to examine the membrane-anchoring ability

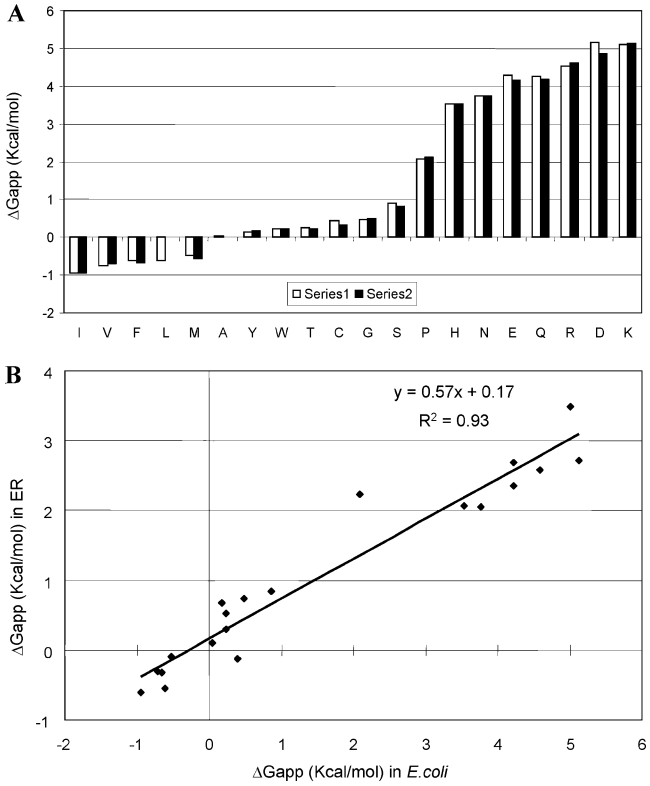

FIGURE 4: ΔG^{aa}_{app} values determined using PCLep constructs. A. ΔG^{Leu}_{app} and ΔG^{Ala}_{app} values were derived from the regression line in Figure 2B; all other values were calculated from the data in Figure 3 (white and black bars show two independent experiments). B. ΔG^{aa}_{app} values determined using PCLep plotted against ΔG^{aa}_{app} values measured in mammalian rough microsomes (*19*).

of different TMH sequences. If the TMH anchors the mature CLep protein in the membrane, the protease will digest the protein into a shorter protected fragment. In contrast, if the mature CLep protein is fully translocated into the periplasm, addition of protease will digest it completely. Cells expressing PCLep with the designed TMH sequences were pulse labeled for 30 s, converted to spheroplasts, and then analyzed by the protease protection assay. As shown in Figure 5A, proteinase K treatment produces resistant fragments in all the tested mutants, including the 19A TMH, consistent with the signal peptide processing results. In addition, the constructs are YidC dependent for membrane insertion, including the 19A construct as depletion of YidC blocks processing of PCLep to the mature protein (Figure 5B, Glc lanes).

A Sec-Dependent PCLep Construct. We also tested whether the PCLep constructs require the SecYEG-targeting factor SecA for insertion, since previous studies have shown that some mutations can render the protein SecA dependent (*34*). To examine SecA dependence, we expressed PCLep constructs and then treated the cells with 3 mM sodium azide (*36*). After 5 min the cultures were pulsed with [^{35}S]-methionine for 30 s. Figure 5C shows that treatment with azide has only a small effect on signal peptide processing of the PCLep constructs (see Figure 5C, upper panel). In contrast, the addition of azide has a dramatic effect on signal peptide processing of the SecA-dependent OmpA protein (see Figure 5C, lower panel). These results demonstrate that the

