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Translocation of N-terminal tails across the plasma membrane

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Previously we have shown that the first hydrophobic domain of leader peptidase (lep) can function to translocate a short N-terminal 18 residue antigenic peptide from the phage Pf3 coat protein across the plasma membrane of *Escherichia coli*. We have now examined the mechanism of insertion of N-terminal periplasmic tails and have defined the features needed to translocate these regions. We find that short tails of up to 38 residues are efficiently translocated in a SecA- and SecY-independent manner while longer tails are very poorly inserted. Efficient translocation of a 138 residue tail is restored and is Sec-dependent by the addition of a leader sequence to the N-terminus of the protein. We also find that while there is no amphiphilic helix requirement for N-terminal translocation, there is a charge requirement that is needed within the tail; an arginine and lysine residue can inhibit or completely block translocation when introduced into the tail region. Intriguingly, the membrane potential is required for insertion of a 38 residue tail but not for a 23 residue tail.

Key words: membrane assembly/membrane protein/protein secretion

Introduction

The biogenesis of integral transmembrane proteins is a complex problem. The mechanism must account for the membrane topology of the protein and explain how hydrophilic regions of the protein get across the apolar phase of the membrane bilayer. Recently the rules that determine the transmembrane topology have been determined (Boyd and Beckwith, 1989; Laws and Dalbey, 1989; von Heijne, 1989; Nilsson and von Heijne, 1990; McGovern *et al.*, 1991). The 'positive inside rule' states that basic residues flanking a transmembrane segment cause retention of that region in the cytoplasm and therefore can control the orientation of the hydrophobic regions. This rule is well-established in bacteria [for reviews see Boyd and Beckwith (1990) and Dalbey (1990)] and appears to apply to eukaryotes (Hartmann *et al.*, 1989) as well. The mechanism by which hydrophilic regions C-terminal to a transmembrane segment get across the membrane has also been investigated. It is found that cleavable leader peptides (Emr *et al.*, 1978; Carlson and Botstein, 1982; Michaelis and Beckwith, 1982) or uncleaved signals (Boyd *et al.*, 1987; Dalbey *et al.*, 1987) can function to initiate translocation of polar domains. While translocation of long C-terminal domains requires the SecA/SecY/SecE machinery, translocation of short domains does not (Kuhn, 1988; Kuhn *et al.*, 1990; Andersson and von Heijne, 1993).

One of the major unresolved questions in membrane assembly is the mechanism of insertion of N-terminal tails across the membrane. Leader peptidase (lep), which has a periplasmic N-terminus (Lee *et al.*, 1992; Whitley *et al.*, 1993), has been used to resolve this question. It has two transmembrane segments, H1 and H2, oriented as shown in Figure 1A (Moore and Miura, 1987). H1 is followed by a polar cytoplasmic loop, P1, and H2 is followed by a large periplasmic C-terminal domain. To study how the N-terminus crosses the membrane, we have used lep in which an 18 residue N-terminal tail of Pf3 bacteriophage coat protein has been inserted at the N-terminus of the protein. We found that neither the SecA or SecY proteins nor the transmembrane electrical potential was required for insertion of the N-terminus, while the transfer of the C-terminal domain of lep had these requirements (Lee *et al.*, 1992).

In this paper, we have used the Pf3−lep hybrid protein as a model system to determine how membrane proteins without leader (or signal) sequences insert their N-terminal tails across the membrane. We find that the insertion of these tails occurs independently of the Sec machinery and does not require an amphiphilic helix such as is found in mitochondrial targeting sequences. Translocation of the N-terminal tail regions is efficient when they are short and when positively charged residues are not present.

Results

Lep has been shown to require the SecA/SecY proteins (Wolfe *et al.*, 1985) as well as the membrane electrochemical potential (Wolfe and Wickner, 1984) for translocation of its C-terminal P2 domain. H2 is the uncleaved signal that initiates translocation of P2 (Dalbey *et al.*, 1987). In most cases, H1 is dispensable for translocation of the P2 domain (Dalbey and Wickner, 1987), suggesting that it may play a passive role in membrane assembly. Recently, we have shown that the 18 residue N-terminal extension Pf3 peptide of Pf3−lep translocates across the membrane in a Sec-independent manner. In this paper, we have identified the features (length, charge or an amphiphilic helix) that are needed in the N-terminal tails for translocation across the membrane.

Length requirement for N-terminal translocation

We have used Pf3−lep to test whether N-terminal tails of 38, 48, 58, 88 and 138 residues can be translocated. We initially made three insertions with 20, 30 and 40 residues introduced between the codons of amino acids 18 and 19 of Pf3−lep. The inserted peptides (see Materials and

