Sec-dependent membrane protein biogenesis: SecYEG, preprotein hydrophobicity and translocation kinetics control the stop-transfer function

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Preprotein translocase catalyzes membrane protein integration as well as complete translocation. Membrane proteins must interrupt their translocation and be laterally released from the translocase into the lipid bilayer. We have analyzed the translocation arrest and lateral release activities of *Escherichia coli* preprotein translocase with an *in vitro* reaction and the preprotein proOmpA carrying a synthetic stop-transfer sequence. Membrane protein integration is catalytic, occurs with kinetics similar to those of proOmpA itself and only requires the functions of SecYEG and SecA. Though a strongly hydrophobic segment will direct the protein to leave the translocase and enter the lipid bilayer, a protein with a segment of intermediate hydrophobicity partitions equally between the translocated and membrane-integrated states. Analysis of the effects of PMF, varied ATP concentrations or synthetic translocation arrest show that the stop-translocation efficiency of a mildly hydrophobic segment depends on the translocation kinetics. In contrast, the lateral partitioning from translocase to lipids depends solely on temperature and does not require SecA ATP hydrolysis or SecA membrane cycling. Thus translocation arrest is controlled by the SecYEG translocase activity while lateral release and membrane integration are directed by the hydrophobicity of the segment itself. Our results suggest that a greater hydrophobicity is required for efficient translocation arrest than for lateral release into the membrane.

Keywords: DFyajC/membrane protein biogenesis/preprotein translocase/SecEYg/stop-transfer sequence

Introduction

Proteins which are transported across the *Escherichia coli* plasma membrane are generally synthesized with a cleavable leader sequence at their N-terminus. The leader sequence guides preproteins to a multisubunit translocase at the membrane (Ito, 1996; Wickner and Leonard, 1996). Many integral membrane proteins are also synthesized with an N-terminal cleavable leader peptide and thus are targeted to the same translocation machinery. These transmembrane proteins are distinguished from the secretory proteins by additional hydrophobic segments termed 'stop-transfer' sequences (von Heijne, 1994, 1997).

A stop-transfer sequence carries the structural and functional information required for stable membrane integ-

ration. Deletion of this structure can convert an integral membrane protein to a secretory protein (Adams and Rose, 1985; Davis *et al.*, 1985). Conversely, a secretory protein can be converted to an integral membrane protein by the addition of a stop-transfer sequence (Davis and Model, 1985). Thus a stop-transfer sequence performs multiple functions: to halt translocation; to specify protein 'lateral' release from the translocase; and to allow stable integration into the lipid bilayer. The level of participation of the translocase in each of these distinct processes is still unknown.

Stop-transfer sequences generally consist of long stretches of hydrophobic amino acyl residues which provide thermodynamically stable contacts with the lipid fatty acyl phase. Theoretically, hydrophobic segments of at least 20 residues are sufficient to traverse a lipid bilayer (Engelman *et al.*, 1986) and most membrane-spanning proteins have hydrophobic segments of this size (Persson and Argos, 1994; von Heijne, 1994). However, artificial segments that are very short but highly hydrophobic can also function as membrane anchors (Kuroiwa *et al.*, 1991; Chen and Kendall, 1995). A recent systematic analysis of the stop-transfer activity of pseudo-random 18 amino acid segments showed a correlation between stop-transfer efficiency and overall hydrophobicity and a threshold value was defined for the stop-transfer function (A.Sääf, E.Wallin and G.von Heijne, submitted for publication). Whether this value defines the minimal hydrophobicity required for stop-translocation, to escape from the translocase or to anchor the protein into the membrane is unknown. A better understanding of these crucial mechanistic distinctions may require *in vitro* biochemical analysis. Indeed, the stop-transfer activity of sequences whose hydrophobicity is near the threshold is difficult to predict. Constraints other than the structure of the stop-transfer sequence, such as temperature, protein folding, lipid composition or the energetics of translocation may affect the partitioning of hydrophobic segments. Unusual transmembrane domains may therefore be overlooked because little is known of the behavior of marginally hydrophobic sequences.

We have used an *E.coli in vitro* translocation assay and artificial single spanning membrane proteins to assay stop-transfer efficiency as a function of hydrophobicity, translocation kinetics and translocase composition. This system has allowed us to dissociate the translocation arrest and lateral release functions of a stop-transfer sequence. For a segment of intermediate hydrophobicity, we find that the translocase activity itself controls the stop-transfer efficiency. Once translocation is arrested, only the hydrophobicity of the preprotein segment specifies lateral release from the translocase into the lipid bilayer.

Results

To analyze the requirements for translocation arrest and membrane integration of hydrophobic proteins, the codons for either five or ten randomly chosen hydrophobic amino acid residues were introduced into the gene encoding the *E. coli* outer membrane protein A (proOmpA), generating the putative membrane-spanning proteins termed MSI and MSII (Figure 1A). The insertions were made into a pre-existing hydrophobic sequence of four residues, and thus increased the size and overall hydrophobicity of this region (Figure 1A). Prediction analysis using the TopPred algorithm (Claros and von Heijne, 1994) with the Persson–Argos hydrophobicity scale (Persson and Argos, 1994) yields a hydrophobic index value of ~1.09 for the region containing the short hydrophobic segment of proOmpA, ~1.27 for the hydrophobic segment of MSI and ~1.44 for the hydrophobic segment of MSII. A systematic analysis of the stop-transfer activities of pseudo-random 18 amino acid segments, both *in vivo* in *E. coli* and in a eukaryotic *in vitro* system, gave a threshold value for stop-transfer activity of ~1.30 (A.Sääf, E.Wallin and G.von Heijne, submitted for publication).