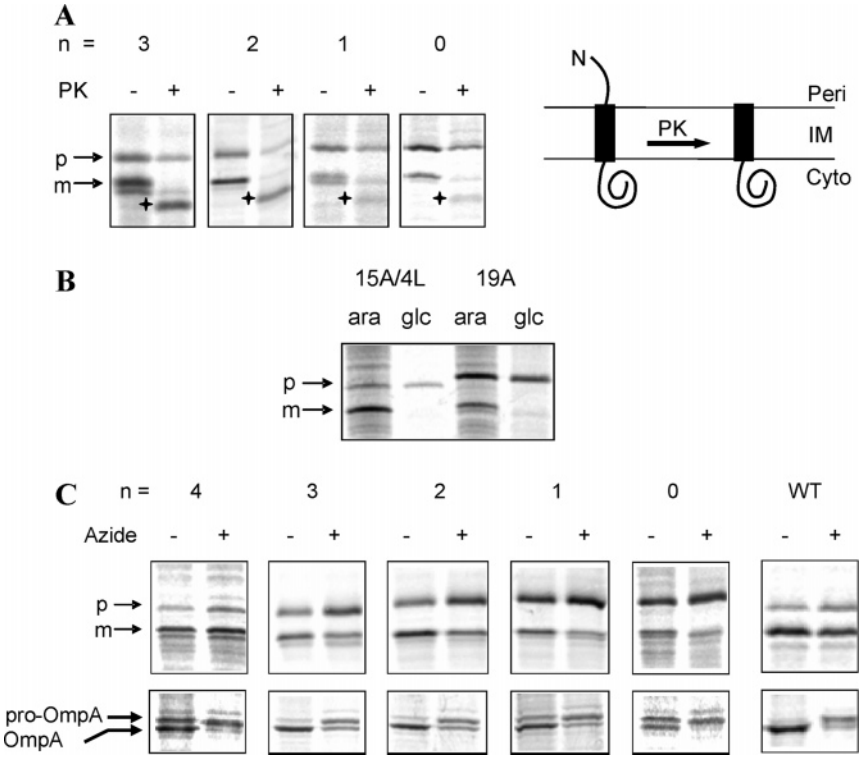

FIGURE 5: Membrane anchoring of PCLep TMH segments and YidC/SecA-dependence of membrane insertion of PCLep constructs. A. Proteinase K (PK) treatment of spheroplasts for PCLep constructs with TMH segments of the composition *n*L/(19 − *n*)A (*n* = 0−3) (see Experimental Procedures). The formation of a protease-protected fragment (+) shows anchoring of the mature CLep in the membrane. B. YidC-dependence of membrane insertion. JS 7131 cells expressing the PCLep(4L/15A) and PCLep(19A) constructs were grown overnight in LB with arabinose (ara lanes) and then diluted 1:50 to LB with arabinose or glucose (glc lanes) to deplete YidC. Cells were grown to $A_{600} \sim 0.6$ (arabinose) and to $A_{600} \sim 0.4$ (glucose), transferred to M9 medium, and grown for an additional 30 min before pulse-labeling, immunoprecipitation, and SDS-PAGE C. SecA-dependence of membrane insertion. MC1060 cells expressing the indicated PCLep constructs were treated with sodium azide. After induction with 1 mM IPTG for 5 min, sodium azide was added to 3 mM final concentration, cells were incubated for 5 min, radiolabeled, and processed for immunoprecipitation and SDS-PAGE. Data for PCLep are shown in the upper panel and for the SecA-dependent protein OmpA in the lower panel.

PCLep constructs insert into the membrane by a SecA-independent route.

Previously, it was shown that a deletion of most of the PCLep TMH and its C-terminal flanking positively charged residues results in the protein becoming Sec dependent for insertion (*37*). We reasoned that this happens because this particular PCLep mutant mimics an exported protein. Like exported proteins, it is synthesized with a cleavable SP and lacks a membrane-anchoring TMH. Since the 19A TMH was still hydrophobic enough to promote some degree of membrane anchoring, we also made a PCLep construct with a more polar 19S TMH sequence. The 19S PCLep was translocated across the membrane, as a processed band was observed (Figure 6A, −PK lane). Insertion was SecA-dependent since the addition of azide completely abolished signal peptide processing (Figure 6B). Protease-accessibility studies showed that the 19S segment is not anchored in the membrane (Figure 6A, +PK lane); the addition of proteinase K completely digested the mature protein and did not give rise to a protected band.