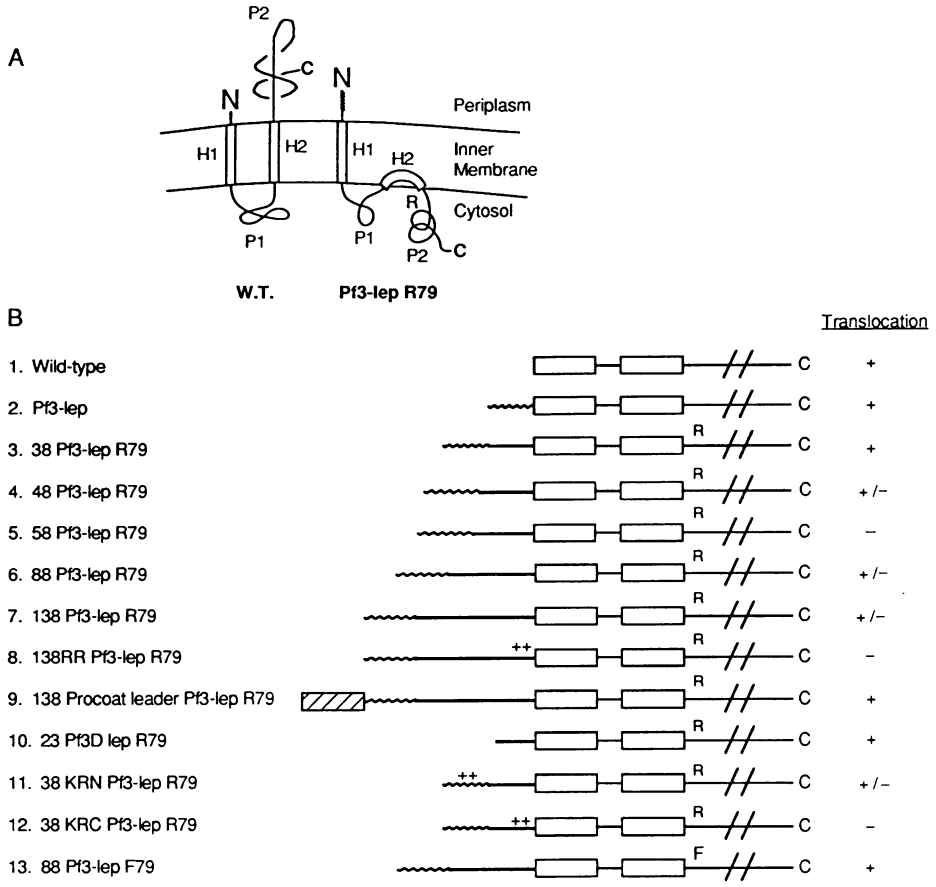

Fig. 1. Mutants used to analyze N-terminal translocation. (**A**) Membrane topology of lep in the plasma membrane. Apolar segments are represented by rectangles and polar segments are depicted by lines. (**B**) Mutants of lep and their membrane assembly characteristics. In these studies, the parent lep construct was Pf3−lep which has the Pf3 epitope at the N-terminus of lep (Lee *et al.*, 1992). Mutants are named by the length of the N-terminal domain that precedes apolar domain (H1) of lep and whether they contain an arginine or phenylalanine at position 79 in the respective wild-type lep sequence. Where indicated, the name also includes additional information; the RR in construct 8 refers to two arginine residues introduced before H1, the procoat leader in construction 9 indicates that the procoat leader sequence is present at the N-terminus, the D in construction 10 indicates a deletion of residues 4–18 of the Pf3 bacteriophage coat sequence; the KRN or KRC in construction 11 and 12 indicate that the lysine (K) and arginine (R) residues are present at the N- or C-terminus of the tail region, respectively

methods for sequences) comprised neutral residues that have a reasonable mixture of polar and non-polar side chains (Andersson and von Heijne, 1993). For the 88 residue tail, 50 randomly selected amino acids with neutral side chains (Andersson and von Heijne, 1993) were inserted (after residue 38) immediately before the H1 of the 38Pf3−lep fusion protein (see Materials and methods). A similar approach was used to make the 138 residue tail where 50 amino acid residues were inserted into the 88 residue tail (of 88Pf3−lep). In all these constructs, Sec-dependent translocation of the large C-terminal domain was prevented by inserting arginine at position 79 (Zhu and Dalbey, 1989). This simplifies the protease mapping studies since the only domain that can insert is the N-terminal tail (see Pf3−lep R79 membrane topology cartoon in Figure 1A).

To monitor N-terminal translocation, we used the well-established protease mapping procedure (Lee *et al.*, 1992). Cells expressing these proteins were pulse-labeled with Trans ^{35}S label for 1 min, then converted into spheroplasts and treated with or without active proteinase K. A portion of the cells was lysed with Triton X-100 prior to digestion with protease. Aliquots were immunoprecipitated with antiserum to lep, outer membrane protein A (OmpA) and ribulokinase (AraB). OmpA, a positive control, was used to assay the efficiency of spheroplast formation. AraB, a cytosolic protein, was used as a negative control to monitor the integrity of spheroplasts.