A mildly hydrophobic segment can induce stable translocation arrest

The introduction of a hydrophobic segment into a normally exported protein such as proOmpA can affect cell viability. Indeed, growth inhibition occurred when eight, 12 or 16 hydrophobic amino acids were introduced into the mature domain of proOmpA (MacIntyre *et al.*, 1988). In our study, synthesis of MSII was particularly lethal and cellular lysis occurred soon after overproduction (data not shown). The reasons for this deleterious effect are unknown but may reflect the massive assembly of MSII into the cytoplasmic membrane. To circumvent cellular lysis, overproduction was performed in a *secY* mutant strain cultivated at non-permissive temperature as described in Materials and methods. After proOmpA, MSI and MSII were purified and solubilized in 8 M urea, then iodinated and their translocation tested in an *in vitro E. coli* translocation reaction (Chen and Tai, 1985) containing the purified chaperone SecB, the translocation ATPase SecA, inner membrane vesicles of *E. coli* (IMVs) and ATP (Figure 1B). As control for translocation arrest without release into the lipid phase, proOmpA carrying a unique cysteine at position 245 (Figure 1A) was conjugated *via* a DTT-cleavable cross-linker (SPDP) to bovine pancreatic trypsin inhibitor (BPTI). The large covalently folded structure of BPTI blocks translocation and thereby forms an arrested intermediate at the point of coupling (Schiebel *et al.*, 1991; Duong and Wickner, 1997b).

Both proOmpA (36 kDa) and its leader peptidase-matured form OmpA were fully translocated into the lumen of the vesicles and were inaccessible to protease (Figure 1B, lane 1). The translocation of pOA-BPTI was arrested near residue 245, and this arrested intermediate yielded a protease-protected fragment of 26 kDa (termed I₂₆, lane 4). Protein MSII and its matured form (lane 3) were also arrested in translocation at position I₂₆, corresponding to the position of the inserted hydrophobic segment, though (as shown below) these arrested species were no longer translocation intermediates. Protein MSI,

Fig. 1. Apolar segments anchor proOmpA in the membrane. (A) The extra hydrophobic amino acids introduced into proOmpA between position 235 and 236 are in bold characters. Note that the proOmpA used throughout this study bears a unique cysteine at position 245 (Duong and Wickner, 1997b). The hydrophobicity index of the region containing the apolar amino acids (boxed segment) is indicated and was calculated using the Persson–Argos hydrophobicity scale and the TopPred II algorithm (Claros and von Heijne, 1994) with the same parameters used by Sääf *et al.* (1998) (full window size of 21 residues and core window size of 15 residues). (B) The hydrophobic segments of MSI and MSII, but not proOmpA, anchor these proteins in the membrane. The translocation mix (TL buffer with BSA, SecB and SecA as described in Materials and methods) contained iodinated proOmpA, MSI, MSII or pOA-BPTI (~0.5 µg/ml, 60 000 c.p.m.) and SecYEG IMVs (50 µg/ml). After 5 min at 37°C with ATP (2 mM), the translocated products protected from proteinase K digestion were analyzed by '15%' SDS–PAGE and fluorography. As a further test of membrane-insertion, translocation reactions were also mixed with an equal volume of extraction solution (10 M urea, 300 mM KCl) and incubated on ice for 30 min. Membranes were re-isolated by ultracentrifugation and the sediments were resuspended and treated with proteinase K. The translocated, urea-resistant proteins were analyzed by SDS–PAGE and fluorography. (C) MSI and MSII are stably inserted into the membrane. Membranes bearing either pOA-BPTI, MSI or MSII, generated as in (B), were re-isolated by ultracentrifugation and resuspended in translocation mix containing 2 mM ATP, 10 mM DTT and an excess (20 µg/ml) of pOA, MSI or MSII, respectively. Further translocation was allowed by re-incubation at 37°C for the indicated times. Samples were treated with proteinase K and analyzed.

with an apolar segment of threshold hydrophobicity, displayed an intermediate behavior in that some of this protein was arrested and some was fully translocated (lane 2). The I₂₆ form of MSI generally formed a triplet of bands which may represent a partial digestion or a membrane anchoring at two slightly different positions. For each of these preproteins, proOmpA, MSI, MSII and pOA-BPTI, the formation of the proteinase K-protected material required ATP (not shown).

To determine whether the hydrophobic segments of MSI and MSII could promote release from the translocase

and stable membrane association or whether they only slowed translocation and remained engaged within the translocase, we examined the stability of these intermediates over time. Membranes bearing radiolabeled pOA-BPTI, MSI and MSII (as in Figure 1B) were re-isolated and incubated with fresh ATP, SecB, SecA and an excess of unlabeled pOA-BPTI, MSI and MSII, respectively (Figure 1C). The I_{26} intermediate generated with pOA-BPTI completed translocation within 5 min of the removal of BPTI by DTT cleavage of the cross-linker. In contrast, no forward movement of MSI or MSII occurred after even a 30 min incubation. Thus, the apolar segments of MSI and MSII arrest translocation in a stable fashion.

A second way to investigate the stability of these intermediates is to test their physical association with the membrane. Urea and high salt concentration efficiently extract translocation intermediates which are still in a proteinaceous environment (Schiebel *et al.*, 1991; Borel and Simon, 1996). Thus membranes bearing translocated proteins, generated as in Figure 1B (lanes 1–4), were incubated in 5 M urea and 150 mM KCl for 30 min on ice, then re-isolated and treated with proteinase K (Figure 1B, lanes 5–8). Only the I_{26} intermediate generated with pOA-BPTI could be extracted from the IMVs by this treatment (Figure 1B, lane 8, also see Figure 6 below). Thus, unlike pOA-BPTI, the I_{26} forms of MSI and MSII are stably arrested and associated with the membrane, presumably in the lipid bilayer.