Analysis of the Reverse Signal-Anchor Sequence in Pf3 Coat. To verify the generality of the results obtained with PCLep but using a protein that does not insert as a helical hairpin, we repeated some of the experiments above using the rSA sequence of the Pf3 coat protein. Like M13 procoat, Pf3 coat inserts by the YidC pathway (*22*) and spans the membrane once with its amino-terminus in the periplasm (Figure 7A). Pf3 coat, however, lacks a SP. We fused the

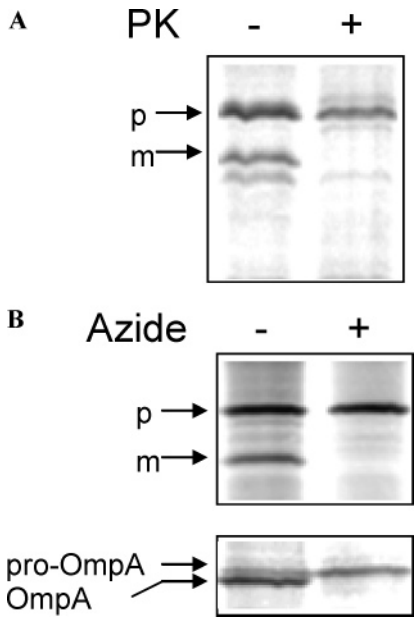

FIGURE 6: The insertion pathway of PCLep depends on the overall hydrophobicity of the TMH segment. A. PCLep(19S) was analyzed for membrane insertion using the proteinase K assay (see Figure 5). Note the weak, proteinase K-resistant background band. This band is slightly smaller than mature CLep and also appears in some of the previous figures. B. SecA dependence of membrane insertion of PCLep(19S) (upper panel) and OmpA (lower panel). MC1060 cells expressing PCLep(19S) was analyzed for SP processing as described in Figure 5C.