Figure 2A shows that a 38 residue N-terminal tail is efficiently translocated across the membrane. Quantification of the bands showed that 92% of 38Pf3−lep R79 is digested by proteinase K and 55% was converted into a slightly shorter form, indicated by the arrow. This protected fragment, lacking the tail region, runs at the same position as the wild-type lep (data not shown). In this study, the mature OmpA was almost completely digested, indicating that most of the cells were converted to spheroplasts. In our gel system, OmpA runs in two forms, heat modified and unmodified (Schnaitman, 1974; Hindennach and Henning, 1975). AraB was not digested by the external protease, which demonstrates that there was no detectable lysis. In contrast, the N-terminal tail of 48Pf3−lep R79 is inefficiently translocated. The majority of this protein is resistant to protease and only a small fraction of the shifted band was produced (Figure 2B). A further increase in the size of the tail to 58 residues prevented translocation entirely (Figure 2C); 58Pf3−lep R79 is completely inaccessible to proteinase K. We next investigated whether

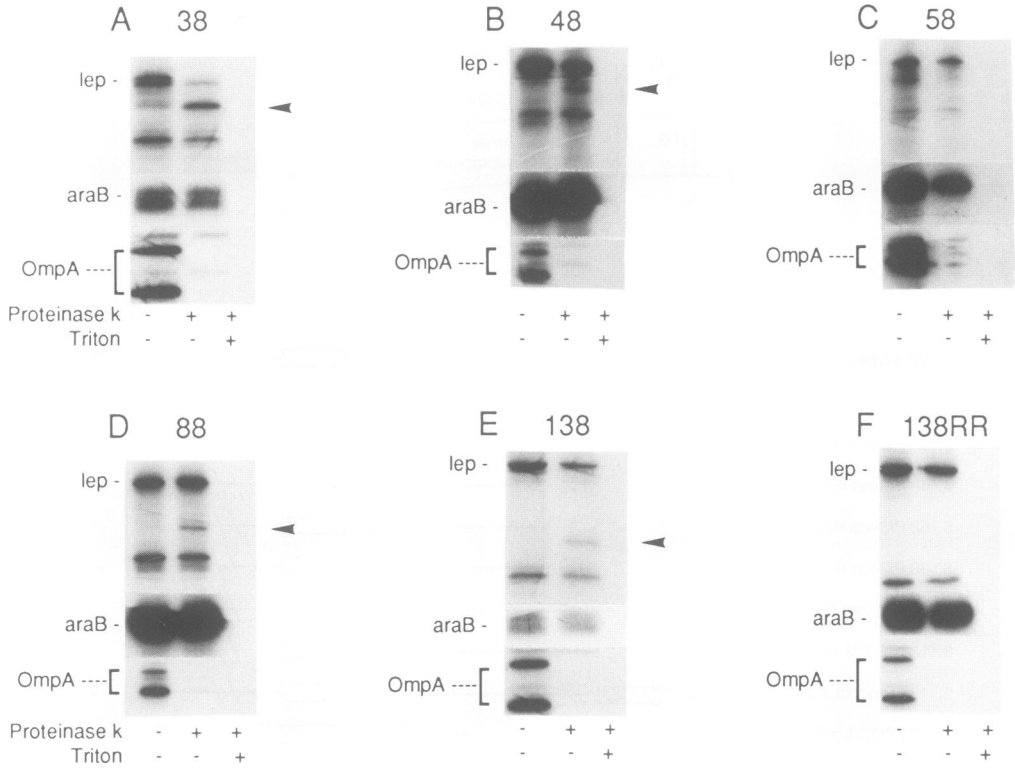

Fig. 2. Protease mapping to monitor the translocation of the N-terminal tails of the various Pf3−lep constructs. Exponential growing cells of MC1061 with plasmids expressing Pf3−lep with a 38 (**A**), 48 (**B**), 58 (**C**), 88 (**D**), and 138 (**E** and **F**) N-terminal tail were pulse-labeled with 100 μCi of Trans ^{35}S label for 1 min and then converted into spheroplasts as described in the Materials and methods. Aliquots (150 μl) were incubated on ice for 60 min with 15 μl of 10 mg/ml proteinase K or with inactive proteinase K (5 μl of 0.1 M PMSF and 15 μl of 10 mg/ml proteinase K) or with 15 μl of proteinase K and 15 μl of Triton X-100. Active proteinase K was then inactivated with 5 mM PMSF. The samples were then acid precipitated and then immunoprecipitated with antisera to lep (lep), OmpA and ribulokinase (araB). In F, the 138 amino acid tail was within a protein that had two arginine residues immediately before H1 of Pf3−lep.

increasing the size of the tail would restore translocation since von Heijne and colleagues have shown that while intermediate lengths of C-terminal domains are poorly translocated, longer ones are efficiently translocated (Andersson and von Heijne, 1993); these longer loops allow the protein to use the Sec machinery. However, most of 88Pf3−lep R79, which has an 88 residue tail (Figure 2D), was resistant to protease, indicating translocation of the N-terminus was severely inhibited; only a small quantity of the shifted band was detected in the proteinase K lane (see arrow). This was also observed for the 138Pf3−lep R79, with a 138 residue tail (Figure 2E). Quantification of the bands revealed that 50% of lep with a 138 residue tail was digested by proteinase K and 3.5% of the original radiolabeled lep was converted into the translocated product (see arrow). We believe that there is some genuine translocation of the long tails, albeit low, and that the shorter protected fragment (as indicated by the arrows) is not an artifact because we did not observe a protease-protected fragment when two arginines were inserted immediately before H1 to block translocation (Figure 2F).