Apolar segments do not 'block' the translocase

Although the apolar segments of MSI and MSII are sufficient to stop translocation and to provide stable association with the membrane, these proteins might not have been released from the translocase. We therefore examined the capacity of the translocase to undergo multiple translocation cycles. Saturation of the translocation reaction occurred with $\sim 4 \mu\text{g/ml}$ of pOA-BPTI (Figure 2A). Since pOA-BPTI is not released but is stoichiometrically arrested in the translocase (Bassilana and Wickner, 1993), the number of active translocation sites was $\sim 540 \text{ pmol/mg}$ of SecYEG-enriched IMVs (see the legend of Figure 2A), in agreement with the previous estimates of high-affinity SecA binding sites (Douville *et al.*, 1995; Duong and Wickner, 1997a). In contrast, the translocation reaction was linear at up to $20 \mu\text{g/ml}$ of added proOmpA and the same saturation curves were obtained when MSI or MSII were used as substrates (Figure 2A). Since each translocation site can translocate a supra-stoichiometric quantity of MSI and MSII, as seen for proOmpA (Bassilana and Wickner, 1993), these proteins must have been catalytically released from the translocase in a lateral fashion into the lipid bilayer.

To test the effectiveness of release from the translocation site, we examined whether the membrane integration of MSI and MSII can affect the subsequent translocation of proOmpA. Translocation reactions were performed with unlabeled preproteins in excess to the translocation sites and the membranes were then re-isolated and incubated with [^{125}I]proOmpA (Figure 2B). Neither proOmpA, MSI nor MSII, which were translocated during the first incubation, were able to prevent the subsequent translocation of [^{125}I]proOmpA (lanes 1, 2 and 3, respectively). In contrast, pOA-BPTI blocked the translocation sites and no translo-

Fig. 2. MSI and MSII are efficiently released from the translocase. **(A)** Membrane integration of MSI and MSII is catalytic. [^{125}I]-labeled proOmpA, MSI, MSII and pOA-BPTI ($0.5 \mu\text{g/ml}$; $60\,000 \text{ c.p.m.}$) were pre-mixed with their respective unlabeled forms ($0\text{--}36 \mu\text{g/ml}$). Proteins were incubated with the translocation mix and IMVs ($50 \mu\text{g/ml}$) for 5 min at 37°C with 2 mM ATP. After proteinase K treatment, arrested and translocated [^{125}I]-proteins were analyzed by SDS-PAGE, visualized by fluorography and quantitated by scanning densitometry. Assuming that the radioactivity is uniformly distributed in the preproteins, the density values obtained for the intermediates I_{26} were multiplied by 1.4 to convert them to fully translocated material. The maximal amount of translocated pOA-BPTI was $\sim 2.7 \text{ pmol}$ per $5 \mu\text{g}$ IMVs. Thus, $\sim 540 \text{ pmol}$ of pOA-BPTI saturate the translocation sites of 1 mg IMVs. **(B)** MSI and MSII do not block the translocation sites. Unlabeled proOmpA, MSI, MSII or pOA-BPTI ($20 \mu\text{g/ml}$; supra-stoichiometric to the estimated number of translocation sites) were incubated with the translocation mix and IMVs ($50 \mu\text{g/ml}$) for 10 min at 37°C with 2 mM ATP. Membranes were re-isolated by ultracentrifugation, resuspended in the translocation mix and re-incubated at 37°C with 2 mM ATP and 10 mM DTT, except where indicated. After 5 min, [^{125}I]proOmpA ($\sim 0.5 \mu\text{g/ml}$; $60\,000 \text{ c.p.m.}$) was added and the reaction further incubated for 5 min. Translocated [^{125}I]proOmpA was analyzed by proteolysis, SDS-PAGE and fluorography. **(C)** ProOmpA, MSI and MSII support similar translocation ATPase activities. Translocation ATPase was measured as described by Douville *et al.* (1995). ProOmpA and derivatives ($10 \mu\text{g/ml}$) were incubated with the translocation mix, IMVs ($50 \mu\text{g/ml}$) and 2 mM ATP. The P_i release was measured at the indicated times and the values were corrected for the ATP hydrolysis in the absence of proOmpA. To complete the translocation of pOA-BPTI, DTT (10 mM) was added after 10 min of incubation.

cation of [125 I]proOmpA was observed (lane 4). The addition of DTT, which frees proOmpA from BPTI (Figure 1C), allowed clearance of the translocation sites and subsequent translocation of [125 I]proOmpA (Figure 2B, lane 5). Thus, as seen for proOmpA, both MSI and MSII are effectively released from the translocation site and the translocase remains functional.

Finally, we examined the ability of these preproteins to stimulate the translocation ATPase activity of SecA. SecA couples the energy of ATP hydrolysis to preprotein translocation (Lill *et al.*, 1990). Since proOmpA, MSI and MSII are catalytically released from the translocase, they support similar translocation ATPase activities which last until the completion of translocation or membrane integration (Figure 2C, open symbols). In contrast, as reported by Schiebel *et al.* (1991), pOA-BPTI is arrested in the translocase and supports a linear ATPase reaction for at least 40 min (Figure 2C, filled triangles). The addition of DTT induces a rapid cessation of this activity (Figure 2C, filled squares) at a time which corresponds to the completion of translocation (Schiebel *et al.*, 1991).

SecYEG is sufficient for membrane integration

The membrane domain of the *E. coli* preprotein translocase is composed of six distinct subunits, SecY, E, G, D, F and YajC (Duong and Wickner, 1997a). While SecYEG and SecA are sufficient for efficient preprotein translocation (Hanada *et al.*, 1994), other subunits or proteins may be required for membrane integration. Thus, the translocation of MSI and MSII was tested in liposomes containing the purified SecYEG trimeric complex (Brundage *et al.*, 1990). The ratio between the translocated and the arrested forms was identical to that seen in IMVs (Figure 3A), thus indicating that the hydrophobic segments of MSI and MSII are as efficiently recognized by the pure SecYEG-translocase. Due to the absence of leader peptidase, only the precursor forms of these proteins were obtained. Moreover, because SecA is not stably inserted and a limited backward translocation occurs in the absence of SecDFyajC (Duong and Wickner, 1997b), pOA-BPTI was arrested in SecYEG proteoliposomes at an earlier position than that seen in IMVs (lanes 7 and 8). With either SecDFyajC-depleted IMVs (data not shown) or SecYEG proteoliposomes, the I_{26} forms of MSI and MSII did not undergo this backward movement and were arrested at the same position as seen in wild-type IMVs (lanes 4 and 6). This observation also suggests that MSI and MSII must have escaped from the SecYEG-translocase and become stably integrated into the lipid bilayer of the proteoliposomes. To confirm their release from SecYEG, translocation was first performed with unlabeled preproteins, then with [125 I]proOmpA (Figure 3B), as described above (Figure 2B). Neither proOmpA, MSI nor MSII, translocated during the first incubation, blocked the subsequent translocation of [125 I]proOmpA (lanes 1, 2 and 3, respectively). We conclude that the SecYEG translocase is sufficient for the membrane integration of the MSI and MSII proteins.