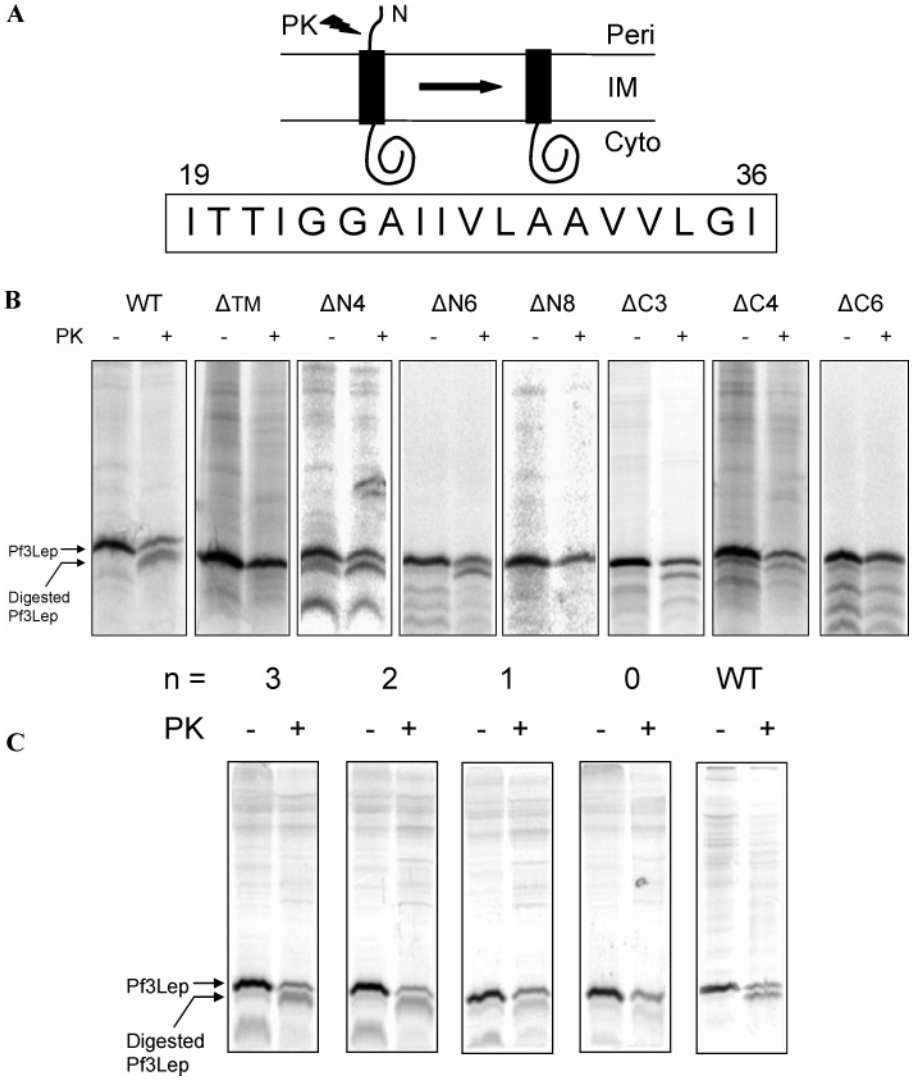

FIGURE 7: Membrane insertion of Pf3Lep with deletions in the rSA sequence and with designed rSA sequences. A. Membrane topology of Pf3Lep. The rSA sequence is shown. B. Membrane insertion of Pf3Lep constructs with deletions in the rSA sequence assayed by proteinase K resistance. Cells expressing Pf3Lep were radiolabeled, and membrane integration was analyzed by proteinase K assay as described in Figure 5A. C. Pf3Lep with designed rSA sequences of the composition *n*L/(19 − *n*)A (*n* = 0−3). Cells expressing Pf3Lep were radiolabeled and membrane integration was analyzed by the proteinase K assay.

Lep P2 domain to the carboxy-terminus of the Pf3 coat, allowing us to monitor the membrane anchoring of the protein.

The rSA of Pf3 coat consists of 18 residues. A deletion analysis was used to examine the minimum length of the rSA sequence that is required for insertion (Figure 7B). Cells expressing Pf3Lep were pulse labeled with [^{35}S]-methionine for 30 s and then subjected to the protease accessibility assay. The addition of proteinase K converts Pf3Lep to a shifted protected band; this conversion is not observed with Pf3Lep ΔTM, lacking the rSA. Pf3Lep with an amino-terminal deletion in the rSA of 4 and 6 residues, but not 8, inserts efficiently across the membrane. Membrane insertion can occur with a carboxyl-terminal deletion of 3 residues, but is inhibited by larger deletions. Thus, a hydrophobic stretch of 12 residues is sufficient for membrane insertion of Pf3Lep.

We next analyzed the same designed *n*L/(19 − *n*)A rSA sequences with Pf3Lep that we used with PCLep. Two to three leucines in the 19 residue segment were found to be sufficient for membrane insertion (Figure 7C). Although a precise quantitation is not possible in this case, the results nevertheless are very similar to what we observed with PCLep.

Analysis of Stop-Transfer Segments in Sec-Dependent Leader Peptidase. To complete the study, we also compared the sequence requirements for membrane insertion of the TMH in PCLep and the rSA sequence in Pf3Lep to those required for membrane insertion of a ST sequence in the Sec-dependent Lep protein. The latter has been extensively analyzed previously using *in vitro* translation of Lep constructs in the presence of dog pancreas rough microsomes (*19*, *38*). As a test of ST sequence recognition by the *E. coli* SecYEG translocase, we expressed Lep constructs carrying engineered ST sequences of the general design GGPG-[*n*L/(19 − *n*)A]-GPGG, Figure 8A. In order to measure the degree of membrane insertion of the ST sequence, we fused alkaline phosphatase (PhoA) to the C terminus of Lep; as PhoA is active only when localized in the periplasm (*39*), the PhoA activity measured on whole cells is a good indicator of whether the ST sequence is inserted into the inner membrane or translocated into the periplasm.