Sec-independent insertion of N-terminal tails

We wanted to test whether there is a correlation between the size of the N-terminal tails and the requirement for the Sec apparatus. It has been previously shown that translocation of C-terminal hydrophilic domains require the SecA/SecY/SecE machinery if the segments exceed a certain length (Andersson and von Heijne, 1993). First, we examined whether translocation is SecA-dependent by treating the cells with sodium azide prior to pulse-labeling (Oliver *et al.*, 1990); azide has been shown to inhibit the SecA ATPase activity. Figure 3A shows that the N-terminal tail of 38Pf3−lep R79 is translocated, suggesting that SecA is not required for translocation of the tail. Under these experimental conditions, the translocation of OmpA is completely blocked, as indicated by the fact that the two forms of pro-OmpA that run on our gel system (Schnaitman, 1974; Hindennach and Henning, 1975) are protease resistant. Likewise, in azide-treated cells, 48 Pf3−lep R79, which has a 48 residue tail, translocates its N-terminus across the membrane to the same extent as observed with no azide treatment (compare Figures 3B and 2B). Translocation of the 138 residue tail occurs to the same extent in azide treated cells as it does in cells without azide (compare Figures 3C and 2E). As a second approach, we examined translocation in a temperature-sensitive (ts) host containing the SecA and SecY ts genes. Protease accessibility experiments were performed at the non-permissive temperature to assay for translocation. Figure 3D and E, shows that translocation of the 38 residue N-terminal tail can occur even when the function of the SecA and SecY is impaired; 38Pf3−lep R79 was digested by proteinase K and converted into the shorter fragment (see arrows in Figure 3D and E) under conditions where the translocation of pro-OmpA and its processing to OmpA was impaired.

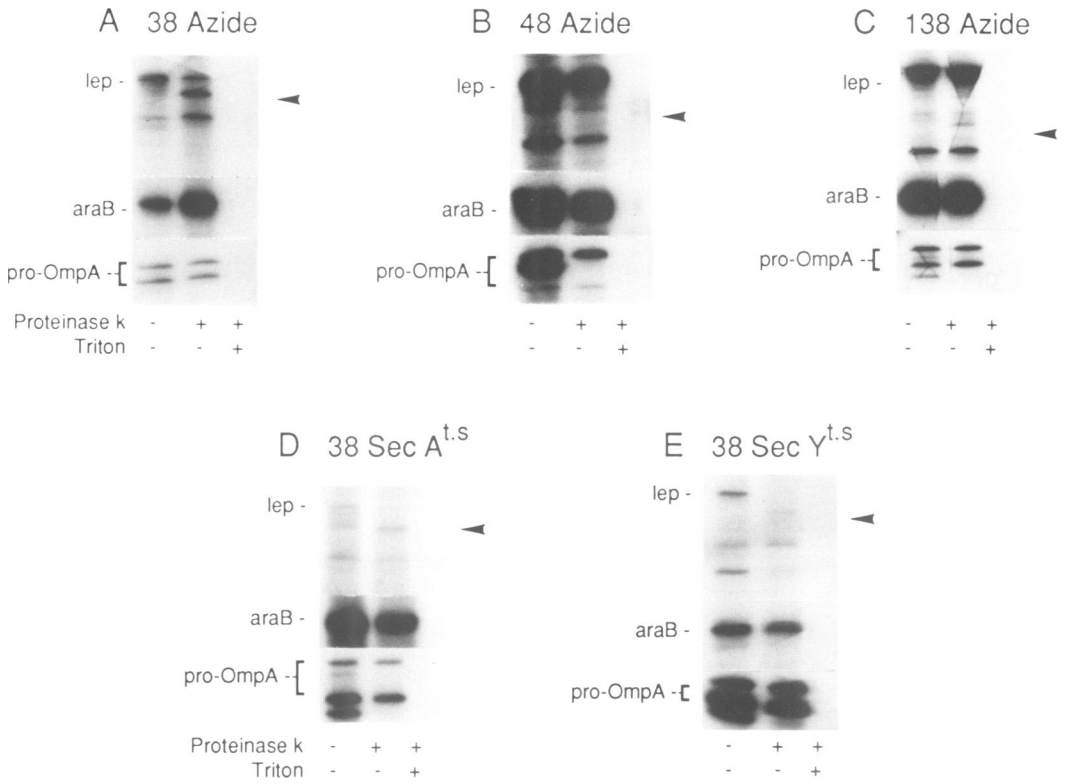

Fig. 3. Translocation of the N-terminal tails do not depend on the Sec proteins. (A–C) MC1061 cells bearing a plasmid encoding Pf3–lep with a 38 (**A**), 48 (**B**) and 138 (**C**) residue tail were grown to the mid-log phase. After 5 min of induction of plasmid encoded proteins by arabinose (0.2%, final concentration), cells were treated with 3 mM azide for 5 min, then labeled with 100 μCi of Trans ^{35}S label for 1 min. Aliquots were analyzed for protease mapping, as described in Figure 2. (**D**) *Escherichia coli* CJ105-bearing the plasmid expressing the 38 residue tail was grown to the mid-log phase at 30°C, shifted to 42°C and induced with 0.2% arabinose for 1 h, and then labeled with 100 μCi of ^{35}S label for 1 min. (**E**) *Escherichia coli* CJ107 bearing the plasmid expressing the 38 residue tail was grown and labeled as described in D. After pulse labeling, the cells were converted to spheroplasts and analyzed as described in Figure 2.