Translocation kinetics control the partitioning of MSI

The dual localization of MSI, membrane-anchored or translocated into the lumen of the IMVs or the proteolipo-

Fig. 3. SecYEG is sufficient to support membrane protein integration. (A) SecYEG proteoliposomes (PL) and IMVs support similar translocation arrest activities. Translocation and membrane integration of [125 I]-labeled proOmpA, MSI, MSII or pOA-BPTI (~0.5 μ g/ml, 60 000 c.p.m.) diluted in translocation mix was tested with SecYEG proteoliposomes (10 μ g of reconstituted SecYEG/ml) or with IMVs from wild-type *E. coli* BL21 (100 μ g of IMVs proteins/ml) for 10 min at 37°C in the presence of ATP (2 mM). Proteinase K protected material was analyzed by SDS-PAGE and fluorography. (B) ProOmpA, MSI and MSII are released from SecYEG translocase. Unlabeled proOmpA, MSI, MSII or pOA-BPTI (20 μ g/ml) were incubated with SecYEG proteoliposomes (10 μ g of SecYEG/ml), the translocation mix and 2 mM ATP for 10 min at 37°C. Proteoliposomes were re-isolated by ultracentrifugation, resuspended in translocation mix and re-incubated at 37°C with 2 mM ATP and 10 mM DTT, except where indicated. After 5 min, [125 I]proOmpA (0.5 μ g/ml; 60 000 c.p.m.) was added and the incubation continued for 5 min. Translocated [125 I]proOmpA was analyzed by SDS-PAGE and fluorography.

somes, suggests that factors other than SecYEG and the hydrophobicity of MSI may modulate the stop-transfer activity. These factors may also differentially affect the stop-translocation and translocase-release steps. We therefore investigated the effects of the translocation conditions on the partitioning of MSI.

The translocation of proOmpA, MSI and MSII was assayed in the presence of the PMF-generating substrate NADH or the uncoupler CCCP (Figure 4A). One of the known effects of the PMF is to accelerate preprotein translocation (Geller *et al.*, 1986; Driessen, 1992). Indeed, proOmpA translocation was stimulated by NADH (lanes 1 and 2). In contrast, the translocation of MSII was not affected (lanes 5 and 6), suggesting that the membrane integration step is not sensitive to the PMF. However, when the translocation of MSI was tested, the membrane potential promoted an increase of the fully translocated form (lanes 3 and 4). To confirm that the PMF does not inhibit membrane insertion, we tested the stability of the I_{26} form of MSI during a chase reaction with an excess of unlabeled substrate (Figure 4B) as described in Figure 1C. Whether formed in the presence or the absence of PMF, no chase of the I_{26} form of MSI occurred, indicating that there had been membrane integration and that this form is not a translocation intermediate. Thus the PMF does not inhibit membrane integration *per se* but rather

Fig. 4. The PMF favors translocation over membrane integration of MSI. **(A)** [125 I]-labeled proOmpA, MSI, MSII or pOA-BPTI (0.5 μ g/ml, 60 000 c.p.m.) were incubated for 10 min at 37°C with the translocation mix, non-stripped IMVs (50 μ g/ml), ATP (1 mM), the uncoupler CCCP (20 μ M) or the PMF-generating nucleotide NADH (5 mM). Proteinase K-inaccessible material was analyzed by SDS-PAGE and fluorography. **(B)** Once anchored, MSI is stable even in the presence of PMF. Translocation/membrane integration of [125 I]MSI was performed as above, in the presence of CCCP or NADH, for 5, 10 or 15 min. After 15 min, an excess of proOmpA (20 μ g/ml) was added and the incubation continued for the indicated total time. Proteinase K-inaccessible material was analyzed by SDS-PAGE and fluorography.

favors the translocation of MSI into the lumen of the vesicle.

We next examined whether a limiting ATP concentration (4 μ M), which decreases the rate of translocation (Shiozuka *et al.*, 1990), favors the membrane integration of MSI. ProOmpA itself pauses in its translocation at I_{26} at such ATP levels (Figure 5A, lanes 1–3) and this effect has been attributed to the short hydrophobic segment present at this position (Figure 1A; Sato *et al.*, 1997a). However, proOmpA- I_{26} and lower intermediates such as I_{16} are not stably arrested since they can undergo forward translocation upon the addition of 2 mM ATP (lanes 4–6). In contrast, the MSI- I_{26} species formed at low ATP levels (lanes 7–9) did not translocate forward after the addition of 2 mM ATP (lanes 10–12). The small amount of fully translocated MSI (lanes 10–12) rather came from lower translocation intermediates such as I_{16} (lanes 7–9). Thus slow translocation kinetics favor the partitioning of MSI into the membrane.