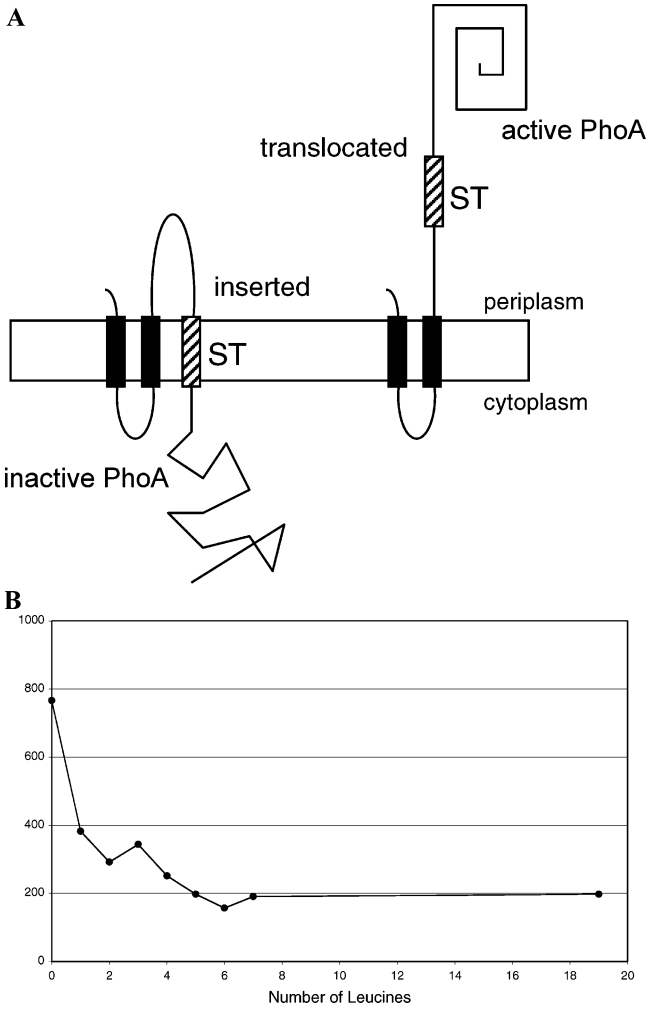

FIGURE 8: Membrane insertion of designed ST sequences in the Sec-dependent protein Lep. A. ST sequences (hatched) of different composition were engineered into the model protein *E. coli* Lep as shown (*19*). The degree of membrane integration of the ST sequence was assessed by measuring the enzymatic activity of the C-terminal PhoA fusion partner. B. PhoA activities measured for Lep constructs with ST sequences of the overall composition GGPG-[*n*L,(19 − *n*)A]-GPGG.

Results for $n = 0-7$ and 19 are shown in Figure 8B. Although we do not know if the correlation between PhoA activity and the degree of membrane integration of the stop-transfer segment is strictly linear, it is nevertheless clear that the 19A segment is inserted poorly, while segments with $n > 3$ are close to 100% inserted into the membrane. This is similar to the results obtained with PCLep and Pf3Lep discussed above.

DISCUSSION

All membrane proteins, whether from prokaryotic or eukaryotic cells, contain topogenic sequences that determine their membrane topology (*1*). In this work, we have used the YidC-dependent PCLep and Pf3Lep proteins as model proteins to study the sequence features that promote membrane insertion and lateral release of a transmembrane segment from the YidC translocase into the lipid phase. PCLep and Pf3Lep absolutely require YidC for membrane insertion (*22*, *24*). PCLep, unlike Pf3 Lep, is synthesized as a precursor form containing a cleavable SP that is proteolytically removed during membrane insertion.