Translocation of the large N-terminal domain is restored by the addition of a leader sequence

While translocation of short tails occurs very efficiently, translocation of long domains is very poor. Thus, it was of interest to test whether the addition of a leader sequence could restore translocation of a long N-terminal domain. To accomplish this, the leader sequence of procoat was inserted at the N-terminus of the 138 construct just before the Pf3 sequence (see Materials and methods). Procoat leader 138Pf3–lep R79, which contains the leader sequence, translocates across the membrane (Figure 4A) and is digested by proteinase K; a protected fragment was generated (see arrow) corresponding to the size of the wild-type protein. Careful inspection of the gel reveals the protein runs as a doublet. The faster running species, which is accessible to proteinase K, corresponds to the protein which had translocated across the membrane and is cleaved by chromosomally encoded lep; the slower running species, which is resistant to proteinase K, corresponds to the cytosolic precursor form of the protein that is not cleaved by lep. These studies show that translocation of a large N-terminal domain can be very efficient when there is a leader sequence preceding it.

We next investigated whether Sec-dependent translocation occurs when a leader sequence is present by treating the cells with sodium azide prior to pulse labeling (Oliver *et al.*, 1990). As seen in Figure 4B, the precursor form of 138 procoat leader Pf3–lep R79 is observed, in addition to the mature form, when azide is added to the cells (compare +/– azide lanes). In addition, translocation is dependent on the membrane potential, as pretreatment with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which abolishes the membrane potential, leads to the accumulation of the precursor form.

Role of the amphiphilic helix

Having shown that short tails can translocate efficiently without the function of the Sec proteins, we next examined what features may be needed in the N-terminal tails to translocate them across the membrane. One can imagine that the N-terminal tail may need an amphiphilic helix that can promote its own translocation similar to the way the mitochondrial amphiphilic sequences function (Allison and Schatz, 1986). Indeed, residues 1–18 of the Pf3 peptide, which are located in the N-terminal tail of the Pf3–lep constructs, form an amphiphilic helix when plotted on a 'helical wheel' and have been proposed to play a 'positive role' in promoting the translocation of the N-terminus of the Pf3 coat protein (Saier *et al.*, 1989). Oligonucleotide-directed mutagenesis was used to delete the Pf3 sequence from the tail region. Cells synthesizing 23Pf3–lep R79, which lack the Pf3 amphiphilic helix, were pulse-labeled with Trans ^{35}S label for 1 min. As observed with lep with the 38 residue tail, proteinase K degraded the protein and converted it into a slightly smaller band. This indicates that the N-terminal tail can

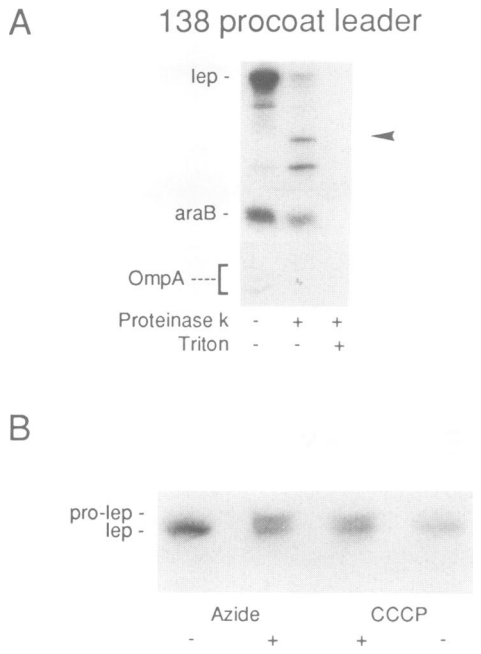

Fig. 4. The addition of a leader sequence restores efficient translocation of the 138 amino acid N-terminal tail. (**A**) *Escherichia coli* strain MC1061 containing the plasmid that encodes a hybrid protein comprising the procoat leader sequence joined to the 138Pf3−lep R79 (with a 138 residue tail) was analyzed for translocation of the N-terminal domain, as described in Figure 2. (**B**) Cells encoding the 138 procoat leader Pf3−lep R79 were grown to the mid-log phase and treated with or without azide, as described in Figure 3. The cells were then acid-precipitated, immunoprecipitated with antisera to lep and analyzed by SDS−PAGE and fluorography. For the membrane potential study, cells expressing 138 procoat leader Pf3−lep R79 were treated with or without 5 μl of 10 mM CCCP for 45 s and labeled with 100 μCi of Trans ^{35}S label for 1 min and analyzed as described for the azide study.

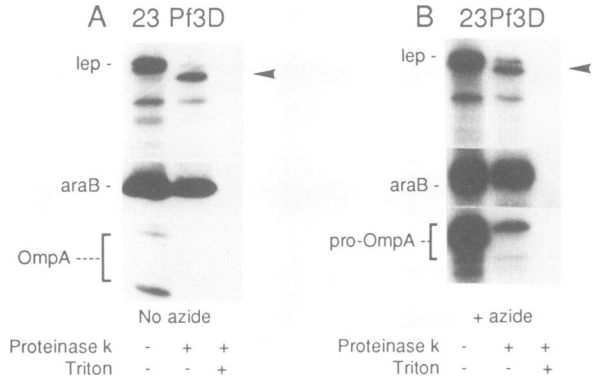

Fig. 5. The Pf3 epitope is not required for translocation of the N-terminal tail across the membrane. (**A**) *Escherichia coli* strain MC1061 expressing 23Pf3D−lep R79 was pulse-labeled for 1 min and analyzed for N-terminal translocation, as described in Figure 2. (**B**) In the azide study, MC1061 cells (1 ml) synthesizing 23Pf3D−lep was treated with 60 μl of 50 mM NaN_3 (3 mM, final concentration). After 5 min, cells were labeled with 100 μCi of Trans ^{35}S label for 1 min and then analyzed for translocation as described in Figure 2.