To arrest the translocation of MSI at 2 mM ATP, we coupled BPTI to the unique cysteine located near the C-terminal end of the hydrophobic segment of MSI (Figure 1A). As seen for pOA-BPTI, the addition of BPTI arrested any forward translocation of MSI (Figure 5B) and most of the protein was recovered at the position I_{26} . We then tested the stability of this synthetic intermediate after the removal of BPTI. While the addition of DTT to pOA-BPTI allowed rapid and complete forward translocation (Figures 1A and 5B), MSI- I_{26} which had been liberated from BPTI remained stably arrested at the position I_{26} during a 30 min incubation period (Figure 5B). Thus the synthetic arrest created by BPTI allowed the hydrophobic

Fig. 5. The kinetics of translocation control the membrane integration of MSI. **(A)** A low ATP concentration favors membrane integration. [125 I]proOmpA or [125 I]MSI (0.5 μ g/ml; 60 000 c.p.m.) was incubated with the translocation mix, IMVs (50 μ g/ml), 4 μ M ATP and an ATP-regenerating system (5 mM creatine phosphate, 10 μ g/ml creatine kinase). After 5, 10 or 15 min incubation at 37°C, half of the samples received 2 mM ATP and the incubation was prolonged for 5 min. Proteinase K-inaccessible material was analyzed by SDS-PAGE and fluorography. **(B)** Once arrested, MSI completely integrates into the membrane. Iodinated pOA-BPTI and MSI-BPTI (~1 μ g/ml, 50 000 c.p.m.) were incubated with translocation mix, IMVs (50 μ g/ml) and 2 mM ATP. After 15 min, unlabeled proOmpA (20 μ g/ml) and 10 mM DTT were added and the incubation continued for the indicated times. The translocation of [125 I]-labeled proOmpA, MSI, MSI-BPTI and pOA-BPTI in the absence of DTT is shown in the left four lanes. Note that, even in the absence of DTT, a small proportion of the pOA-BPTI and MSI-BPTI are fully translocated. Since this material migrates as proOmpA, it may result from some uncontrolled release of BPTI before translocation. **(C)** The release of MSI from the translocase occurs after removal of the BPTI molecule. Unlabeled proOmpA, MSI, MSI-BPTI or pOA-BPTI (10 μ g/ml) were incubated with the translocation mix, IMVs (50 μ g/ml) and 2 mM ATP for 10 min at 37°C. Membranes were re-isolated by ultracentrifugation, resuspended and re-incubated at 37°C with 2 mM ATP and 10 mM DTT where indicated. After 5 min, [125 I]proOmpA (0.5 μ g/ml; 60 000 c.p.m.) was added and the incubation continued for 5 min. Translocated [125 I]proOmpA was analyzed by SDS-PAGE and fluorography.

segment of MSI to interact with the membrane in a stable and permanent fashion. Taken together, these results show that the kinetics of translocation itself modulate the stop-transfer efficiency of a mildly hydrophobic segment. Moreover, since MSI has the intrinsic capacity to integrate into the membrane, as seen by the reduction of its translocation by CCCP, low ATP or BPTI, it appears that a higher hydrophobicity is required to stop translocation than is needed to allow anchoring of MSI in the membrane.

Membrane integration requires physiological temperature but not ATP hydrolysis

We expected that MSI-BPTI would not block the translocase but would be released in a catalytic manner into the

membrane. However, as with pOA-BPTI (Figure 2A), the translocation of MSI-BPTI was only stoichiometric (data not shown). Moreover, the subsequent translocation of [125 I]proOmpA was inhibited by a prior incubation with MSI-BPTI (Figure 5C, lane 4) while the addition of DTT allowed clearance of the translocation site (lane 6). Thus the BPTI molecule prevents lateral release of MSI from the translocase. It is possible that BPTI directly blocks the membrane-release function of the translocase. Alternatively, BPTI may not allow the hydrophobic segment of MSI to completely enter the translocase and hence to be laterally released into the membrane. This latter possibility could be responsible for the slight size difference observed between the I_{26} forms of MSI and MSI-BPTI (Figure 5B, lanes 2 and 3). Nevertheless, since MSI-BPTI is stably arrested even after the removal of its BPTI (Figure 5B, lanes 9–12), we were able specifically to analyze the requirements of the translocase-release step.

Membranes bearing MSI-BPTI or pOA-BPTI were treated with urea/KCl. As expected for proteins still engaged in a proteinaceous environment, the I_{26} forms of MSI-BPTI and pOA-BPTI (Figure 6, lanes 1 and 2) were fully extracted from the membrane (lanes 3 and 4). In contrast, when DTT was added before the urea treatment, to allow proOmpA to complete its translocation and MSI to enter the membrane, neither proOmpA nor MSI were extracted by urea/KCl (lanes 5 and 6). To test whether physiological temperature is essential for the lateral release from the translocase, incubation with DTT was performed on ice. The removal of BPTI by DTT occurs at 4°C (lane 9) and can be blocked by the prior addition of the oxidant ferricyanide (lane 10). At this temperature, proOmpA cannot complete translocation and is urea-extractable (lane 7). Since MSI, liberated from BPTI, was also fully extracted under these conditions (lane 8), we conclude that the protein had not entered the lipid bilayer at this temperature.

To test whether ATP hydrolysis by SecA is required for the release reaction, membranes bearing MSI-BPTI and pOA-BPTI were first treated with apyrase and then with DTT (Figure 6, right panel). After apyrase treatment, and hence in the absence of ATP, proOmpA cannot complete translocation and remains extractable with urea/KCl (lane 11). In contrast, even in the absence of ATP, MSI freed of BPTI becomes stably integrated into the membrane and is urea resistant (lane 12). Only the removal of BPTI from the MSI was required for the release reaction to occur. Without BPTI release, the MSI-BPTI remained extractable by urea (lane 14). Thus, once the hydrophobic segment of MSI has entered the translocase, ATP binding and hydrolysis by SecA is not necessary for the release and stable anchoring of MSI in the membrane. During translocation, SecA undergoes cycles of membrane insertion and deinsertion at the translocation site which are partly coupled to preprotein movement (Economou and Wickner, 1994; Duong and Wickner, 1997b). Since apyrase locks SecA in the membrane-inserted state by preventing its deinsertion (Economou and Wickner, 1994) and since a true translocation intermediate, proOmpA-BPTI, is still extracted by salt and urea when SecA is locked in the inserted state by apyrase treatment (lanes 11), our results indicate that the SecA deinsertion from the translocase is not essential for the lateral escape of MSI. These findings agree with observations of Sato *et al.* (1997b) who studied a proOmpA derivative with a distinct apolar segment. They reported that locking SecA in the inserted state with AMP-PNP did not block the formation of an alkali-resistant state, which may correspond to membrane integration (Sato *et al.*, 1997b).