We find that the length of the transmembrane segment in both model proteins can be shortened dramatically without significantly affecting insertion and membrane anchoring (Figure 1C; Figure 7B). The minimal PCLep TMH was found to be 11 residues long, while in Pf3Lep a 12-residue rSA sequence was needed. The minimal PCLep TMH contains some polar residues but is quite hydrophobic with 4 valines and 3 isoleucines. The minimal Pf3Lep rSA is even more hydrophobic and contains 3 valines, 3 isoleucines, and 2 leucines (Figure 7B).

To determine the contribution of individual amino acids to the overall membrane insertion efficiency of a transmembrane segment, we substituted artificially designed peptide segments for the authentic PCLep TMH (Figure 3). We find that a 3L/16A segment promotes efficient membrane insertion (Figure 2). A 19A segment is close to the insertion threshold, whereas a 19S segment does not support membrane insertion (Figure 6A). Similar results were obtained for the Pf3Lep rSA (Figure 7).

The contribution to the apparent free energy of membrane insertion (ΔG^{aa}_{app}) of all 20 amino acid was measured using the PCLep TMH. We adapted an approach recently described by von Heijne and co-workers (*19*). ΔG_{app} for different PCLep TMH segments was determined from the insertion ratio ($K_{app} = f_p/f_u$, where f_p is the fraction of signal-peptidase processed protein and f_u the fraction of uncleaved PCLep) and the relation $\Delta G_{app} = -RT \ln K_{app}$. ΔG^{aa}_{app} values were then obtained from a series of designed TMH segments as described in the legend to Figure 3. Our analysis shows that hydrophobic amino acids promote membrane insertion, polar residues are weakly inhibitory, and charged residues strongly inhibit membrane insertion, Figure 4A. These results compare well with earlier results reported for Sec61-mediated membrane insertion of a ST sequence into the mammalian ER (*19*): the relative order of the ΔG_{app} values for the different amino acids is similar in the bacterial and mammalian systems although the absolute difference between the ΔG_{app} contributions for the most hydrophobic and most hydrophilic residues is somewhat larger in the former, as shown in Figure 4B.

As a final comparison, we also tested the sequence-composition dependence of ST function in a SecYEG-dependent, Lep-based construct previously used for comparable studies in the mammalian ER membrane (*19*). In this case, the topology reporter PhoA (*39*) was used to measure the efficiency of membrane integration, Figure 8. As for PCLep and Pf3Lep, the 19A ST sequence was poorly inserted, while 19-residue Leu-Ala ST sequences with four or more Leu residues were quantitatively inserted into the inner membrane.

It has been reported that a 21-residue stretch composed of only alanines or 18 alanines and 3 leucines does not function as a ST sequence when inserted into the SecYEG-dependent protein PhoA, whereas a 21 amino acid stretch containing 5 leucines and 16 alanines does (*40*). It thus appears that the sequence context can have some influence on the results. In the Lep constructs studied here, the ST sequence is 'insulated' by flanking GGPG...GPGG stretches designed to minimize the effects of the surrounding sequence.

Finally, we have studied the sequence determinants that control whether a protein is targeted to the YidC or the Sec-pathway. We find that substituting the TMH of PCLep with a 19S segment results in full translocation into the periplasm via the Sec pathway, in contrast to the YidC-dependent membrane insertion of PCLep constructs with hydrophobic TMH segments (Figure 6). Apparently, the hydrophobicity of the mature part of the protein determines the choice of insertion pathway.

Taken together, the results reported here define the sequence characteristics required for lateral release of a hydrophobic transmembrane segment from the YidC translocase. Irrespective of model protein (PCLep, Pf3Lep, or Lep-ST) and type of membrane-insertion signal (SP+TMH, rSA, or ST), the results are similar, and are also similar to results previously obtained in a mammalian system (*19*). It therefore appears that the underlying physical chemistry of membrane-protein assembly may be similar across species. Future studies will be directed toward elucidation of the mechanisms by which different translocases are able to decode the topogenic information in the nascent polypeptide.

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