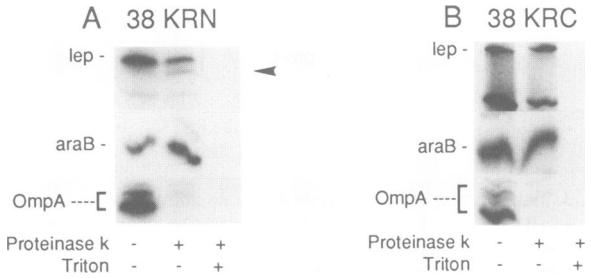

Fig. 6. Introduction of positively charged residues hinder or block N-terminal translocation. *Escherichia coli* strain MC1061 containing the plasmid that encodes for 38Pf3−lep R79 with a lysine and arginine residue introduced near the N-terminus (**A**) or the C-terminus of the tail (**B**) was analyzed for its N-terminal translocation.

translocate across the membrane in the absence of an amphiphilic helix. We also confirmed via helical wheel plots that, after the deletion was made, the newly generated sequence does not contain a good candidate for an amphiphilic sequence. We next examined whether translocation is Sec-dependent by treating cells with azide to inhibit the SecA ATPase. Even in the presence of azide, translocation of the 23 residue tail is very efficient. As a control, we also confirmed that the precursor of OmpA accumulates in these cells and is resistant to proteolysis (Figure 5B).

Positively charged residues impede or block translocation when introduced into the tail region

Because N-terminal tails usually have very few positively charged residues (Lewis *et al.*, 1990; Traxler *et al.*, 1993), we asked whether basic residues prevent translocation. To test this, we inserted two positively charged residues either near the N- or C-terminus of the tail region. Translocation of the 38KRNPf3−lep R79 can occur, although inefficiently (Figure 6A), even with positively charged residues located near the N-terminus. Translocation is completely blocked when two positively charged residues were introduced near the C-terminal end of the tail immediately before H1; 38KRCPf3−lep R79 was inaccessible to proteinase K in a 1 min pulse and no shifted band the size of the wild-type lep was detected; the lep band for the KRC mutant was at the same intensity as the KRN mutant in the no protease lane. These results reveal that positively charged residues hinder N-terminal translocation and the degree of inhibition depends on their relative position to the hydrophobic domain.

Role of the membrane potential in N-terminal translocation.

Previously we showed that the N-terminus of Pf3−lep inserts across the membrane in a membrane potential independent manner (Lee *et al.*, 1992). In that study, the tail was of approximately 18 residues in length. Here we tested whether protein derivatives with tails of different sizes can also insert across the membrane independent of the potential. Exponentially growing cells were pretreated with CCCP in order to collapse the membrane electrochemical potential, labeled with Trans ^{35}S label for 1 min, converted to spheroplasts and then treated with proteinase K for 60 min. Figure 7 shows that 23Pf3D−lep R79, which lacks the Pf3 peptide, is completely digested with protease and converted to a slightly smaller fragment (see arrow). This indicates that translocation of the 23 residue tail is very efficient in CCCP-treated cells. In this study, the potential had been dissipated because OmpA, which requires the membrane potential, accumulates in a protease-resistant precursor form (pro-OmpA). In contrast to the results with the 23 residue tail, translocation of the

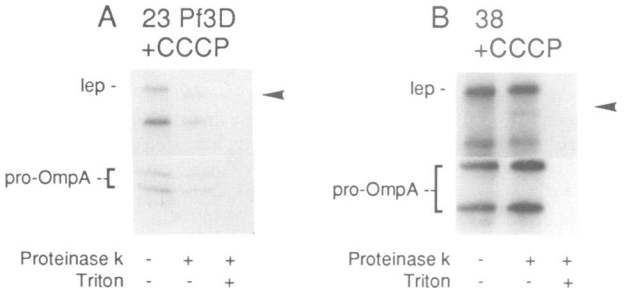

Fig. 7. The membrane potential is required for translocation of the 38 residue tail, but not the 23 residue tail. *Escherichia coli* MC1061 bearing a plasmid encoding 23DPf3−lep R79 with a 23 residue tail (**A**) or 38Pf3−lep R79 with a 38 residue tail (**B**) was grown to the mid-log phase and induced with 0.2% arabinose for 1 h at 37°C. The cells (1 ml) were then treated with 5 µl of 10 mM CCCP for 45 s and labeled with 100 µCi of Trans ^{35}S label for 1 min. Protease mapping was performed on spheroplasts as described in Figure 2.