Discussion

In vitro analysis of the post-translational mechanism of membrane proteins biogenesis has required an efficient

Fig. 6. Release of MSI from the translocase requires physiological temperature but not ATP. (A) To test the effect of temperature, iodinated pOA-BPTI and MSI-BPTI (1 µg/ml, 50 000 c.p.m.) were incubated with the translocation mix, IMVs (50 µg/ml) and 2 mM ATP for 15 min at 37°C, followed by addition of unlabeled proOmpA (20 µg/ml). Reactions corresponding to lanes 1–4 were chilled on ice. Reactions 5 and 6 were further incubated for 5 min at 37°C with 10 mM DTT before chilling on ice. Reactions 7 and 8 were chilled on ice before incubation with DTT (10 mM, 10 min at 0°C). Reactions 3–8 were then treated with the extraction solution (10 M urea, 300 mM KCl) as described in Figure 1B and the membranes re-isolated. Reactions 1–8 were then treated with proteinase K. To control for the effective removal of BPTI by DTT on ice, MSI-BPTI was incubated with the translocation mix, IMVs and DTT for 10 min at 0°C and the reduction was then quenched by addition of 50 mM potassium ferricyanide (lane 9). When ferricyanide was added before DTT, no removal of BPTI occurred (lane 10). (B) ATP hydrolysis is not required for the release reaction. Reactions 11–14 were prepared as described in (A) but stopped by the addition of apyrase (20 U/ml; 2 min). DTT (10 mM) was added to reactions 11 and 12 before chilling on ice. The membrane insertion was tested by urea/KCl treatment, followed by re-isolation of the membranes and proteinase K digestion as described above.

reconstitution of membrane integration. We find that (i) membrane integration takes place in a catalytic manner with multiple turnovers (Figure 2A); (ii) once inserted into the membrane, proteins remain stably anchored (Figure 1C) and are resistant to urea extraction (Figures 1B and 6); (iii) membrane-integrated proteins do not block the translocation sites and thus do not sequester any components required for normal translocation (Figure 2B); and (iv) the induced ATPase activity and the translocation efficiency (Figure 2A and C) are similar for the artificial membrane protein and its 'parental' protein, which has no highly hydrophobic segment. Taken together, these results show a successful reconstitution of the membrane integration process. They agree with a pioneering *in vitro* study which has been recently reported (Sato *et al.*, 1997b), but exploit the greater solubility and translocation stability of MSI and MSII to allow further exploration of the membrane assembly mechanism.

The first steps of the process, i.e. protein targeting and translocation, are directed by the same translocase for both membrane-anchored and fully translocated proteins. Genetic experiments have indeed established the SecY and SecA-dependence of the biogenesis of leader peptide-bearing membrane proteins (Schatz and Beckwith, 1990). However, membrane protein biogenesis also requires that transmembrane segments can be distinguished from other regions of the protein during translocation and that transmembrane segments can move laterally out of the translocase and into the surrounding lipid. In addition to SecA, the translocase is composed of six distinct membrane subunits (Duong and Wickner, 1997a) and other proteins such as FtsH have been proposed to participate in membrane protein biogenesis (Akiyama *et al.*, 1994a,b). Moreover, since SecDFyajC controls the stability of translocation intermediates such as proOmpA I₂₆ and I₁₆ (Duong and Wickner, 1997b), these subunits might have been expected to have a role during the stop-translocation process. However, we find that translocation arrest and lateral release can be efficiently achieved with only a few pure components: the preprotein bearing a hydrophobic segment, the chaperone SecB, the peripheral membrane ATPase SecA, ATP, the purified trimeric complex SecYEG and lipids.

The efficiency of membrane integration depends primarily on the hydrophobicity of the apolar segment itself. MSII, with 14 consecutive hydrophobic amino acids, is completely integrated into the membrane while the parental protein, proOmpA, is fully translocated. Several analyses of the limits of variation of membrane-spanning segments (Zerial *et al.*, 1987; MacIntyre *et al.*, 1988; Kuroiwa *et al.*, 1990, 1991; Lee and Manoil, 1994; Chen and Kendall, 1995) have demonstrated that overall hydrophobicity, rather than the exact amino acid composition, determines the threshold for stop-transfer function (A.Sääf, E.Wallin and G.von Heijne, submitted for publication). However, it was unknown whether this threshold defines the minimal hydrophobicity required to arrest translocation or whether it specifies exit from translocase and entry into the lipid bilayer. These distinct steps, as well as the contribution of factors beyond overall hydrophobicity, are difficult to analyze separately *in vivo* or with an *in vitro* co-translational membrane integration assay. In the latter case, the

stop-transfer efficiency depends on the choice of translation system (Spiess *et al.*, 1989).

We have analyzed the membrane integration process for a protein whose hydrophobicity is near the stop-transfer threshold, allowing both partitioning into the membrane and translocation into the lumen of the vesicle. Hydrophobic α -helical segments of 20 residues are theoretically required to span the 30 Å thickness of the membrane interior (Engelman *et al.*, 1986). However, provided that the aminoacyl residues are sufficiently apolar, very short segments can span the membrane (Kuroiwa *et al.*, 1991). Such hydrophobic segments may drag adjacent polar residues into the membrane or may create a local distortion of the lipid bilayer. Nevertheless, when not fully translocated, the 10 hydrophobic amino acyl residues of MSI were sufficient to irreversibly integrate the protein into the membrane. This suggests that the function of stop-transfer segments may be more limited by their capacities to stop translocation than by their ability to stably anchor proteins in the lipid bilayer.