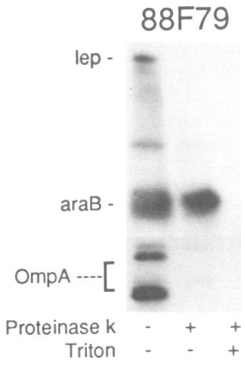

Fig. 8. Translocation of the P2 domain can occur even when translocation of the N-terminal domain is severely impaired. MC1061 expressing 88Pf3−lep F79 , which has a 88 residue tail and a functional signal, was pulse-labeled with Trans ^{35}S label for 1 min and analyzed for C-terminal translocation using protease mapping, as described in Figure 2.

38 residue tail is inhibited by CCCP and only a small amount of the translocated product is observed (see arrow).

Translocation of the P2 domain can occur when insertion of the N-terminus is blocked

Thus far in this paper, all the protein constructs have not been able to initiate C-terminal translocation because an arginine had been inserted after the internal signal at position 79; this renders H2, the uncleaved signal, non-functional (Zhu and Dalbey, 1989). To gain more insight into the mechanism of membrane insertion, we asked if the C-terminal domain of lep could translocate with a functional signal even when N-terminal translocation was severely inhibited. To do so, we substituted in the 88 residue tail lep construct the arginine at position 79 back to phenylalanine, which is found in the wild-type protein. Figure 8 shows that 88Pf3−lep F79 is accessible to added proteinase K indicating that the P2 domain translocates across the membrane. This shows that translocation of C-terminal domains does not require prior translocation of N-terminal domains.

Discussion

The major conclusion in this paper is that short N-terminal tails can be efficiently translocated across the membrane and the translocation does not require the Sec machinery. Interestingly, we do observe inefficient translocation of large domains and, to our surprise, this translocation is still Sec independent. Thus, it does not appear that the Sec machinery can engage N-terminal loops and help promote them across the membrane, indicating that there is specificity and that the Sec apparatus can only catalyze insertion in the N- to C-terminal direction, not in the C- to N-terminal direction. In bacteria, if one needs efficient translocation of a long N-terminal tail, then one needs to add a leader sequence to these proteins (Figure 4A) and then the proteins translocate across the membrane in a SecA-dependent manner (Figure 4B).

These results on the translocation of N-terminal tails contrast with those found for translocation of C-terminal domains where translocation of short C-terminal domains is Sec independent and translocation of very long domains occurs only with the help of the SecA and SecY proteins (Andersson and von Heijne, 1993). We believe that our results, which show only efficient translocation of short tails, may explain why N-terminal tails are often short in bacteria for membrane proteins made without a leader sequence. For example, the longest reported N-terminal tail is found in the pro W protein which is 100 residues long (Traxler *et al.*, 1993). The next longest known tail in *Escherichia coli* is the subunit A from the F_1F_0-ATP synthase, which is ~40 residues (Lewis *et al.*, 1990). In eukaryotes, though, one often finds very long translocated N-terminal tails in membrane proteins without leader peptides, but they are exceedingly rare in prokaryotes (Hennessey *et al.*, 1993).

What are the features within the N-terminal tail that allow it to translocate across the membrane without assistance of the Sec machinery? Examination of the tail sequences show that they usually have very few positively charged residues, implying that positively charged residues may hinder the translocation event. For instance, the 100 residue tail of proW and the 40 residue tail of subunit A of the F_1F_0-ATP synthase have only three and one positively charged residue(s), respectively. Indeed, we find that translocation is hindered or abolished altogether when positively charged residues are introduced into a protein with a 38 residue tail (Figure 6); the closer the charges are to the hydrophobic domain the more severe their effects are on translocation. It has also been proposed that N-terminal tails may require an amphiphilic helix which promotes translocation analogous to those sequences that direct nuclear-encoded proteins into mitochondria. Nevertheless, we find that translocation proceeds very efficiently even in the absence of the putative amphiphilic helix, ruling out that the N-terminal tails contain a novel 'translocation' sequence that promotes translocation.

Although the shorter N-terminal tails of 18 and 23 residues were translocated in the absence of the membrane potential (Figure 7; Lee *et al.*, 1992), the 38 residue tail was translocated only in the presence of the potential. It is therefore possible that the energy that is gained from inserting a hydrophobic domain out of the aqueous phase and into the apolar phase of the bilayer is sufficient for translocation of very short tails of membrane proteins. In the case of a longer N-terminal tail, this driving force is not adequate for translocation and the potential is required.

We observed that the C-terminal domain of lep can

translocate even when the N-terminal tail cannot (Figure 8). Similar findings have been reported for MalF that spans the membrane eight times. In the MalF protein, the second, third and fourth uncleaved signal sequences can initiate translocation even when the first signal sequence cannot (Ehrmann and Beckwith, 1991), showing that the N-terminus of the protein does not have to insert into the membrane in a specific orientation and is then passively followed by the remaining polar and apolar domains that sequentially insert, as suggested for eukaryotic membrane proteins (Hartmann *et al.*, 1989).

In summary, we have defined some of the features needed to translocate the N-terminal tails across the membrane and have shown that the insertion mechanism does not entail the Sec machinery. One of the major unanswered questions is whether the membrane insertion occurs spontaneously across the lipid bilayer or whether another proteinaceous machinery is utilized besides the Sec apparatus.

Materials and methods

Strains and plasmids

Escherichia coli strain MC1061 [Δ*lacX74*, araD139, Δ (*ara, leu*)*7697*, *galU*, *galk*, *hsr*, *hsm*, *strA*], JM103 [Δ(*lacpro*)*thi*, *strA*, *supE*, *endA*, *sbcB*, *hsdR*, *traD36*, *proB*, *lacIqZM15*] were from our laboratory. CJ105 (*secAts51*) and CJ107 (*secYts24*) have been described (Wolfe *et al.*, 1985). The pING-1 plasmid (Johnston *et al.*, 1985), which contains the arabinose promoter and the arabinose regulatory elements was obtained from Dr Gary Wilcox (Ingene, Inc.).

Materials

Restriction enzymes, T4 kinase and T4 DNA ligase were purchased from Bethesda Research Laboratories. PMSF was from Sigma. DNA polymerase I (Klenow fragment) and Proteinase K were from Boehringer Mannheim. Trans ^{35}S label, a mixture of 85% [^{35}S]methionine and 15% [^{35}S]cysteine, 1000 Ci/mmol, was from ICN K and K Laboratories.

DNA techniques

Oligonucleotide-directed mutagenesis was performed as described by Zoller and Smith (1983) with some modifications (Dalbey and Wickner, 1987). The template used in this study was M13mp8 containing the gene coding for Pf3−lep (Lee *et al.*, 1992) or various derivatives of it. After mutagenesis, the template was isolated from the phage of single plaques and the mutants were identified by DNA sequencing (Sanger *et al.*, 1977). All DNA preparations were performed as described in Maniatis *et al.* (1982). Transformation followed the calcium chloride method of Cohen *et al.* (1973). To construct proteins with 38, 48 and 58 residue tails, the spacer peptides were exactly as used by Andersson and von Heijne, (1993) and were inserted between residues 18 and 19 of Pf3−lep: TQVLNAPTSGGQSLNAPTSG (20 residues), TQVLNAPTSGTQVLNAPTSGGQSLNAPTSG (30 residues) and TQVLNAPTSGVTQLNASGTPTQVLNAPTSGGQSLNAPTSG (40 residues). The 88 residue tail protein derivative (88R) was constructed by engineering a unique restriction site into the gene encoding 38Pf3−lep R79 with a 38 residue tail as a template. First, a unique *Kpn*I site was introduced into the 38 residue tail immediately before the first transmembrane segment. Next, the gene that codes for 50 randomly selected amino acids (TQVLNAPTSGVTQLNASGTPNSQTLVVGG-ATQVLNAPTSGGQSLNAPTS) that contain a mixture of polar and apolar side chains was inserted at the *Kpn*I opened site. To accomplish this, two *Kpn*I sites were introduced into both the 5′ and 3′ ends of the DNA segment encoding these 50 amino acids (Andersson and von Heijne, 1993) and then, after digesting with *Kpn*I, the 150 nucleotide DNA fragment was cleaved from the vector, purified and inserted by T4 ligation into the *Kpn*I opened vector, which contains the gene that encodes the 38 residue tail. The correct orientation of the fragment was determined by DNA sequencing. In a similar fashion, the 138 N-terminal tail of 138Pf3−lep R was created. First, after destroying by oligonucleotide-directed mutagenesis one of the *Kpn*I sites on the M13mp8 vector that encode the 88Pf3−lep R79, the vector was opened up with *Kpn*I and used to ligate the purified 150 nucleotide DNA fragment to form the 138 residue construct. The 138 procoat leader Pf3−lep R79, which contains the procoat leader sequence, was created by inserting the procoat lep (MKKSLVLKASVAVATLVPMLSFA) at the N-terminus of 138Pf3−lep in two steps. In the first step, oligonucleotide-directed mutagenesis was used to insert the DNA that encodes MKKSLVLKASVA into M13 encoding 138Pf3−lep R79 with the 138 residue tail. In the second step, VATLVPMLSFA was inserted as well. The 23DPf3−lep R79 construct was made by performing oligonucleotide-directed deletion using the gene encoding the 38Pf3−lep as a template. The 15 amino acids VITDVTGQLTAVQAD that were deleted correspond to residues 4–18 of the Pf3 sequence.

Protease mapping studies

One millilitre cultures of cells was grown at 37°C to the early log phase in M9 minimal medium (Miller, 1972) and was supplemented with 0.5% fructose and 50 μg/ml of each amino acid (except methionine). At an A_{600} of ~0.2, the plasmid-encoded proteins were expressed by 0.2% arabinose. The cells were then labeled with Trans ^{35}S label for 1 min, chilled on ice, then pelleted by centrifugation. After resuspending in 0.25 ml of ice-cold buffer A (0.1 M Tris−acetate, pH 8.2, 0.5 M sucrose and 0.5 M EDTA), the cells were treated with lysozyme (80 μg/ml, final concentration) and 0.25 ml of cold water. After a 5 min incubation, 150 μl of 0.2 $MgSO_4$ was added to stabilize the spheroplasts and then they were pelleted by centrifugation. The spheroplasts were resuspended in buffer B (50 mM Tris, 0.25 M Sucrose, 10 mM $MgSO_4$) and were then ready for the protease mapping studies. Proteinase K was quenched with PMSF and then the samples were analyzed by immunoprecipitation (Wolfe *et al.*, 1982), SDS−PAGE and fluorography (Ito *et al.*, 1980).

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