To test this hypothesis, we have analyzed the contribution of factors other than the hydrophobic sequence itself. We find that, for a given hydrophobic segment near the threshold for stop-transfer function, the kinetics of translocation determine the final localization of the protein. A high rate of translocation, seen with 2 mM ATP and PMF, facilitates complete translocation, while a low rate, as seen with 4 μ M ATP, favors membrane integration. Since the artificial stabilization of a mildly hydrophobic segment within the translocase, caused by BPTI, allows for the complete transfer of MSI to the lipid bilayer, the membrane integration of MSI may be in kinetic competition with translocation. This concept is consistent with previous studies of the microsomal translocase (Kuroiwa *et al.*, 1990, 1991). These authors reported that positive charges at the end of a mildly hydrophobic segment facilitated membrane integration while negative charges increased translocation. As a rule, positively charged residues are more difficult to translocate across a membrane and may undergo electrostatic interactions with the negatively charged anionic phospholipids (Andersson and von Heijne, 1994; von Heijne, 1994; von Klompenburg *et al.*, 1997). Taken together, these results show that the threshold for stop-transfer activity is modulated by the translocase itself. Since even a mildly hydrophobic segment has the capacity to span the lipid bilayer, this agrees with the hypothesis that a greater hydrophobicity is required for the translocation arrest process than for membrane anchoring. Thus, the threshold value for stop-transfer function probably specifies the hydrophobicity required for efficient translocase-dependent membrane assembly.

The mechanism of preprotein lateral release from the translocase has been analyzed in the microsomal system using site-specific crosslinking between membrane lipids and nascent polypeptides (Martoglio *et al.*, 1995; Mothes *et al.*, 1997). These studies showed that hydrophobic segments became lipid exposed soon after entering the translocation channel, whereas polar segments remained in an aqueous environment. In our study, the use of MSI conjugated to BPTI allowed us to uncouple translocation arrest from lateral release and to analyze the specific requirements of membrane integration. We find that

physiological temperature is essential for the release, which may reflect either a temperature-dependent rearrangement of the translocase subunits to allow the lateral escape of the hydrophobic segment or a need for lipid fluidity of the bilayer to accept the polypeptide chain. In either case, the release reaction does not require either ATP hydrolysis by SecA or SecA deinsertion from the membrane. It is noteworthy in this context that inserted SecA is largely shielded from the lipid phase during translocation and that SecA ATPase activity is regulated by anionic phospholipids (Lill *et al.*, 1990; Eichler *et al.*, 1997). It is also remarkable that the forward translocation of proOmpA does not require SecA deinsertion and can be driven solely by the energy of the PMF (Schiebel *et al.*, 1991; Duong and Wickner, 1997b). Structural analysis of the translocase may illuminate the pathway taken by polar and hydrophobic segments. However, the ability to promote the lateral release solely by temperature in the absence of added energy sources suggests that membrane integration is passive and depends only on the hydrophobicity of the preprotein segment.

Why are most natural stop-transfer sequences so much more hydrophobic than necessary to anchor a protein in the membrane? Thermodynamic considerations had suggested that a segment of 20 glycine residues would suffice to remain in a lipid bilayer (Engelman *et al.*, 1986), yet this is well below the threshold of stop-transfer activity. It is also noteworthy that the hydrophobicity and length of the stop-transfer sequences of proteins with a single membrane span is somewhat greater than that of leader sequences which initiate translocation (von Heijne, 1986; von Heijne and Gavel, 1988). This 'extra' hydrophobicity may simply be required for efficient arrest of the movement of the translocating polypeptide chain but may not be essential for integration into the lipid bilayer *per se*. Thus, while stop-transfer sequences generally act in an absolute manner, specifying a single topologic form, mildly hydrophobic sequences might allow for alternate distributions of a protein within the cell. When present in multi-spanning membrane proteins, such sequences may also modulate the final topology adopted by the protein. The biological relevance of a stop-transfer function modulated by the translocation kinetics may be illustrated by the unusual behavior of the prion protein PrP, which partitions both across and into the endoplasmic reticulum membrane (Yost *et al.*, 1990). The mildly hydrophobic sequence of PrP is preceded by a sequence rich in hydroxylated and basic residues termed a 'stop transfer effector' (STE). The STE apparently lowers the threshold hydrophobicity required for membrane integration in the same manner as C-terminally flanking basic residues (see above). Since a STE is also present in the secreted apolipoprotein B (Chuck and Lingappa, 1993), where it induces a pause in translocation but not release into the membrane, a process analogous to the stepwise translocation of proOmpA (Schiebel *et al.*, 1991), the STE may modulate the time interval during which this mildly hydrophobic segment of PrP can enter the membrane (Nakahara *et al.*, 1994). The biological role for 'leaky stop-transfer' is also illustrated by the NADH-cytochrome *b₅* reductase, which partitions into both the outer membrane and the inter-membrane space of mitochondria. The efficiency of its marginally hydrophobic stop-transfer sequence depends on the elec-

trochemical gradient across the inner membrane (Hahne *et al.*, 1994). The electrochemical gradient may indirectly regulate the partitioning of the protein by affecting the translocation kinetics across the outer membrane.

Translocase-dependent membrane integration appears to be determined by two factors, the hydrophobicity of the segment to be integrated and the activation energy barrier associated with the further translocation of this segment. This second factor can be modulated by the energetics of translocation. The favorable physical properties of MSI and MSII and the reconstitution of their membrane integration in a defined reaction may allow further definition of the translocation arrest and lateral release mechanism.

Materials and methods

Materials

[¹²⁵I]Na (~17 mCi/μg) was purchased from Amersham. Proteinase K, creatine kinase, creatine phosphate, CCCP and NADH were from Boehringer Mannheim. Potato apyrase (grade VIII), ATP, DTT, lipid-free BSA, BPTI and potassium ferricyanide were from Sigma. Iodogen and SPDP were from Pierce. SecA (Cunningham *et al.*, 1989) and SecB (Weiss *et al.*, 1988) were purified as described. The construction of pHis₆-EYG and the purification of the His₆-SecEYG trimeric complex by Ni²⁺-NTA affinity chromatography will be described elsewhere. Inner membrane vesicles (IMVs) from *E. coli* strain BL21 (*hsdS*, *ompT*⁻, *gal*) and BL21 pHA-EYG (plasmid pBAD22 carrying *HA-secE*, *secY* and *secG*) were prepared as described (Douville *et al.*, 1995). To render the IMVs proton-permeable, the F₁ subunit of the F₀-F₁ ATPase was removed in 6 M urea (30 min, 4°C). This treatment also removed endogenous SecA (Cunningham *et al.*, 1989).

Introduction of hydrophobic segments into proOmpA

The sequence coding for the hydrophobic segments was inserted into the plasmid pOmpA-C245, a pTrc99A-derivative (Pharmacia) coding for proOmpA with a single cysteine at position 245 (Duong and Wickner, 1997b). To create MSI, the codons for five hydrophobic amino acids (VAAVL; Figure 1A) were introduced between codons 235–236 using primers complementary to the 5' and 3' ends of OmpA-C245, 5'-GAAACAGACCATGGAATTC (*EcoRI*, sense) and 5'-CCGCCA-AAACAGCCAAGCTT (*HindIII*, anti-sense), respectively, and the mutagenic primers (*PstI*, anti-sense) 5'-CTGCAGCTACAACCTACGG-AACCGTCTTTC and (*PstI*, sense) 5'-CTGCAGTACTGGTTCTG-GGTTACACC. The restriction sites are in italics and the newly introduced sequence is underlined. After PCR-amplification, the DNA fragments were digested with the corresponding restriction enzymes and cloned into the *EcoRI*–*HindIII* sites of pTrc99A. Correct insertion was verified by sequencing the relevant coding regions. To create MSII, the codons for 10 hydrophobic amino acids (AVLVIAAVLA; Figure 1A) were introduced between codons 235–236 using the complementary primers '*EcoRI*' and '*HindIII*' described above and the mutagenic primers (*PstI*, anti-sense) 5'-CTGCAGCGATAACCAGTACAGCAACTACGGAACCGTCTTTC and (*PstI*, sense) 5'-CTGCAGTACTGGCAGTTCTGGGTTACACC. The PCR-amplified fragments were digested with the appropriate restriction enzymes, cloned into the *EcoRI*–*HindIII* sites of pTrc99A and the relevant coding region was sequenced.

Synthesis, purification and iodination of proOmpA derivatives

ProOmpA C245 and MSI were expressed and purified as described (Crooke *et al.*, 1988). To avoid the cell lysis problems associated with the overexpression of MSII, plasmid pTrc-MSII was transformed into the *secY* thermo-sensitive strain CJ107 (Wolfe *et al.*, 1985). The cells were cultivated at permissive temperature to OD_{600nm} ± 0.4, then shifted to 42°C for 30 min and the synthesis induced with 1 mM IPTG for 120 min. The MSII protein, accumulated as inclusion bodies, was purified first as described (Crooke *et al.*, 1988). Both proOmpA, MSI and MSII were further purified on Pharmacia FPLC MonoQ resin (in 8 M urea, 1 mM 2-mercaptoethanol, 50 mM glycine, pH 10). Proteins were eluted from the column with a gradient of 0–0.5 M NaCl. BPTI was coupled to the unique cysteine of proOmpA-C245 or MSI using the cross-linker SPDP (Schiebel *et al.*, 1991) and the conjugates were

purified from the adducts on Pharmacia FPLC MonoS (Duong and Wickner, 1997b). ProOmpA-C245 and derivatives (100 µg) were iodinated as described (Duong and Wickner, 1997b) using $^{125}\text{I}[\text{Na}]$ (1 µl; 500 µCi). The iodinated proteins were recovered in 8 M urea, 50 mM Tris-HCl, pH 7.9. [^{125}I]proOmpA and derivatives ($\sim 1.2 \times 10^6$ c.p.m./µg) were stored at -80°C and used within 2 months.

Translocation assay

Except where indicated, each translocation assay was performed in 100 µl of 'translocation mix': TL buffer (50 mM KCl, 5 mM MgCl_2 , 50 mM Tris-HCl, pH 7.9) containing BSA (200 µg/ml), SecB (40 µg/ml) and SecA (10 µg/ml). ProOmpA or derivatives were diluted from 8 M urea to the desired concentrations. Except where indicated, SecYEG-enriched IMVs were subsequently added to a final concentration of 50 µg/ml. After a 1 min preincubation at 37°C , translocation was started by the addition of 2 µl of 0.1 M ATP. Translocation was arrested by chilling on ice. Samples were treated with proteinase K (1 mg/ml; 15 min, 0°C) and the digestion was stopped by adding 1/3 volume of ice-cold 50% TCA. After 30 min on ice, the TCA-precipitated material was collected by centrifugation (16 000 r.p.m., 10 min, 4°C , Beckman microfuge). The sediment was suspended in 1 ml of acetone and centrifuged. Acetone was removed by aspiration and the pellet dried at 37°C for 10 min. Proteins were resuspended in SDS-sample buffer and analyzed by '15%' SDS-PAGE and fluorography (Brundage *et al.*, 1990). Re-isolation of the IMVs was performed by layering the translocation reaction over 1/4 volume of 0.8 M sucrose in TL buffer followed by ultracentrifugation (10 min, 4°C , 73 000 r.p.m., Beckman TLA120 rotor). Sediments were resuspended on ice with 100 µl of translocation mix (TL buffer containing BSA, SecB and SecA as above).

Miscellaneous

Protein concentration was determined using the Bradford reagent (Biorad) with BSA as a standard. Autoradiography of [^{125}I]-labeled polypeptides was performed at -80°C with intensifying screens. Densitometry was performed using a Silverscan III scanner (La Cie Ltd) and IPLabH software.

Acknowledgements

We thank Professor G.von Heijne for communicating prepublication results and Professor A.Pugsley for comments on the manuscript. We are also grateful to M.Rice Leonard for expert assistance, A.Price, K.Sato, C.Ungermann, Z.Xu for helpful discussions and critical reading of the manuscript and J.Eichler and Phoebe Tzou for encouragement throughout the course of this work. This work was supported by a grant from the National Institute of General Medical Sciences.

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Received October 23, 1997; revised November 17, 1997;
accepted November 19, 1997